Table 1. Plasma levels of anti-ADAMTS13 autoantibodies

Age, years	Gestational weeks	FFP infusion	ADAMTS13 activity, %	ADAMTS13 inhibitor, BU/ml	ADAMTS13 IgG type antibody, units/ml	Clinical status
21	*	–	<0.5	1.4	42.9	TTP bout
22	*	–	<0.5	1.7	35.0	remission
27	*	–	3.7	0.8	48.9	remission
27	*	–	1.9	1.6	33.3	remission
30	8	–	<0.5	<0.5	28.2	pregnancy
30	10	+	6.5	0.5	34.4	pregnancy
30	11	+	4.5	0.5	31.2	pregnancy
30	13	+	3.4	0.5	19.9	pregnancy
30	15	+	3.3	0.8	30.2	pregnancy
30	20	+	3.2	0.9	23.7	pregnancy
30	24	+	2.9	<0.5	21.6	pregnancy
30	29	+	2.3	0.6	13.9	pregnancy
30	33	+	3	<0.5	19.7	pregnancy
30	38	+	2.9	<0.5	14.9	pregnancy
30	39	+	6.9	0.5	16.1	pregnancy
30	*	–	1.9	0.6	13.9	1 month after delivery
32	*	–	5.2	<0.5	15.4	1.5 years after delivery
32	*	–	1.8	<0.5	9.8	2 years after delivery

microangiopathic hemolytic anemia in childhood that are reversible by infusions of fresh frozen plasma (FFP) (early-onset phenotype) [3]. On the other hand, patients with the 'late-onset phenotype' are diagnosed with USS in adulthood, usually during episodes of infectious disease or pregnancy [3]. Moatti-Cohen et al. [4] reported that the rate of USS is much higher in pregnancy-onset TTP patients than in all adulthood-onset TTP patients.

We previously described 43 USS patients in Japan up to the end of March 2011 [3]. Among them, 9 patients developed bouts of TTP and were correctly diagnosed with USS in association with pregnancy [5]. These pregnancies often result in premature delivery or fetal loss. Recent papers have reported successful delivery with FFP infusion therapy in patients with USS diagnosed prior to pregnancy [6, 7]. However, a detailed therapeutic protocol including FFP infusions for pregnant women with USS has not yet been established.

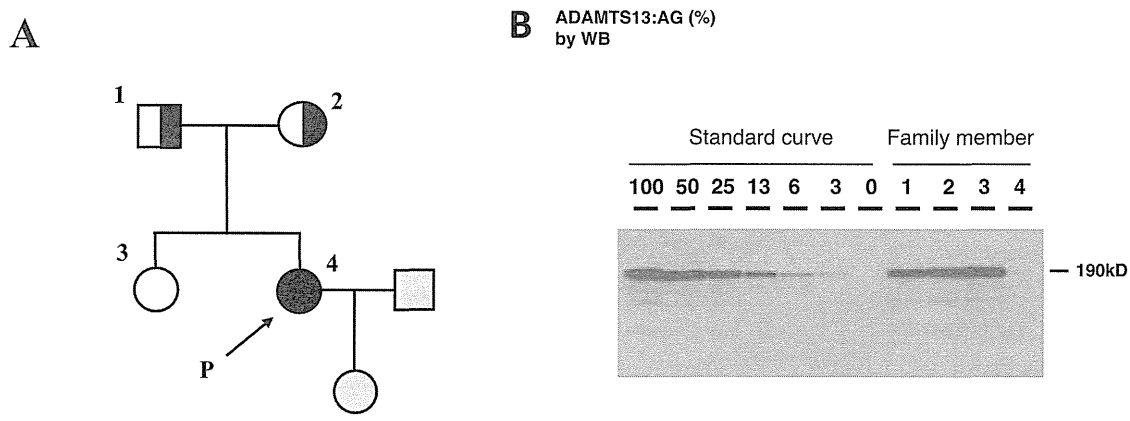
Here, we report a USS patient with low titers of neutralizing (inhibitory) and non-neutralizing (binding) antibodies against ADAMTS13 who successfully underwent delivery with the use of gradually increasing FFP infusions as the pregnancy progressed. The intervals between and volumes of FFP infused were determined by close monitoring of levels of ADAMTS13 activity and its inhibitor.

Material and Methods

Until 2005, ADAMTS13 activity was analyzed by a VWF multimer assay with a detection limit of 3% of normal controls [2, 8]. Since 2005, a highly sensitive chromogenic ADAMTS13-act-ELISA [9] with a detection limit of 0.5% of normal was developed and replaced the VWF multimer assay. Thus, we re-examined ADAMTS13 activity in stored plasma samples using this act-ELISA and reported the results by the act-ELISA in this study. Plasma ADAMTS13 inhibitor titers were also re-examined using the chromogenic ADAMTS13-act-ELISA in heat-inactivated plasma at 56 °C for 30 min. One Bethesda unit (BU) of inhibitor was defined as the amount of inhibitor that reduces ADAMTS13 activity to 50% of control [10]. ADAMTS13 inhibitor titers were defined as: <0.5 BU/ml (negative), 0.5–1.0 BU/ml (marginal), and ≥1.0 BU/ml (positive). Plasma levels of ADAMTS13 antigen were determined using a quantitative sandwich ELISA assay [11]. Plasma ADAMTS13 antigen was also analyzed by quantitative and qualitative western blotting (WB) under reducing conditions [12]. Densitometric analysis of ADAMTS13 antigen was performed for the 190 kDa band using NIH imageJ (developed by the National Institutes of Health, *http://rsb.info.gov/nih-image/*). Plasma anti-ADAMTS13 IgG antibody titers (binding antibody) were determined by TECHNOZYM® ADAMTS-13 INH (Technoclone, Vienna, Austria) according to the manufacturer's instructions. In this assay, plasma IgG levels less than 12 units/ml were defined as negative, 12–15 units/ml were considered borderline, and levels greater than 15 units/ml were defined as positive. *ADAMTS13* gene analyses [13] were performed with the permission of the Ethics Committees. The pathogenicity of missense mutations was analyzed in silico using PolyPhen-2 (*http://genetics.bwh.harvard.edu/pph2/*) to predict the functional significance of missense mutations. Written informed consent for *ADAMTS13* gene analysis was obtained from the patient and her family.

Case Report

Proband LL4 is a female born in 1981. Her parents and elder sister are apparently healthy. She did not have any episodes of severe neonatal jaundice requiring exchange blood transfusion. At 14 years of age, she developed thrombocytopenia and acute renal failure requiring hemodialysis during an upper respiratory tract infection. She had similar episodes during upper respiratory tract infections at the ages of 15, 16, 17, and 20 years. These bouts were ameliorated by FFP infusion. At 21 years of age, she was admitted to a local hospital complaining of diarrhea and high-grade fever. She was diagnosed with TTP based on the pentad of hemolytic anemia, thrombocytopenia, acute renal failure, fever, and mild neurological symptoms. Her condition improved with FFP administration. Soon after this episode, she got married. When the patient was 27 years old, detailed investigation including *ADAMTS13* gene analysis was performed in all members of her family. At 28 years of age, she underwent an elective termination at 6 weeks of gestation after the risk of developing TTP was taken into consideration. She has never received prophylactic FFP infusions without the presence of thrombocytopenia.



C

ADAMTS13:AC (%)	ADAMTS13:AG (%)		*ADAMTS13 gene*					
ELISA	ELISA	WB	Thr339	Cys438	Gln448	Pro475	Pro618	Gly909
-1	27	23	37	T/T	C/S	Q/Q	P/P	P/P	G/G
-2	34	50	51	T/R	C/C	Q/E	P/S	P/A	G/R
-3	57	77	97	T/T	C/C	Q/Q	P/S	P/P	G/G
-4	3.7	1.2	<3	T/R	C/S	Q/E	P/P	P/A	G/R

Fig. 1. Pedigree and ADAMTS13 analysis of USS-LL4 and her family. **A** The propositus (denoted as P), USS-LL4, is the second child of nonconsanguineous parents. Squares and circles indicate males and females, respectively, and shaded symbols represent individuals who were not examined. Half-black symbols indicate asymptomatic carriers. **B** WB analyses of plasma ADAMTS13 antigen (AG) in the patient's family members are shown. **C** ADAMTS13 activity (AC) was determined using activity ELISA, and ADAMTS13 AG levels were measured using ELISA and WB. Results are shown as percentages of normal values. Identified mutations in ADAMTS13 are depicted using one-letter amino acid abbreviations.

ADAMTS13 Activity, Antibody, and Antigen Analysis

Plasmas obtained at 21 and 22 years of age showed severely decreased ADAMTS13 activity (<0.5 % of normal) and low titers of ADAMTS13 inhibitor (1.4 and 1.7 BU/ml, respectively) (table 1). In addition, ADAMTS13 binding IgG antibodies were found in both samples. These results indicated that this patient might have acquired TTP or USS with the presence of ADAMTS13 inhibitor. As shown in figure 1C, plasma ADAMTS13 antigen levels as analyzed by ELISA were 1.2% of normal values in the patient at 27 years of age. Further, plasma levels of ADAMTS13 antigen analyzed by WB were <3% of normal values in the patient.

ADAMTS13 Gene Analysis

We found 6 missense mutations (p.T339R, p.C438S, P.Q448E, p.P475S, p.P618A, and p.G909R) in this family (fig. 1C). Of these, p.T339R, p.Q448E, p. P475S, and p.P618S have been previously reported as single nucleotide polymorphisms (SNPs) in the Japanese population [14]. This patient had two mutations (p.C438S and p.G909R) that appear to be disease-causing mutations that have never been previously reported. We analyzed these two mutations using PolyPhen-2 to predict their effects on ADAMTS13. Both mutations were predicted to be 'probably damaging.' Thus, the patient was a compound heterozygote for two mutations in the *ADAMTS13* gene: p.C438S (c.1313G>C, exon 12) was inherited from her father and p.G909R (c.2725 G>A, exon 21) was inherited from her mother.

Clinical Course in Pregnancy

Although the patient had low levels of ADAMTS13 inhibitor, we diagnosed this patient with USS based on the results of the genetic analysis. Taking into account the risk of TTP, she chose elective abortion for her first pregnancy at 28 years of age. However, when she became pregnant again at the age of 30, she strongly hoped to have a child. After thorough discussions between the hematologists and obstetricians, we decided to continue the pregnancy with close monitoring of her condition and her fetus.

The patient's plasma ADAMTS13 activity was under 0.5% and ADAMTS13 inhibitor was negative at 8 weeks of gestation without FFP infusion. Starting at 9 weeks of gestation, 4 units of FFP were infused (480 ml / 92 kg body weight = 5.2 ml/kg). Between 11 and 17 weeks of gestation, the patient received 6 units of FFP (97 kg, 7.4 ml/kg) biweekly. In this period, ADAMTS13 activity was 3–4% of normal just before FFP infusion. At 17 weeks, she had fever with an upper respiratory infection. Her platelet count suddenly decreased to 141 × 10^9/l. Therefore, she received 6 units of FFP on the next day. Subsequently, 6 units of FFP were infused weekly, with plasma levels of ADAMTS13 activity measured before and after FFP infusion. After 32 weeks of gestation, the volume of FFP infusion increased to 8 units (103 kg, 9.3 ml/kg) per week. In addition to FFP infusion, she took low-dose aspirin (100 mg/day) between 9 and 34 weeks of gestation.

As shown in figure 2, this regimen maintained her platelet count over 200 × 10^9/l. Plasma levels of ADAMTS13 before FFP infusion were 3–5% of normal, and levels after FFP infusion were approximately 10%. The maximum level of ADAMTS13 inhibitor was 0.9 BU/ml at 20 weeks of gestation. Until 29 weeks of gestation, the levels of inhibitor were relatively high. However, after 30 weeks of gestation, inhibitor levels over 0.5 BU/ml were not observed except at 35 weeks (0.6 BU/ml). After delivery, ADAMTS13 inhibitor levels over 0.5 BU/ml were not detected. Moreover, levels of ADAMTS13 binding antibodies before pregnancy were over 30 units/ml (table 1). These levels gradually decreased as the pregnancy progressed, similar to levels of ADAMTS13 inhibitor. At 39 weeks of gestation, she gave birth to a healthy baby girl by cesarean section. She received 8 units of FFP on the day of surgery and 6 units on postoperative days 1, 3, and 5. Prophylactic FFP infusion was then stopped. After delivery, plasma levels of ADAMTS13 activity were maintained between 1.8 and 5.2%, and both ADAMTS13 inhibitor titers and IgG antibodies were almost undetectable on three different occasions without FFP infusion (table 1).

The birth weight of her baby was 3,474 g. External malformations were not found. The ADAMTS13 activity of the umbilical cord was 35.5%, and the level of inhibitor was 0.7 BU/ml. Pathological examination of the placenta revealed only mild infarcts in the periphery and at the insertion of the umbilical cord.

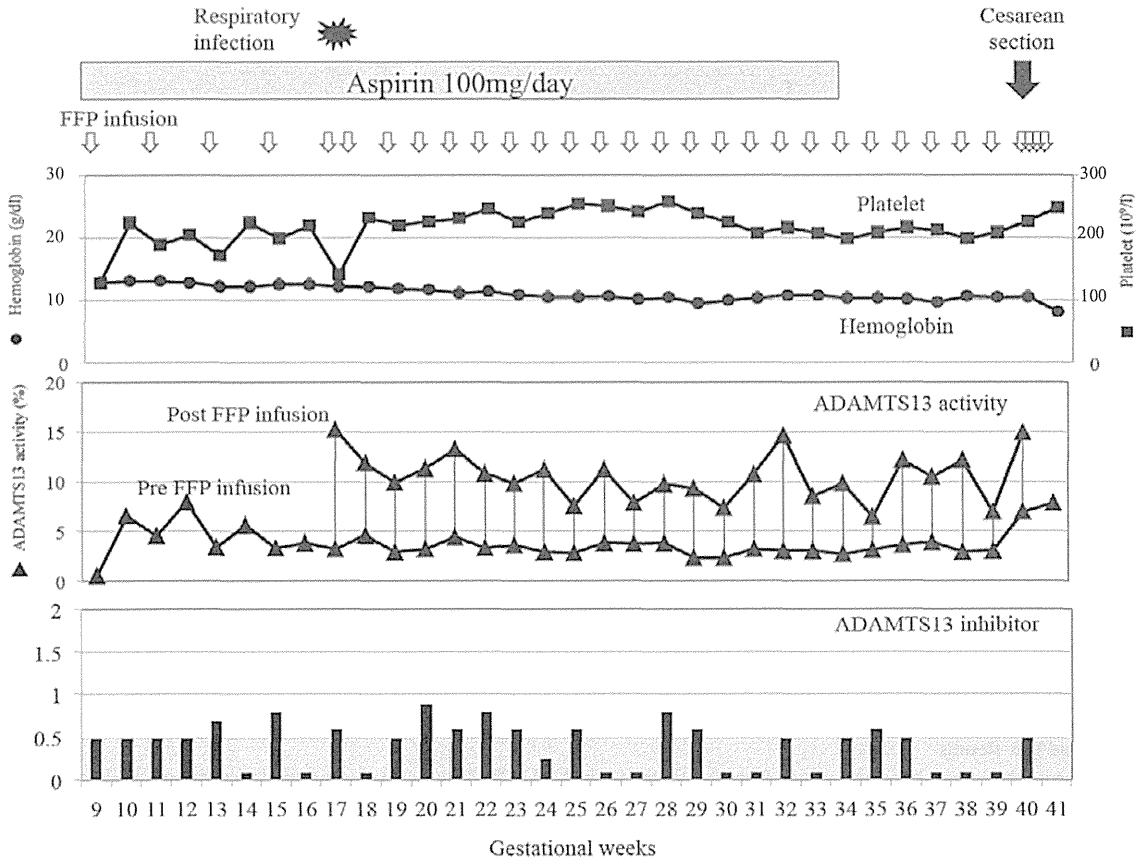


Fig. 2. Clinical course of USS-LL4 during pregnancy. FFP infusions were started at 5 ml/kg biweekly. At 17 weeks of gestation, she had an episode of respiratory infection. Her platelet count decreased from 200 to 140 × 10^9 /l. Subsequently, the interval between FFP infusions was shortened to 1 week. Plasma levels of ADAMTS13 activity before FFP infusion were 3–5% and those after FFP infusion were approximately 10%. Platelet counts were maintained over 200 × 10^9 /l. The patient took low-dose aspirin between 9 and 34 weeks of gestation. At 39 weeks, she gave birth to a healthy baby girl via cesarean section.

Discussion

We diagnosed patient described here with USS with low titer of ADAMTS13 antibodies based on the results of *ADAMTS13* gene analysis. When the patient first became pregnant at 28 years of age, we chose an elective termination due to the risk of developing TTP. However, in her second pregnancy at 30 years of age, we decided to continue the pregnancy with close monitoring of her condition and her fetus. Since plasma VWF levels increase with gestational age even in normal pregnancy [15], much more ADAMTS13 supplementation may be necessary in late gestation in USS patients. Thus, the therapeutic protocol for this patient involved dose escalation of FFP infusions, and the interval between infusions was gradually shortened with the progression of pregnancy, with frequent monitoring of ADAMTS13 activity levels.

In addition to FFP infusion, we used low-dose aspirin between 9 and 34 weeks of gestation. In our USS registry, one patient with USS (USS-L2) successfully gave birth to 4 babies (including twins) with taking low-dose aspirin during pregnancy [5]. Another patient successfully treated with FFP infusion and low-dose aspirin was reported by another group from our registry in Japan [6]. Antiplatelet agents such as aspirin, dipyridamole and ticlopidine, have been used in the acute treatment of acquired TTP. British treatment guidelines for TTP recommend low-dose aspirin during platelet recovery (platelet count >50 × 10^9/l) for patients with acquired TTP. Fetal loss in patients with USS is presumably caused by the disturbance of utero-placental circulation by platelet thrombi. Although there is only anecdotal evidence, low-dose aspirin in addition to FFP infusion may be effective in pregnant patients with USS.

In this patient, we identified the presence of both ADAMTS13 inhibitors and ADAMTS13 binding antibodies before pregnancy. Kentouche et al. [16] reported a similar patient in whom ADAMTS13 inhibitors were detected during pregnancy. However, binding ADAMTS13 antibodies were not detected by a commercially available assay (Technoclone) in stored samples in which ADAMTS13 inhibitors were detected. In contrast, both ADAMTS13 inhibitor and binding antibodies were detected in our patient (table 1).

We were concerned about increasing ADAMTS13 antibody titers with frequent antigen stimulation associated with FFP infusions. Thus, plasma levels of ADAMTS13 inhibitor and ADAMTS13 activity were analyzed each week before and after FFP infusion. However, increases in ADAMTS13 inhibitor were not observed as her pregnancy progressed. On the contrary, plasma levels of ADAMTS13 inhibitor decreased to marginal levels (<0.5–0.9 BU/ml) during pregnancy, and so far have not increased again after delivery. ADAMTS13 IgG antibodies also decreased to almost undetectable levels after delivery (table 1).

Regarding this interesting phenomenon, it is generally said that during pregnancy a mother has a natural intra-uterine allograft (fetus), which is regularly not rejected, indicating that immunological tolerance is up-regulated during this period [17]. In fact, it

has been reported that rheumatoid arthritis (RA) disease activity is often transiently lower during pregnancy [18]. However, unlike RA, in which disease activity flares up in 90% of patients within the first 3 months postpartum unless appropriate medications are given before delivery [19], both neutralizing and non-neutralizing antibodies against ADAMTS13 in our USS patient have not increased after delivery. Although we cannot fully explain this interesting phenomenon at present, it is possible that an immunological reset after delivery might be involved [16, 17]. So far, we have observed the patient for over 2 years after delivery, but much longer observation may shed a light on this difficult question.

Acknowledgments

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Disclosure Statement

YF is a member of clinical advisory boards for Baxter BioScience.

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ORIGINAL ARTICLE

Analysis of patients with atypical hemolytic uremic syndrome treated at the Mie University Hospital: concentration of *C3* p.I1157T mutation

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Abstract Atypical hemolytic uremic syndrome (aHUS) is caused by abnormalities of the complement system and has a significantly poor prognosis. The clinical phenotypes of 12 patients in nine families with aHUS with familial or recurrent onset and ADAMTS13 activity of ≥20 % treated at the Mie University Hospital were examined. In seven of the patients, the first episode of aHUS occurred during childhood and ten patients experienced a relapse. All patients had renal dysfunction and three had been treated with hemodialysis. Seven patients experienced probable triggering events including common cold, influenza, bacterial infection and/or vaccination for influenza. All patients had entered remission, and renal function was improved in 11 patients. DNA sequencing of six candidate genes, identified a *C3* p.I1157T missense mutation in all eight patients in six families examined and this mutation was causative for aHUS. A causative mutation *THBD* p.D486Y was also identified in an aHUS patient. Four missense mutations, *CFH* p.V837I, p.Y1058H, p.V1060L and *THBD* p.R403K may predispose to aHUS manifestation; the remaining seven missense mutations were likely neutral. In conclusion, the clinical phenotypes of aHUS are various, and there are often trigger factors. The *C3* p.I1157T mutation was identified as the causative mutation for aHUS in all patients examined, and may be geographically concentrated in or around the Mie prefecture in central Japan.

Keywords aHUS · C3 mutation · Trigger factor · Renal failure · Thrombotic microangiopathy

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Introduction

Hemolytic uremic syndrome (HUS) [1] is characterized by the presence of microangiopathic hemolytic anemia, thrombocytopenia, renal impairment with symptoms similar to those of thrombotic thrombocytopenic purpura (TTP) [2–4]. Approximately 10 % of cases are classified as atypical due to the absence of Shiga toxin-producing bacterial infection as a trigger [5]. Compared to typical HUS, atypical HUS (aHUS) is considered to be caused by abnormalities of the complement system and has a much poorer prognosis and higher mortality, with up to half of patients progressing to end-stage renal disease [6].

The alternative pathway of the complement system is a natural defense system against invasive microbial attack, in which complement component C3 (C3), the central complement protein, is hydrolyzed to C3b and directly binds to the microbe for opsonization or the subsequent activation of the complement pathway [7]. When C3b binds to host cells, further activation of the complement system is stringently limited by several endogenous complement regulatory proteins present on the surface of the host cells [8]. Complement factor H (CFH) and membrane cofactor protein (MCP or CD46) are cofactors for the proteolytic degradation of C3b by complement factor I (CFI). Thrombomodulin, an endothelial anticoagulant glycoprotein encoded by *THBD*, also functions as a cofactor for CFI-mediated C3b inactivation [9]. The uncontrolled activation of the alternative pathway of the complement system plays an important role in the pathogenesis of aHUS. More than half of patients with aHUS have mutations in the genes involved in the alternative pathway of the complement system [5]. Mutations with loss-of-function of regulators (CFH, CFI, MCP and THBD) [9–12] and gain-of-function of key complement components (C3 and CFB) [13, 14] have been found to predispose patients to the development of aHUS. A normal plasma level of complement proteins does not preclude the presence of mutations in these genes. More importantly, genotype–phenotype correlations of aHUS have clinical significance in predicting renal recovery and transplant outcomes [12].

We previously reported the clinical characteristics and genetic variations of ten aHUS patients in nine family, in whom two aHUS patients in one family have been treated at the Mie University Hospital [15]. In the present study, we examined the clinical phenotypes of 12 aHUS patients in nine families, including previously reported those two patients in one family, all treated at the Mie University Hospital. We also performed the genetic analysis in six aHUS patients, in total, identified at the Mie University Hospital. Unexpectedly, we found that all six patients shared the same genetic mutation, *C3* p.I1157T missense mutation.

Materials and methods

Twelve aHUS patients in nine families, all treated at the Mie University Hospital, were investigated in this studies. We included two previously reported patients (patients JJ1 and JJ2 [15]) in one family as patients IV-1 and IV-2, respectively, because they have been treated at the Mie University Hospital. Six of the patients were sporadic and three were familial. The diagnosis of aHUS was made based on the simultaneous occurrence of microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure without Shiga toxin [16]. In addition, patients with familial or recurrent aHUS, which is associated with an ADAMTS13 activity of more than 20 % to completely exclude TTP due to ADAMTS13 deficiency, and a survival of more than 1 year, were selected in this study. The study protocol was approved by the Mie University Graduate School of Medicine and the National Cerebral and Cardiovascular Center, and written informed consent was obtained from all of the participants.

ADAMTS13 activity assay

The ADAMTS13 activity was measured using a FRETS-VWF73 peptide (Peptide Institute, Japan) according to the method reported by Kokame et al. [17, 18].

Hemolytic assay

The hemolytic assay was performed at the Department of Blood Transfusion Medicine, Nara Medical University [15]. Resuspended sheep red blood cells (Japan Lamb, Japan) were incubated with a dilution series of a patient plasma sample at 37 °C for 30 min, and the level of hemoglobin released from the red blood cells was measured by the absorbance at 414 nm [19]. The hemolysis obtained from normal plasma spiked with monoclonal antibody against CFH (200 μg IgG/ml, final) was defined as a 100 % hemolysis as the control. The result of hemolysis in patient plasma was expressed as follows; enhanced (≥50 % of the control), moderate (15–50 %) and no hemolysis (<15 %).

Mutation screening

Genomic DNA was extracted from the peripheral blood leukocytes. The coding exons and the intronic flanking regions of *CFH* (NM 000186.3), *C3* (NM 000064.2), *MCP* (NM 002389.4), *CFI* (NM 000204.3), *CFB* (NM 001710.5) and *THBD* (NM 000361.2) were sequenced at Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, as previously described [15]. The A of the ATG translation initiation start site was designated as

+1 position and the initial Met was denoted as +1. Multiplex ligation –dependent probe amplification analysis was used to screen the gene deletion using a commercially available kit (MLPA kit P236-A2, MRC-Holland, the Netherlands) as previously described [15].

Results

The clinical features of 12 aHUS patients in nine families are summarized in Table 1. All of the patients showed no signs for infection with Shiga toxin-producing *Escherichia coli*. Nine families were non-consanguineous with each other. The first episode of aHUS occurred during childhood (≤10 years of age) in seven patients, while five patients experienced their first episode at more than 20 years of age. Ten patients had experienced relapse, with a varying number of relapse events. All patients had renal dysfunction and three patients had been treated with hemodialysis (HD), although they were being weaned from this treatment. Three patients had central nervous symptoms. Seven patients experienced probable triggering events, such as the common cold, influenza, bacterial infection or vaccination for influenza.

The laboratory data of 12 patients with aHUS are summarized in Table 2. The platelet count was markedly reduced in all patients, with the exception of patient IV-2 and hemoglobin levels, ranging from 5.9 to 9.8 g/dl. The levels of creatinine and lactate dehydrogenase were increased in most patients with aHUS, while the total bilirubin levels were slightly increased. The plasma ADAMTS13 activity was within the range of 40–100 % in all patients. The patients were treated with plasma exchange, transfusion of fresh frozen plasma, steroid or infusion therapy or the administration of anti-platelet, anti-hypertensive or antibiotic agents. All patients had remission, in addition, the renal function improved in 11 patients and worsened in one patient (IV-2). "When patient VI-1 developed relapse, he was treated with eculizumab and his symptoms promptly improved.

Genetic analyses of six candidate genes and the gene deletion have been performed in six aHUS patients and found that all patients had a causative mutation, p.I1157T, in *C3*, and the same mutation has been previously identified in two aHUS patients in one family (patients IV-1 and IV-2) treated at the Mie University Hospital, as summarized in Table 3. A causative mutation *THBD* p.D486Y previously identified in aHUS patients in Europe and North America [7] was also identified in an aHUS patient, I-2. Gene deletion of *CFH* and *CFHRs* were not found in the aHUS patients. DNA sequencing identified additional 12 missense mutations. Among them, two rare missense

Table 1 Subjects

	Age	Sex	Age of first episode	Relapse	Outcome	Renal dysfunction	HD	CNS symptoms	Trigger for aHUS
I-1	38	F	6	6	Survive	Positive	ND	Positive	Common cold
I-2	68	F	20	2	Survive	Positive	ND	Negative	–
II	35	F	5	6	Survive	Positive	ND	Negative	Influenza
III	12	M	1	2	Survive	Positive	Weaning	Negative	Common cold, infection
IV-1	36	M	2	7	Survive	Positive	Weaning	Positive	Common cold, infection
IV-2	71	M	70	0	Survive	Positive	Weaning	Negative	–
V	38	F	21	3	Survive	Positive	ND	Positive	Common cold, vaccine[a]
VI-1	9	M	9	1	Survive	Positive	ND	Negative	Infection
VI-2	45	M	38	0	Survive	Positive	ND	Negative	–
VII	2	M	1	1	Survive	Positive	ND	Negative	–
VIII	22	F	3	7	Survive	Positive	ND	Negative	–
IX	28	M	24	7	Survive	Positive	ND	Negative	Common cold

IV-2 the father of IV-1, *HD* hemodialysis, *ND* not done, *CNS* central nerve system

[a] Vaccine for influenza

Table 2 Laboratory data of the patients with aHUS

	Platelet ($\times 10^8$/ml)	Hemoglobin (g/dl)	Creatinine (mg/dl)	LDH (U/l)	T-Bil (mg/dl)	ADAMTS13 (%)
I-1	1.9	7.4	1.7	972	1.9	76.3
I-2[a]						100
II	3.5	5.9	4.7	4485	3.5	88.8
III	2.6	5.9	4.7	4465	2.4	53.8
IV-1	2.8	6.7	10.6	1280	1.1	92.5
IV-2	9.5	9.6	8.0	398	1.2	96.0
V	3.5	9.8	1.4	928	3.5	92.5
VI-1	1.4	6.6	1.8	3160	1.4	67.5
VI-2	1.0	7.4	1.9	2850	1.8	40.0
VII	2.6	7.2	10.9	696	2.6	97.5
VIII	2.2	6.0	0.85	1098	2.2	100
IX	1.5	7.9	2.1	1780	1.9	ND

T-Bil total bilirubin, *ND* not done

[a] Previous data not available

mutations, *CFH* p.Y1058H and p.V1060L, and two low-frequency missense mutations, *CFH* p.V837I and *THBD* p.R403K, might predispose to aHUS, and the remaining seven missense mutations were likely neutral. Patient IV-1 who has *C3* p.I1157T developed aHUS at 2 year of age and experienced seven recurrences of aHUS (Table 1). He had acute renal failure and was treated with HD. Patient IV-2, a patient IV-1's father, who also has *C3* p.I1157T, developed aHUS after undergoing nephrectomy at 70 years of age and was treated with HD. Both patients and patient VI exhibited a mildly elevated hemolytic activity, however other five patients with *C3* p.I1157T did not show an elevated activity (Table 3).

Discussion

In Japan, the frequency of Shiga toxin-producing *Escherichia coli* (STEC)-HUS is approximately 40 % of all cases of thrombotic microangiopathy (TMA), according to the national questionnaire survey of TMA [20, 21], indicating that the frequency of STEC-HUS is lower in Japan than in Europe and North America. There are few methods for detecting abnormalities in the regulatory complement system. Therefore, the hematologists diagnosed the patients with suspected aHUS as having TTP. Other doctors also diagnosed the patients as having renal disease due to TMA. Therefore, there are a few reports of aHUS in Japan. In the present study, cases of fatal TMA were excluded due to the difficulty of confirming the subject's past and family history. Although all patients evaluated in the present study survived, the outcomes of aHUS were not always good. It has recently been reported that the terminal complement inhibitor eculizumab, which is approved for the treatment of paroxysmal nocturnal hemoglobin-uria, improves aHUS [22]. Eculizumab was also approved for the treatment of aHUS in Japan in September 2013. The drug binds with high affinity to human complement protein C5 and blocks the generation of proinflammatory C5a and the membrane attack complex, C5b-9 [22, 23]. "Although eculizumab is expensive, its use promptly improved the relapse in patients VI-1. Patients III, IV-1 and IV-2, who had been tread with HD, should receive eculizumab if they exhibit a relapse. Treatment with eculizumab is recommended in the acute phase in patients with mild aHUS, such as that involving C3 mutations, and as prophylaxis in those with severe aHUS, such as that involving CFH mutations [24].

In the present study, we examined the genetic abnormalities in six aHUS patients and identified the *C3* p.I1157T mutation as a causative mutation in the patients. We have previously performed the genetic analysis in aHUS patients and identified the same mutation in three patients, two of whom were referred from the Mie University Hospital [15]. Thus, as summarized in Table 3, eight aHUS patients treated at the Mie University Hospital carried the *C3* p.I1157T mutation. This finding was unexpected because our previous genetic analysis in 10 aHUS patients revealed the heterogeneous phenotype-genotype correlation [15]. The *C3* p.I1157T mutation might be geographically concentrated in or around the Mie prefecture located in central Japan.

A causative mutation *THBD* p.D486Y previously identified in aHUS patients in Europe and North America was also identified in the present study. The allele frequency of this mutation was 0.011 in the Japanese population referred from the 1000 Genomes database [25] and the recombinant mutant thrombomodulin with this mutation showed defective C3b inactivation [9]. The significance of remaining missense mutations was unknown. Both p.Y1058H and p.V1060L in the short consensus repeat-18 domain of CFH were rare mutations. They were not

Table 3 Genetic analysis of eight aHUS patients in the six families

Patients			I-1	I-2	II	III	IV-1	IV-2	V	VI
Hemolytic assay			−	−	−	−	±	±	−	±
Missense mutations	rs number	MAF								
CFH										
c.184G>A	rs800292	0.416				p.V62I			p.V62I	p.V62I
c.2509 G>A	rs55807605	0.011						**p.V837I**		
c.2808G>T	rs1065489	0.455	p.E936D (homo)	p.E936D	p.E936D (homo)	p.E936D	p.E936D (homo)	p.E936D	p.E936D	p.E936D
c.3172T>C	rs55679475	None			**p.Y1058H**	**p.Y1058H**				**p.Y1058H**
c.3178G>C	rs55771831	None			**p.V1060L**	**p.V1060L**				**p.V1060L**
CFI										
c.603A>C	rs145769028	0.028					p.R201S	p.R201S		
c.1217G>A	rs74817407	0.096				p.R406H				
C3										
c.3470T>C		None	**p.I1157T**	**p.I1157T (homo)**	**p.I1157T**	**p.I1157T**	**p.I1157T**	**p.I1157T**	**p.I1157T**	**p.I1157T**
CFB										
c.94C>T	rs12614	0.112	p.R32W	p.R32W	p.R32W		p.R32W	p.R32W (homo)		
c.95G>A	rs641153	0.073								p.R32Q
THBD										
c.1208G>A	rs41400249	0.006							**p.R403K**	
c.1418C>T	rs1042579	0.253				p.A473V			p.A473V	
c.1456G>T	rs41348347	0.011		p.D486Y						
CNV of CFH and CFHRs					Normal	Normal	Normal	Normal	Normal	

Hemolytic assay, − no hemolysis; ± moderate hemolysis. Bold and underlined: definite causative mutation [13], Bold: rare and low-frequency potentially predisposing mutation; homo, homozygote, The A in the ATG translation initiation start site is designated as the +1 position and the initial Met denotes +1

MAF minor allele frequency taken from the 1000 genome database (http://www.1000genomes.org/data), *CFH* complement factor H, *CFI* complement factor I, *C3* complement component 3, *CFB* complement factor B, *THBD* thrombomodulin, *CNV* copy number variation, *CFHRs* CFH related genes

identified in the 1000 Genomes database [25] but were found as somatic mutations in human cancers in the COSMIC database [26]. Both p.V837I in the short consensus repeat-14 domain of CFH and p.R403K in the EGF-like 4 domain of THBD were low-frequency mutations with the minor allele frequency of 0.011 and 0.006, respectively [25]. It can be assumed that, in addition to the main genetic mutation, *C3* p.I1157T, the environmental factors and/or other genetic variations are required for the manifestation of aHUS as a second hit. Rare or low-frequency missense mutations in the aHUS patients identified in the present study may predispose to the aHUS manifestation. The remaining seven missense mutations were likely neutral.

Sheep red blood cells are rich in sialic acid and are capable of binding CFH from the plasma to protect themselves against human complement attack. Therefore, the hemolytic assays are frequently used to evaluate the function of CFH-related abnormalities [18, 27]. Generally, plasma samples containing CFH mutant with mutation in the C-terminal domains or auto-antibodies against CFH would exhibit an increased hemolytic activity. In the present study, patients with the *C3* p.I1157T mutation showed negative and weak hemolytic activity, indicating that this mutation does not directly influence the hemolytic assay.

In conclusion, we found that the clinical phenotypes of aHUS patients are various, and there are often trigger factors. The *C3* p.I1157T mutation was found in eight aHUS patients examined as the causative mutation for aHUS, and would be geographically concentrated in or around the Mie prefecture in central Japan.

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Conflict of interest All authors have no conflict of interest to report.

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ORIGINAL ARTICLE

Effect of ADAMTS-13 on cerebrovascular microthrombosis and neuronal injury after experimental subarachnoid hemorrhage

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Summary. *Background:* Microthrombosis and reactive inflammation contribute to neuronal injury after subarachnoid hemorrhage (SAH). ADAMTS-13 cleaves von Willebrand factor multimers, and inhibits thrombus formation and, seemingly, inflammatory reactions. *Objective:* To investigate the effect of ADAMTS-13 in experimental SAH. *Methods:* A total of 100 male C57/BL6 mice were randomly assigned to four groups: sham (n = 15), SAH (n = 27), vehicle (n = 25), and ADAMTS-13 (n = 23; 100 μL per 10 g of body weight of 100 μg of ADAMTS-13 per 1 mL of 0.9% NaCl; 20 min after SAH). Neurologic performance was assessed on days 1 and 2 after SAH. Animals were killed on day 2. The amounts of subarachnoid blood, microthrombi, apoptosis and degenerative neurons were compared. The degree of neuronal inflammation and vasospasm was also compared. In five mice each (SAH and ADAMTS-13 groups), bleeding time was assessed 2 h after SAH. *Results:* Systemic administration of ADAMTS-13 achieved significant amelioration of microthrombosis and improvement in neurologic performance. ADAMTS-13 reduced the amount of apoptotic and degenerative neurons. A tendency for decreased neuronal inflammation was observed. ADAMTS-13 did not show any significant effect on vasospasm. The degree of systemic inflammation was not changed by ADAMTS-13 administration. ADAMTS-13 neither increased the amount of subarachnoid blood nor prolonged the bleeding time. *Conclusions:* ADAMTS-13 may reduce neuronal injury after SAH by reducing microthrombosis formation and neuronal inflammation, thereby providing a new option for mitigating the severity of neuronal injury after SAH.

Keywords: ADAMTS13, mouse; cerebral vasospasm; microcirculation; subarachnoid hemorrhage; thrombosis.

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Introduction

Subarachnoid hemorrhage (SAH), resulting from a ruptured intracranial aneurysm, is a common and frequently devastating condition, accounting for 5–10% of all strokes, with an incidence of 7–20 per 100 000. Despite considerable advances in diagnosis and treatment, the outcome for patients with SAH remains poor. The optimal treatment of neuronal injury after aneurysmal SAH remains challenging. Cerebral vasospasm (CVS), which is a morphologic narrowing of large cerebral arteries, has been regarded as the major cause of neurologic deterioration and consequent poor outcome [1–3]. As neurologic deterioration often occurs in a delayed fashion, owing to ischemia, the term 'delayed cerebral ischemia' (DCI) is frequently used. However, recent evidence suggests that treatment of CVS does not necessarily prevent the occurrence of DCI, with a consequent poor outcome [2,4]. The dissociation between CVS and outcome became evident in well-designed clinical trials using the endothelin-1 receptor antagonist clazosentan [2,3]. Current evidence suggests that the mechanisms underlying neuronal injury are multifactorial [1–4]. The inflammatory response and the

occurrence of microthrombosis are frequently discussed as potential protagonists [4–10]. A complex series of cellular and molecular events are elicited by the breakdown of erythrocytes in the subarachnoid space, leading to an inflammatory response with cytokine release [8,10,11]. Several proinflammatory cytokines have been associated with CVS, DCI, and/or poor outcome, among them interleukin (IL)-6 and high-mobility group protein box 1 (HMGB1) [8,11,12]. Supporting evidence suggests that microthrombosis is an additional explanation for DCI. A correlation between procoagulant factors – in particular, von Willebrand factor (VWF) – and the development of DCI has been reported [4,9]. In patients who were thought to have died because of DCI, significantly high amounts of microthrombi were found in ischemic regions [4]. In more recent animal studies, the occurrence of microthrombi was verified, and the number of microthrombi correlated with the number of apoptotic cells [6,7,13]. Furthermore, inflammatory cytokines, such as IL-6, induce coagulation and might contribute to microthrombosis formation [4,9,14]. Interestingly, clazosentan alleviated CVS but did not affect the occurrence of microthrombi or the amount of neuronal injury after experimental SAH [6,13].

ADAMTS-13 is a VWF-cleaving protease that inhibits thrombus formation. ADAMTS-13 has been described as a key protein in linking thrombosis with inflammation [14,15]. Almost no reports have been published on the role of ADAMTS-13 after SAH, except for one clinical study in which reduced ADAMTS-13 activity was reported in patients with DCI [9]. In experimental ischemic stroke models, ADAMTS-13 deficiency resulted in aggravation, and administration of ADAMTS-13 in amelioration, of neuronal injury [16–19]. In the current study, we investigated whether ADAMTS-13 administration would reduce microthrombosis and neuronal injury in mice after SAH.

Materials and methods

Animals

Male C57/BL6 mice were used (body weight, 22–26 g; Kyudo, Saga, Japan). In the first of two separate experimental series, mice were subjected to experimental SAH, and five mice were killed at on each of days 1, 2 and 3 after SAH to establish a time course for microclot formation. Another five sham-operated mice were killed on day 2. Dead mice were replaced to achieve the aforementioned numbers (n = 5 each). In the second study, a total of 100 were used. The mice were handled according to the guidelines and regulations of the institutional animal care committee of Fukuoka University (Fukuoka, Japan). Experiments were performed in accordance with the good laboratory practice guidelines [20]. Mice were randomly assigned to four groups by a random integer generator, with values between one and four for sham, SAH, vehicle, and ADAMTS-13.

Mouse SAH model

The circle of Willis perforation model was used [5,6]. In brief, mice were anesthetized with isoflurane (Escain; Mylan, Tokyo, Japan) (5% induction; 1.5% maintenance). Surgical procedures were performed in a standard microsurgical setting. In the prone position, a fiber-optic micropressure transducer (Samba Sensors AB, Goteborg, Sweden) was placed in the left temporo-basis. In the supine position, a 5–0 monofilament nylon suture (Ethilon; Eticon, Somerville, NJ, USA) was introduced through the external carotid artery and advanced towards the internal carotid artery (ICA). The filament was quickly withdrawn after perforation of the circle of Willis. In sham mice, the filament was advanced only until the tip was located intracranially, to avoid vessel rupture. Body temperature was kept at 37.5 °C with a feedback-controlled heating pad (NS-TC10; Neuroscience, Tokyo, Japan).

Drug administration

In mice of the ADAMTS-13 group, 100 μL per 10 g of body weight of 100 μg recombinant human ADAMTS-13 (rhADAMTS-13) (6156-AD; R&D Systems, Minneapolis, MN, USA) per 1 mL of 0.9% NaCl was administered 20 min after SAH through the tail vein. In mice of the vehicle group, 100 μL per 10 g of body weight of 0.9% NaCl was administered. The currently applied dose was derived from a previous study on experimental ischemic stroke, where multiple dosages were examined (M. Fujioka and K. Mishima, unpublished data).

Neurologic assessments

Neurologic performance was assessed in a blinded fashion by use of the modified Garcia score and tape removal test on days 1 and 2 after SAH. In brief, the Garcia score examines spontaneous activity, motor function, and reflexes [21]. For the tape removal test, standardized 5 × 5-mm pieces of tape were placed on the plantar surface of the forepaw. The mice were subjected to a maximum of 180 s per side. This adhesive removal test is effective in detecting functional deficits after focal ischemia [19,22].

Bleeding time

In 10 mice (five each in the SAH and ADAMTS-13 groups), bleeding time was assessed 2 h after SAH in a blinded fashion. Under anesthesia, a 3-mm segment of the tail was amputated and immersed in phosphate-buffered saline. The time required for the stream of blood to stop was defined as the bleeding time [19].

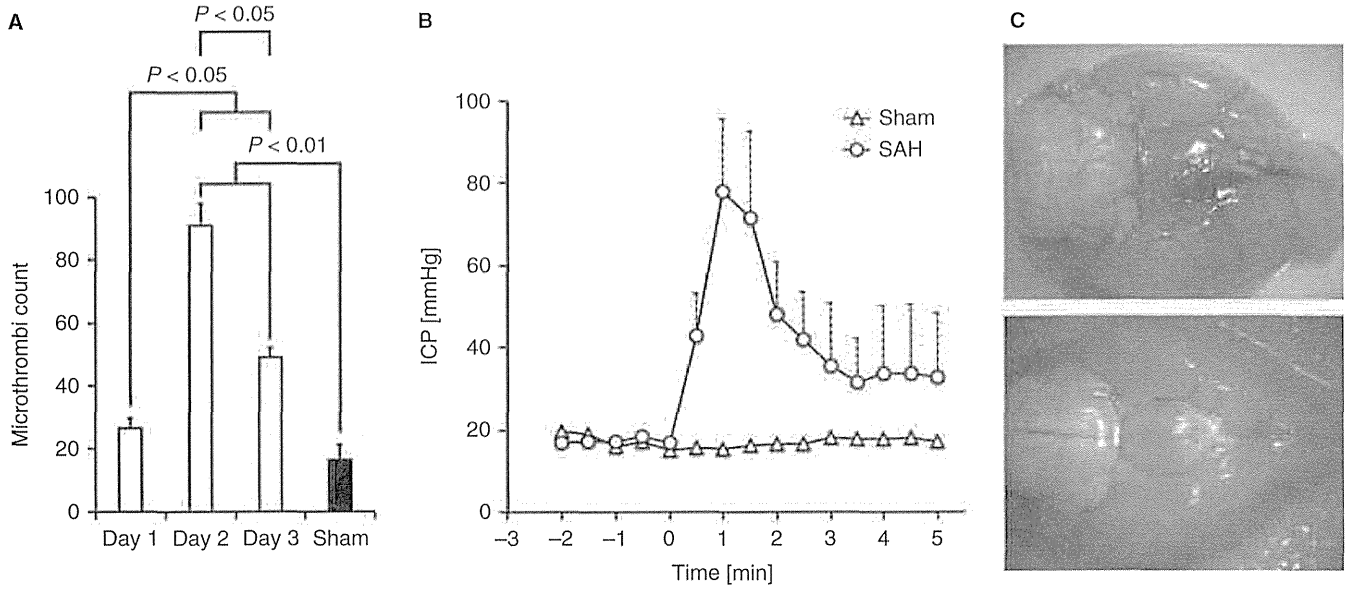

Fig. 1. Time course of microthombosis in experimental subarachnoid hemorrhage. (A) Cumulative numbers of microthrombi shown as bar graphs. The cumulative numbers of fibrinogen-positive microthrombi were significantly higher on days 2 and 3, but not on day 1, in mice subjected to subarachnoid hemorrhage (SAH) than in sham mice. In the SAH group, the cumulative number of microthrombi was higher on day 2 than on days 1 and 3. (B) Mice subjected to SAH showed a sharp increase in intracranial pressure. (C) Representative brain specimen showing successful SAH with hematoma in the basal cisterna (upper picture). No hemorrhage was present in sham mice (lower picture).

Killing of the mice and sampling

In the second experimental series, 51 mice (sham, $n = 10$; SAH, $n = 13$; vehicle, $n = 13$; ADAMTS-13, $n = 15$) were killed under deep anesthesia on day 2 after SAH by transcardial perfusion fixation (10 mL of ice-cold 0.9% NaCl followed by 50 mL of 4% paraformaldehyde). Day 2 was chosen as the time point at which the peak occurrence of microthrombosis was observed in the first experiment (Fig. 1A), in concordance with a previous study [6]. The brains were removed, photographed, and postfixed. Severity of bleeding was assessed with a grading system as previously described [23]. Automated degreasing, dehydration (RH-12; Sakura Seiki, Nagano, Japan) and paraffin embedding were then performed. Transcardial blood sampling was performed prior to killing of the mice. Blood samples were centrifuged at 1500 × *g* for 15 min at 4 °C, and the supernatant was stored at − 30 °C. In 25 mice (sham, $n = 5$; SAH, $n = 8$; vehicle, $n = 6$; ADAMTS-13, $n = 6$), the brains were removed without fixation and homogenized in a protein extraction solution (Pro-Prep; iNtRON Biotechnology, Sungnam, Korea). The samples were centrifuged at 20 000 × *g* for 45 min at 4 °C, and the supernatant was stored at − 80 °C.

Histologic examination

Paraffin blocks were sliced into 6-μm sections and mounted on glass slides. Hematoxylin and eosin staining was performed for CVS identification. The distal ICA was photographed at × 200 magnification under a light microscope. The lumen diameter and vessel wall thickness were quantified at four different points along the artery circumference, and averaged with IMAGE J software (Ver. 1.45s; NIH, Bethesda, MD, USA) in a blinded fashion.

Microthrombi were visualized by immunohistochemistry with fibrinogen staining. After antigen retrieval, the slides were incubated in sheep anti-fibrinogen antibody (1 : 200) (LifeSpan, Seattle, WA, USA) for 2 h. This was followed by incubation in biotinylated secondary anti-sheep antibody (1 : 500) for 1 h. Staining was visualized with the VECTASTAIN Kit (Vector, Burlingame, CA, USA), and counterstaining was performed with hematoxylin. Predefined regions of interest (ROIs) – three hippocampal and five cortical regions in each hemisphere – were photographed at × 200 magnification. The cumulative number of microthrombi was counted in a blinded fashion. The particle analyzer function of IMAGE J was used with predefined stereologic counting rules.

HMGB1 and neuronal nuclei (NeuN) double staining was performed to qualitatively evaluate the degree of neuronal inflammation [17,24]. After antigen retrieval, the slides were incubated in rabbit anti-HMGB1 (Abcam, Cambridge, UK) and biotinylated mouse anti-NeuN antibody (Chemicon, Temecula, CA, USA) overnight. This was followed by incubation with DyLight 488-conjugated donkey anti-rabbit (Abcam) and Texas Red-conjugated avidin (NeutrAvidin; Invitrogen, Carlsbad, CA, USA) for 1 h. The ROIs were photographed under a fluorescence microscope, and quantitatively assessed.

For the quantitative assessment of neuronal injury after SAH, Fluoro-Jade C (FJC) staining (Merck Millipore, Billerica, MA, USA) with 4′,6-diamidino-2-phenylindole (Invitrogen) counterstaining was performed, as described

previously [25]. To evaluate the occurrence of apoptotic neurons, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed (Roche Diagnostics, Rotkreuz, Switzerland). Staining was performed according to the manufacturers' instructions. The numbers of FJC-positive and TUNEL-positive cells were determined in ROIs in a blinded fashion.

ELISA

IL-6 (Quantikine mouse IL-6 immunoassay; R&D Systems), HMGB1 (HMGB1 ELISA kit II; Shino-Test Corporation, Tokyo, Japan) and ADAMTS-13 (Quantikine human ADAMTS-13 immunoassay; R&D Systems) were quantitatively measured in blood and homogenized brain supernatant. Total protein in supernatant was calculated with the Bradford protein assay, and cytokine levels were calculated per milligram of total protein. All concentrations below the minimal detection limit were assigned to the detection limit.

Statistical analysis

Nominal variables are given as frequency and percentage, and continuous variables as mean ± standard error of the mean. Nominal variables were compared by use of the chi-squared test. Ordinal variables (Garcia score) were compared by use of a non-parametric ANOVA. Continuous variables were compared by use of an ANOVA, with logarithmic transformation if needed. For comparison within the group, ANOVA for repeated measures was applied (first experimental series). A P-value of < 0.05 was regarded as statistically significant. IBM SPSS Statistics 20.0 (IBM, Armonk, NY, USA) software was used.

Results

Mortality, bleeding time, and degree of hemorrhage

In the first experimental series, the mortality was 20% ($n = 3$) within 72 h. In the second experimental series, a total of 10 mice were excluded (intrasurgical fatality before induction of SAH, $n = 3$; no or questionable induction of SAH, $n = 4$; failed drug administration, $n = 1$; accidental ICA occlusion, $n = 1$; failed tissue sampling, $n = 1$), leaving 15 sham, 27 SAH, 25 vehicle and 23 ADAMTS-13 mice for analysis. The vessel perforation resulted in a sharp increase in intracranial pressure, confirming successful SAH (Fig. 1B,C). At day 2 after SAH, the mortality in the ADAMTS-13 group was lower, although the difference was not statistically significant (sham, $n = 0$ [0%]; SAH, $n = 6$ [22%]; vehicle, $n = 6$ [24%]; and ADAMTS-13, $n = 3$ [9%]; $P = 0.12$). The bleeding time did not differ between the SAH and ADAMTS-13 groups: 103.2 ± 20.8 s vs. 100.8 ± 21.4 s ($P = 0.94$). The degree of hemorrhage was not different between mice in the SAH, vehicle and ADAMTS-13 groups ($P = 0.42$).

Neurologic performance

Mice in the SAH and vehicle groups had worse neurologic performance than those in the sham group, as assessed by the Garcia score and tape removal test (both $P < 0.01$). Administration of ADAMTS-13 significantly improved neurologic performance ($P < 0.05$). The results are shown in Fig. 2.

Time course of microthrombus formation

The first experimental study showed that the cumulative numbers of fibrinogen-positive microthrombi were significantly higher on days 2 and 3, but not on day 1, in mice subjected to SAH than in the sham group. Within the SAH group, the cumulative number of microthrombi was higher on day 2 than on days 1 and 3, in concordance with a previously published study [6]. The results are shown in Fig. 1A.

Effect of ADAMTS-13 on the occurrence of microthrombosis and CVS

Immunohistochemical examination showed the presence of microthrombi in both the cortical region and the hippocampal region at day 2 after SAH (Fig. 3A). The cumulative numbers of microthrombi were significantly higher in the SAH, vehicle and ADAMTS-13 groups than in the sham group ($P < 0.05$). Administration of ADAMTS-13 significantly reduced the numbers of microthrombi in both the cortex and the hippocampus, as compared with SAH and vehicle ($P < 0.01$). However, the number remained significantly higher than in the sham group in the cortex ($P < 0.05$). The results are shown in Fig. 3B. Signs of CVS could be seen in mice subjected to SAH (Fig. 3C). The lumen diameter/wall thickness ratio was significantly higher in the sham group than in the SAH, vehicle and ADAMTS-13 groups ($P < 0.05$). Administration of ADAMTS-13 did not resolve the morphologic signs of CVS (Fig. 3C,D).

Effect of ADAMTS-13 on inflammation

Immunohistochemical examination showed translocation of HMGB1 from the NeuN to the cytoplasm in the SAH and vehicle groups. In the ADAMTS-13 group, translocation was less often observed, indicating amelioration of neuronal inflammation (Fig. 4). Mice developing hemorrhage showed higher cerebral HMGB1 levels than those in the sham group. In the ADAMTS-13 group, the increase in cerebral HMGB1 was less pronounced. However, only a statistical tendency could be found ($P = 0.10$). Cerebral IL-6 levels showed a similar pattern, but without statistical significance ($P = 0.29$). Systemic IL-6 levels were significantly increased in mice subjected to SAH ($P < 0.05$). However, systemic IL-6 levels in mice

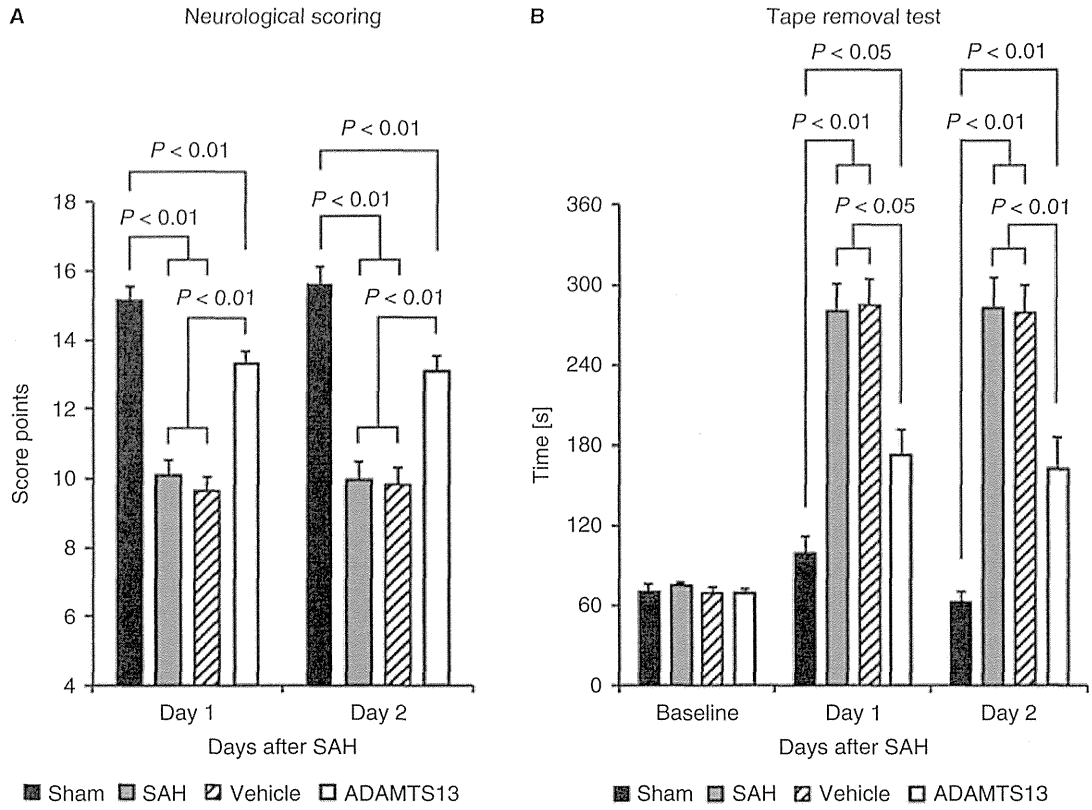

Fig. 2. Neurological scorings. Neurologic assessment by Garcia score (A) and tape removal test (B) shown as bar graphs. All three groups subjected to subarachnoid hemorrhage (SAH) had worse neurologic performance than the sham group ($P < 0.01$ and $P < 0.05$). Administration of ADAMTS-13 improved neurologic performance as compared with mice in the SAH and vehicle groups (both $P < 0.01$). However, performance remained significantly worse than that in sham mice (both $P < 0.01$).

subjected to SAH did not differ. Systemic HMGB levels were higher in the SAH and vehicle groups than in the sham and ADAMTS-13 groups, but the difference was not significant ($P = 0.27$). Systemic levels of ADAMTS-13 were under the mean minimal detection limit in the sham, SAH and vehicle groups, whereas the presence of rhADAMTS-13 could be verified in the ADAMTS-13 group. No ADAMTS13 was detected in brain supernatants. The results are shown in Table 1.

Effect of ADAMTS-13 on neuronal injury

In the SAH and vehicle groups, neuronal cell death and degeneration were detected to the same extent in the parietal and temporal cortex as in the hippocampal regions by FJC staining. Administration of ADAMTS-13 resulted in a decreased number of FJC-positive cells (Fig. 5). TUNEL-positive cells, which appeared to be neurons, were found in the outer two layers of the temporal and parietal cortex of SAH and vehicle mice. ADAMTS-13 administration reduced the number of apoptotic cells (Fig. 5).

Discussion

In the current study, important pathologies could be reproduced. These include CVS, microthrombosis, neuronal injury, and neurologic deficits. Administration of rhADAMTS-13 after SAH produced significant amelioration of microthrombosis and improvement in neurologic performance. The numbers of apoptotic and degenerative neurons were decreased. A tendency for there to be decreased neuronal inflammation was noted. ADAMTS-13 administration did not have any significant effect on CVS of the large arteries, and the degree of systemic inflammation was not significantly affected. Increased rhADAMTS-13 levels on day 2 confirmed its successful administration, in view of its reported circulatory half-life of 48–72 h [26]. ADAMTS-13 prevents microvascular thrombosis by cleaving ultralarge (UL) VWF multimers, which are extremely thrombogenic, into smaller, less active, multimers. Deficiency of ADAMTS-13 causes thrombotic thrombocytopenic purpura, which is associated with the occurrence of microthrombosis followed by ischemic complications, such as cerebral infarction [27]. The VWF–ADAMTS-13 axis modulates inflammation, and has recently been described as a 'new link between thrombosis and inflammation' [15]. ADAMTS-13 deficiency increases VWF-dependent leukocyte rolling, adhesion and extravasation in experimental inflammation [15,18]. In experimental ischemic stroke, a couple of studies have shown that ADAMTS-13 plays a major role in determining the extent of cerebral injury:

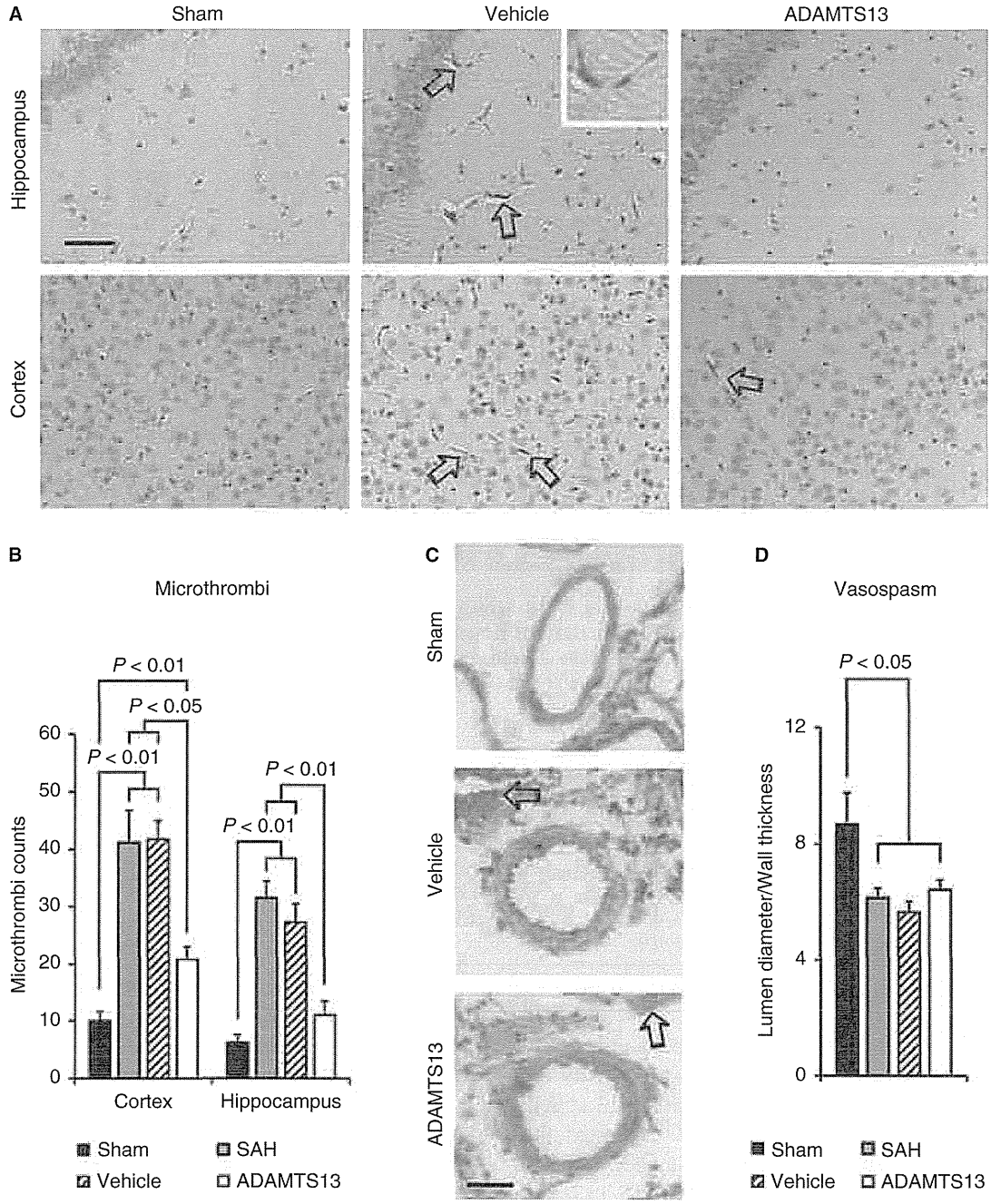

Fig. 3. Microthrombosis and morphologic vasospasm. (A) Representative images from histochemical visualization of microthrombi (arrows). Scale bar: 100 μm. (B) Numbers of microthrombi shown as bar graphs. Administration of ADAMTS-13 significantly decreased the number of microthrombi (arrow). (C) Representative images of the distal internal carotid artery, showing morphologic signs of cerebral vasospasm in mice of the vehicle and ADAMTS-13 groups. Note the presence of subarachnoid blood (arrows). Scale bar: 50 μm. (D) Bar graphs showing a significantly higher lumen diameter/wall thickness ratio in all three groups subjected to subarachnoid hemorrhage (SAH) than in the sham group.

ADAMTS-13 deficiency resulted in neurologic worsening [16,17,19], increased infarct size [16–19], increased leukocyte infiltration [16,18,19], and increased the expression of proinflammatory cytokines, such as IL-6 and HMGB1 [17–19]. Importantly, infusion of rhADAMTS-13 improved neurologic performance, reduced infarct size and neither prolonged bleeding time nor promoted brain hemorrhage [19]. Further experiments using ADAMTS-13-deficient mice with prior immunodepletion of neutrophils and VWF-deficient mice demonstrated a causal role for inflammation in the enhanced brain injury that occurred in the presence of ADAMTS-13 deficiency [18]. As mentioned above, almost no reports are available on the role of ADAMTS-13 in neuronal injury after SAH, except for one clinical observational study. In the latter, patients with DCI had a more marked decrease in ADAMTS-13 activity, and a more profound increase in VWF activity. The results suggest that microthrombosis

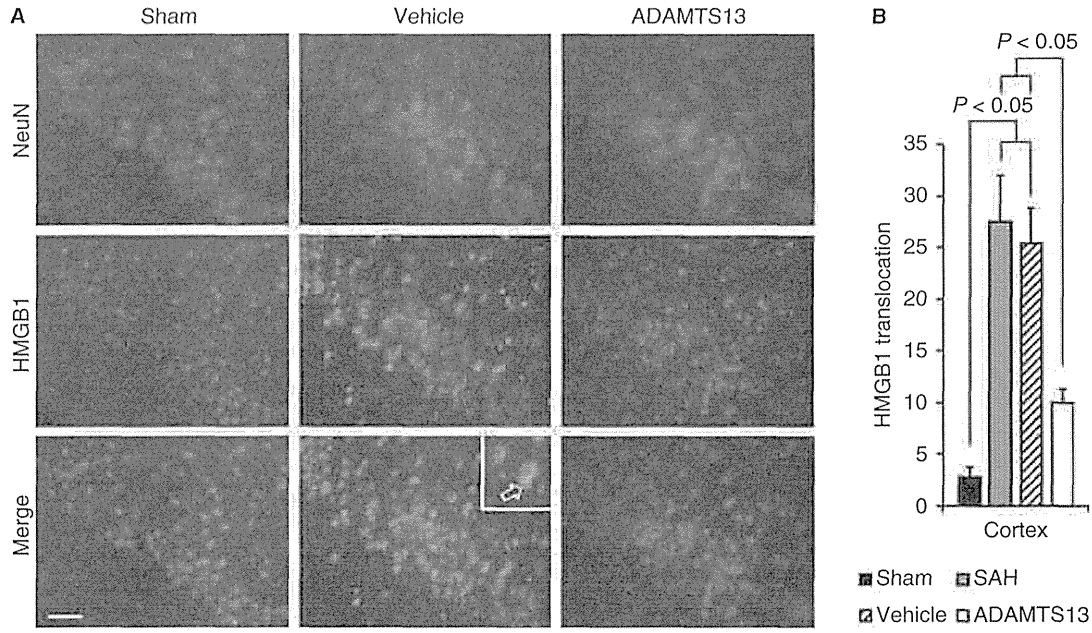

Fig. 4. Translocation of HMGB1. (A) Representative images of high-mobility group protein box 1 (HMGB1) (green)/neuronal nuclei (NeuN) (red) double staining of the ipsilateral cortex. In sham mice, HMGB1 remained predominantly nuclear (left column). Subarachnoid hemorrhage (SAH) induced HMGB1 translocation from the nucleus (middle column, arrow, higher magnification). There was less translocation of HMGB1 in mice of the ADAMTS-13 group (right column). Scale bar: 100 μm. (B) Quantitative results shown as a bar graph.

Table 1 Inflammatory parameter and ADAMTS-13 levels

	Sham	SAH	Vehicle	ADAMTS13	*P*-value
Cerebral HMGB1 (ng mg^{-1})	637.5 ± 150.1	1069.7 ± 128.6	1030.7 ± 168.8	822.4 ± 134.9	0.10
Cerebral IL-6 (fg mg^{-1})	255.5 ± 86.6	525.8 ± 145.2	576.6 ± 200.4	393.0 ± 154.3	0.29
Systemic HMGB1 (ng mL^{-1})	6.3 ± 0.5	9.5 ± 2.0	8.5 ± 2.6	5.5 ± 0.5	0.27
Systemic IL-6 (pg mL^{-1})	6.3 ± 0.9	40.8 ± 9.4	36.2 ± 7.4	33.2 ± 9.7	< 0.05
Systemic ADAMTS-13 (ng mL^{-1})	0.78 ± 0.0	0.78 ± 0.0	0.78 ± 0.0	46.0 ± 20.1	< 0.01

HMGB, high-mobility group protein box; IL, interleukin; SAH, subarachnoid hemorrhage.

plays a role in the pathogenesis of neuronal injury after SAH, because of decreased ADAMTS-13 activity [9]. However, the mechanism by which ADAMTS-13 activity is decreased remains unclear. The author suggested that the activity might be suppressed by IL-6, because IL-6 inhibits the cleavage of UL VWF by ADAMTS-13 [14].

The results of the current study provide further evidence supporting the involvement of ADAMTS-13 in the pathogenesis of microthrombosis and neuronal injury after SAH. Taking the current knowledge – including the present results – into account, the following mechanism might be proposed. An SAH event triggers both a systemic and a compartmental (within the central nervous system) inflammatory response, with cytokine release. This inflammatory response decreases ADAMTS-13 activity. As a consequence, platelet thrombi form, and, without negative feedback regulation by proteolysis of VWF by ADAMTS-13, progressive thrombus growth may narrow and ultimately plug microvascular lumina. The platelet–VWF string directly supports leukocyte transmigration, which results in further amplification and propagation of inflammation, initiating a vicious circle, ultimately leading to neuronal injury.

A meta-analysis showed a trend towards better outcome in patients treated with antiplatelet agents, possibly because of a reduction in the development of DCI. However, the results were not significant, with the authors noting that the hemorrhagic complications of these drugs possibly counterbalanced the beneficial effects [28]. Treatment with antiplatelet agents to prevent DCI or poor outcome is currently not recommended, and more specific strategies are required. In this context ADAMTS-13 might be of interest, as it might not lead to a higher risk of bleeding, on the basis of the current results. However, one has to consider that *in vivo* bleeding time is highly variable, and does not correlate well with a hemorrhagic tendency in daily clinical life. Therefore, a normal *in vivo* bleeding time does not rule out an increase in bleeding caused by ADAMTS-13.

The main limitation of the current study is the use of associated data showing ADAMTS-13 having an effect on numerous parameters. Another limitation is the fact

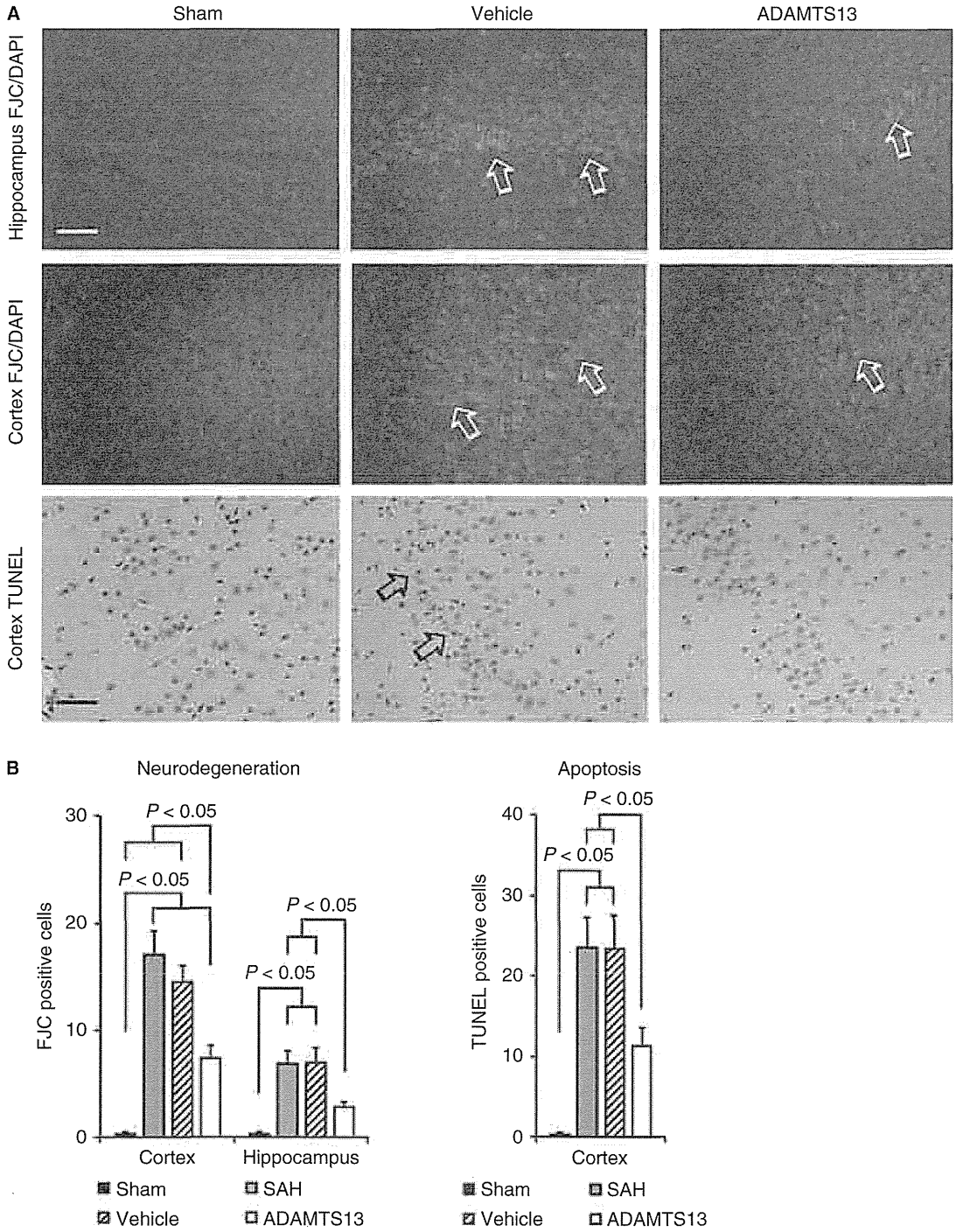

Fig. 5. Neurodegeneration and apoptosis. (A) Representative images of Fluoro-Jade C (FJC) (green)/4′,6-diamidino-2-phenylindole (DAPI) (blue) double staining (upper two rows) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (bottom row). Degenerative neurons were present after subarachnoid hemorrhage (SAH) in both the hippocampus and the cortex (middle column, upper two rows, arrows). TUNEL-positive neurons (brown) were present after SAH in the cortex (middle column, bottom row, arrows). Fewer FJC-positive and TUNEL-positive neurons were observed in mice of the ADAMTS-13 group (right column, arrows). Scale bar: 100 μm. (B) Quantification by counting, shown as bar graphs.

that 10 mice were excluded for a variety of different, but legitimate, reasons. This might have biased the results. Furthermore, the effect of ADAMTS-13 administration on the inflammatory response after SAH remains difficult to interpret. The IL-6 and HMGB1 levels within the brain showed a statistical tendency, i.e. fell short of significance, indicating amelioration of neuroinflammation. This might be explained as follows: (i) cytokine concentrations were measured in the parenchyma and not in the subarachnoid space, i.e. the cerebrospinal fluid (CSF) compartment, where the inflammatory response is suggested to be the strongest – accordingly, IL-6 levels in the parenchyma were

less elevated than in the CSF in patients with SAH [11]; and (ii) ADAMTS-13 leads to amelioration of reactive inflammation, but might not suppress the initial inflammatory response caused by the hemorrhage. Questions remain as to whether the decrease in neuroinflammation is a direct effect of ADAMTS-13 and whether inflammation leads directly to neuronal injury. Taking these findings together, it remains speculative as to how the drug actually works, as with any pharmacologic study. Subsequent studies using ADAMTS-13-deficient mice may provide additional information.

In conclusion, the current study showed that systemic application of ADAMTS-13 resulted in significant amelioration of microthrombosis and an improvement in neurologic performance. Administration of ADAMTS-13 reduced the numbers of apoptotic and degenerative neurons. A tendency for there to be decreased neuronal inflammation was shown. ADAMTS-13 might carry a relatively low risk of hemorrhagic complications. Therefore, we propose that ADAMTS-13 may offer a new option for the prophylaxis of cerebral injury after SAH.

Addendum

C. Muroi designed the study, performed most of the experiments, analyzed the results, and prepared the manuscript. K. Mishima, Y. Fujimura, J. Fandino, E. Keller, K. Iwasaki, and M. Fujiwara provided direction throughout the work, provided the overall experimental design, and edited the manuscript. M. Fujioka and T. Nakano helped to perform the animal experiments, in particular the neuronal assessments. M. Fujioka, K. Mishima, and K. Irie provided direction in histologic staining procedures and ELISA measurements.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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