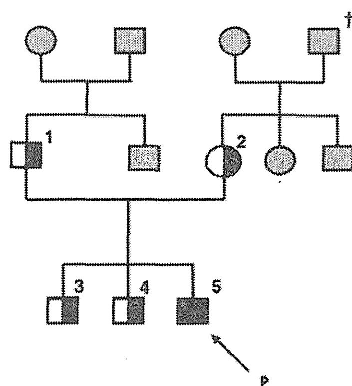


	ADAMTS13 activity (%)	ADAMTS13 antigen (%)		ADAMTS13 gene	
	ELISA	ELISA	WB	p.Arg398	p.Gln723
CC-1	46	24	36	R/R	Q/K
CC-2	30	36	34	R/C	Q/Q
CC-3	40	23	38	R/C	Q/Q
CC-4	23	36	38	R/R	Q/K
CC-5	<0.5	<0.1	<3	R/C	Q/K

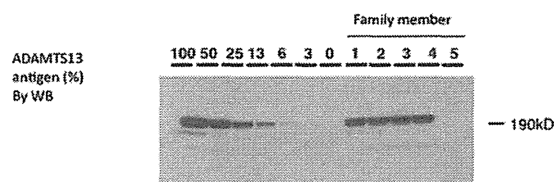


Fig. 2 Pedigree and ADAMTS13 analysis of USS-CC5 and his family. The propositus (denoted as P) USS-CC5 is the third of three offspring of non-consanguineous parents. His parents and two brothers are asymptomatic and in good health. *Squares* and *circles* indicate males and females, respectively and *shaded symbols* show individuals who were not examined. The *cross* denotes a deceased individual. *Half-black symbols* show asymptomatic carriers. ADAMTS13 activities were determined using activity ELISAs and ADAMTS13 levels were measured using antigen ELISAs and Western blotting. Results are shown as percentages of normal values. Identified mutations in ADAMTS13 are depicted as *one-letter amino-acid abbreviations* (*right upper panel*). Western blot analyses of plasma ADAMTS13 antigen in the patient's family members are shown in the *right lower panel*

carriers of one of the two mutations. No single nucleotide polymorphisms causing missense mutations were identified in this patient or her family members.

Plasma levels of ADAMTS13 antigen based on antigen ELISAs were less than 0.1 % of normal values in the patient, whereas her father, mother and older brother showed antigen levels that were 48, 45 and 54 % of normal values, respectively. Further, plasma levels of ADAMTS13 antigen on Western blots were shown to be <3 % of normal values in the patient, and 47, 45 and 54 of normal values in her father, mother and older brother, respectively. Thus, the p.Q449X/p.Q1374Sfs mutations may have resulted in proteins that were not secreted into plasma (Fig. 4).

VWF multimer analysis of the patients with USS during remission

Despite the common features in the two USS cases, including a lack of plasma ADAMTS13 activity and severe jaundice as newborns, the subsequent clinical courses of disease markedly differed. To examine potential proteolytic mechanisms other than ADAMTS13, we performed VWF multimer analysis using plasma samples obtained when the patients were in remission. VWF multimer bands from the patients' plasma samples were each represented by a single symmetrical band, which differed from the triplet structure of bands observed with normal plasma (Fig. 5). Moreover, a predominance of high-molecular weight VWF multimers was noted in the plasma samples from both patients, suggesting that their plasma VWF multimers had not been subjected to any alternative proteolytic modifications.

Discussion

Analysis of the natural history of USS in 43 Japanese patients found that 42 % (18/43) had an episode of severe jaundice as newborns that required exchange blood transfusion, suggesting that jaundice is the earliest clinical sign of bouts of TTP in patients with USS [4]. Although the underlying mechanism has not been fully elucidated, hypoxia can induce the release of UL-VWFMs from vascular endothelial cells by upregulating the production of such inflammatory cytokines as interleukin-6, interleukin-8 and tumor necrotizing factor α [18, 19]. Moreover, newborns can be subjected to hypoxic conditions during prolonged labor, which may induce the release of UL-VWFMs from vascular endothelial cells and cause TTP bouts.

Newborns with USS often show severe jaundice with a negative Coombs test that requires exchange blood transfusion as well as repeated childhood episodes of thrombocytopenia that resolve in response to FFP infusions. Thus, patients with newborn-onset USS have been categorized as having the early-onset phenotype, and are treated throughout their lives with occasional or periodic plasma infusions

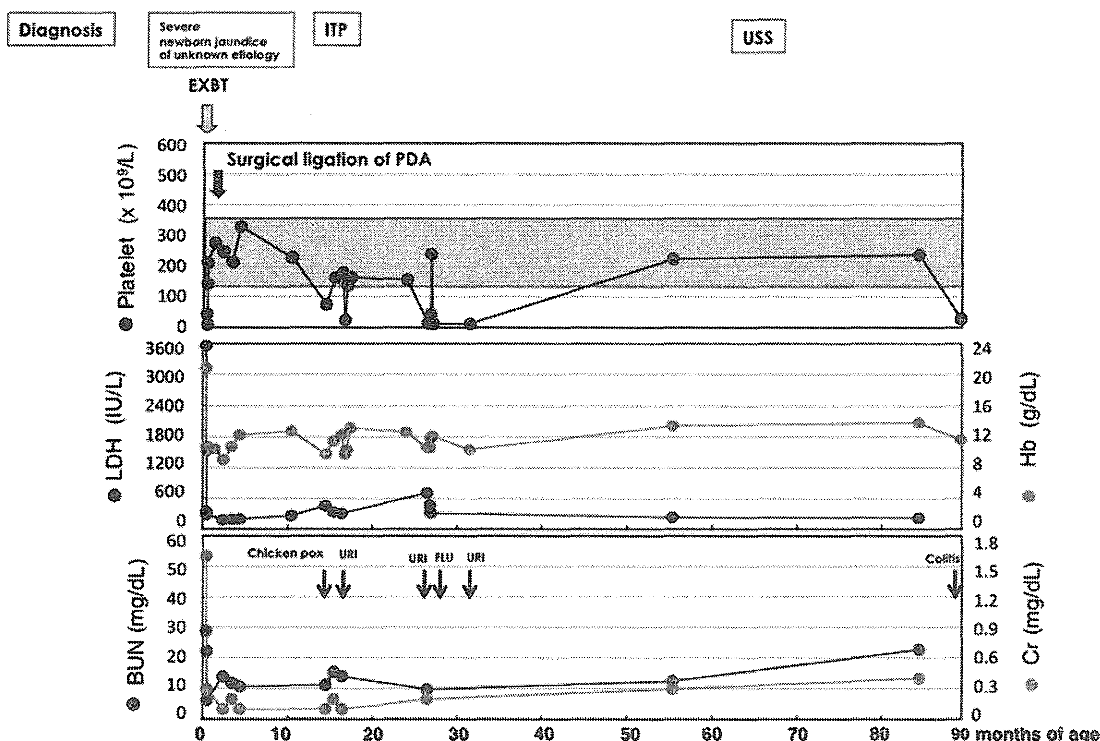
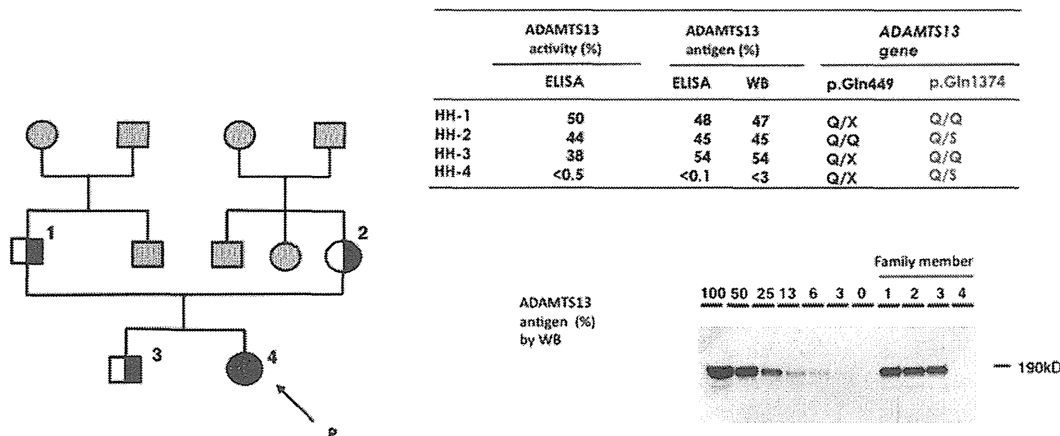


Fig. 3 Laboratory data and the clinical course of USS in patient HH4. In 2003, the proposita was born and transferred to Nihonkai General Hospital because she developed severe jaundice, cyanosis, and thrombocytopenia. After admission, she received exchange blood transfusions and platelet transfusions. At 14 months of age, she received a misdiagnosis of hemophagocytic syndrome associated with a viral infection. Since that time, she developed several episodes of petechiae with fever due to upper respiratory infection and influenza A. After she turned 3 years old, the incidence of petechiae decreased together with less frequent febrile episodes. In 2008, she received a diagnosis of USS based on severely deficient ADAMTS13 activity (<0.5 % of normal values) and no detected ADAMTS13 inhibitor. Interestingly, because her clinical signs and symptoms were mild, she did not receive FFP infusions. She is now 8 years old and has not developed major complications, such as renal insufficiency or neurologic abnormalities. *BUN* blood urea nitrogen, *Cr* creatinine, *Hb* hemoglobin, *PDA* patent ductus arteriosus, *EXBT* exchange blood transfusion, *ITP* idiopathic thrombocytopenic purpura, *USS* Upshaw–Schulman syndrome, *FLU* influenza A infection, *URI* upper respiratory infection

either prophylactically or in response to bouts of TTP [20]. On the other hand, patients categorized as having the late-onset phenotype develop the first episode of TTP after childhood. Patients with USS, however, occasionally show isolated mild thrombocytopenia during childhood, and, therefore, are overlooked or received a misdiagnosis of idiopathic thrombocytopenic purpura. These results indicate that USS with the late-onset phenotype may result from several different pathogenic processes. Generally, however, bouts of TTP in patients with USS are thought to be induced by various triggers, including pregnancy, upregulated cytokine expression during severe infections and excessive alcohol intake, among others [4].

Camilleri et al. [21] reported that a p.R1060W missense mutation in the ADAMTS13 gene was associated with USS with an ethnically specific late-onset phenotype; a Caucasian patient who was homozygous for the mutation showed plasma ADAMTS13 activity levels that were <5 % of normal values. Recently, we reported that a Japanese patient with USS was homozygous for a p.C1024R missense mutation, resulting in an Asian-specific late-onset phenotype. The patient received a correct diagnosis of USS at 77 years old and was shown to have moderately reduced plasma ADAMTS13 activity levels (2.4–3.4 % of normal values) using a sensitive chromogenic assay [13]. In vitro expression studies using HeLa cells transfected with plasmid encoding the p.C1024R mutant revealed that mutant protein was secreted into culture medium but at a significantly lower level than the wild-type protein. Further, the activity of the secreted p.C1024R mutant protein was three times that of the wild-type protein, indicating that p.C1024R was a gain-of-function mutation [22]. These data suggested that the plasma levels of ADAMTS13 activity in this patient prevented TTP during his childhood unless a strong stimulus-induced UL-VWFM release. Even in normal individuals, however, plasma levels of ADAMTS13 gradually decrease with age, in contrast to increasing plasma VWF levels [23]. Thus, in patients with USS, an

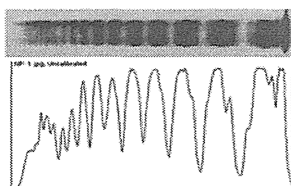
USS
Family HH



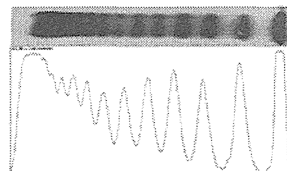
	ADAMTS13 activity (%)	ADAMTS13 antigen (%)		ADAMTS13 gene	
	ELISA	ELISA	WB	p.Gln449	p.Gln1374
HH-1	50	48	47	Q/X	Q/Q
HH-2	44	45	45	Q/Q	Q/S
HH-3	38	54	54	Q/X	Q/Q
HH-4	<0.5	<0.1	<3	Q/X	Q/S

Fig. 4 Pedigree and ADAMTS13 analysis of USS-HH4 and her family. The proposita (denoted as P) USS-HH4 is the second of two offspring of non-consanguineous parents. Her parents and brother are asymptomatic carriers. ADAMTS13 activities were determined using activity ELISAs and ADAMTS13 antigen levels were measured using antigen ELISAs and Western blotting. Results are shown as percentages of normal values. Identified mutations in ADAMTS13 are depicted as *one-letter amino-acid abbreviations* (*right upper panel*). Western blot analyses of plasma ADAMTS13 antigen in the patient's family members are shown in the *right lower panel*

Normal plasma



USS-CC5
Genotype (p.R398C/p.Q723K)



USS-HH4
Genotype (p.Q449X/p.Q1374Sfs)

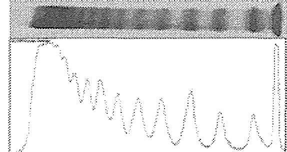


Fig. 5 Plasma VWF multimers from two patients with USS during remission phases. We analyzed VWF multimers in plasma samples obtained when the patients were in remission. Compared with the triplet bands observed in normal plasma (*upper panel*), VWF multimers in the patients' plasma samples consisted of single symmetrical bands (*middle panel* USS-CC5, *bottom panel* USS-HH4). Further, the patients' plasma samples showed high percentages of high-molecular-weight VWF multimers

imbalance in increased substrate levels (highly multimeric VWF) and the reduced ADAMTS13 enzymatic activity generates prothrombotic conditions, leading to more frequent TTP. Thus, mild or moderate deficiency of ADAMTS13 activity in patients with USS may contribute to conditions that allow the late-onset phenotype to develop.

Here, we have described two patients with USS (USS-CC5 and USS-HH4) who both had severe jaundice as newborns, requiring exchange blood transfusions. The subsequent clinical courses of disease in these patients, however, differed; USS-CC5 experienced chronic thrombocytopenia unless he was treated with prophylactic FFP infusions, whereas USS-HH4 developed transient thrombocytopenia only when she had an infection. USS-HH4 is now 8 years of age and has never been treated with FFP infusions. Notably, severe neonatal jaundice due to feto-maternal ABO incompatibility in USS-CC5—uncovered via an indirect positive Coombs test—masked a correct diagnosis of USS. ADAMTS13 gene analyses revealed that USS-CC5 and USS-HH4 were compound heterozygotes of p.R398C/p.Q723K and p.Q449X/p.Q1374Sfs, respectively. Among these mutations, p.Q449X was found in USS patients [16], but p.R398C, p.Q723K, and p.Q1374Sfs have not been previously reported in USS patient. Of these, p.Q723K alone was found as a rare nonsynonymous mutation in 128 normal individuals [6]. Both patients in this study showed plasma ADAMTS13 activity levels that were less than 0.5 % of normal, and ADAMTS13 antigen levels that were less than 0.1 % of normal. Therefore, it was suspected that p.R398C, p.Q723K, and p.Q1374Sfs were not secreted in plasma. Thus, the pathogenesis of the milder clinical presentation of USS-HH4 probably did not reflect the same mechanisms that contributed to the late-onset phenotype observed in the patient who was homozygous for the p.C1024R missense mutation. To determine whether UL-VWFMs were modulated by proteases other than ADAMTS13, we performed VWF multimer analysis

using patient plasma samples obtained during remission; each VWF multimer band in plasma from the two patients was represented by a single symmetrical band, rather than the triplet structure observed in normal plasma, indicating that VWF multimers from the patients had not been subjected to alternative proteolytic modifications.

In patient USS-HH4, the mechanism regulating the interactions between platelets and hyperactive UL-VWFMs without induction of overt TTP is presently unknown. Because we did not observe alternative proteolytic modifications of VWFMs in the patients with USS, we are now interested in potential fluid-phase regulatory mechanisms during high shear stress-induced platelet aggregation (H-SIPA). H-SIPA is dependent on VWFM size, and is inhibited by compounds that disrupt interactions in the VWF–platelet GPIb axis and subsequent platelet activation. Platelet activation during H-SIPA is mediated by endogenous ADP released from platelet δ-granules in microenvironments; therefore, ADP scavengers block H-SIPA without modifying VWFM structures. Indeed, we previously reported that human placental or vascular endothelial cell ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) efficiently inhibited H-SIPA [24]. Further, we also indicated the presence of soluble E-NTPDase in plasma, which is cleaved from the cell surface or generated by alternative splicing [25]. Thus, studies focusing on potential relationships between E-NTDase and H-SIPA would be of great interest, and may help to elucidate the pathogenesis of TTP in patients with USS.

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Conflict of interest Y. Fujimura is a member of clinical advisory boards for Baxter BioScience.

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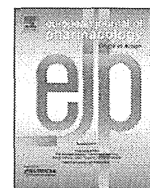

Cardiovascular pharmacology

Cilostazol down-regulates the height of mural platelet thrombi formed under a high-shear rate flow in the absence of ADAMTS13 activity

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ABSTRACT

Cilostazol is an anti-platelet drug that reversibly inhibits phosphodiesterase III (PDE-III), which is ubiquitously expressed in platelets and various tissues. PDE-III converts cyclic adenosine monophosphate (cAMP) to 5'-AMP and up-regulates the intracellular concentration of cAMP, a potent inhibitor of platelet aggregation. Unlike other anti-platelet drugs, cilostazol is unique because patients receiving this drug do not have a significantly prolonged bleeding time, but the reasons for this difference are still unknown. In this study, we have examined how cilostazol inhibits platelet thrombus formation using anti-coagulated normal whole blood in which the platelets were labeled with a fluorescent dye in comparison with the anti-GPIIb/IIIa agent, tirofiban. We used an *in vitro* assay to examine mural platelet thrombus growth on a collagen surface under a high-shear rate flow in the absence of ADAMTS13 activity. These experimental conditions mimic the blood flow in patients with thrombotic thrombocytopenic purpura. Using this model, we clearly determined that cilostazol down-regulates the height of mural platelet thrombi formed on a collagen surface in a dose-dependent manner, without affecting the surface coverage. The concentration of cilostazol used in this study was relatively high (60–120 μM) compared to clinically relevant concentrations (1–3 μM), which may be due to the *in vivo* synergistic effects of PDE-III present in other tissues aside from platelets. Cilostazol does not affect the initial formation of platelet thrombi, but does inhibit the height of thrombi. These results showed a sharp contrast to tirofiban, and address why cilostazol does not significantly prolong bleeding time, despite its strong anti-platelet activity.

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1. Introduction

Cilostazol is a reversible inhibitor of phosphodiesterase III (PDE-III), which is ubiquitously expressed in platelets, vascular endothelial cells and smooth muscle cells. PDE-III up-regulates the concentration of cyclic adenosine monophosphate (cAMP), a potent inhibitor of platelet aggregation. Thus, cilostazol is currently used as an anti-platelet drug and is indicated for intermittent claudication with peripheral arterial occlusion and for ischemic stroke (Thompson et al., 2002; Uchiyama et al., 2009). Earlier studies compared cilostazol to several other common anti-platelet drugs, including aspirin and thienopyridine derivatives such as ticlopidine and clopidogrel, and showed that agonist-induced platelet aggregation was clearly inhibited in patients in a dose-dependent manner (Ikeda et al., 1987). Furthermore, high-shear stress-induced platelet aggregation measured by a cone–plate type aggregometer was inhibited in patients who received ticlopidine, clopidogrel or cilostazol, but was not abolished in patients who were administered aspirin (Minami et al., 1997; Moake et al., 1988; Tanigawa et al., 2000). These anti-platelet drugs had a striking difference in their effects on prolonging the bleeding time, which is an adverse effect of these drugs. Tamai et al. (1999) reported that cilostazol, unlike aspirin or ticlopidine, did not significantly prolong the bleeding time using a computer-assisted quantitative bleeding time apparatus. However, the mechanism by which bleeding time is not prolonged in patients receiving cilostazol has not been addressed.

We recently established an *in vitro* assay that examines mural platelet thrombus growth on a collagen surface under a high-shear rate flow in the absence of ADAMTS13 (a disintegrin-like metalloproteinase with thrombospondin type 1 motifs 13) activity (ADAMTS13:AC; Shida et al., 2008).

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ADAMTS13 is an enzyme that is mainly produced in the liver and specially cleaves the von Willebrand factor multimer (VWFM) at the Tyr1605–Met1606 bond in the subunit to decrease the VWFM size. In the absence of ADAMTS13:AC, unusually large VWFM (UL-VWFM) is produced exclusively in vascular endothelial cells and then accumulates in the circulation and induces generalized microvascular thrombosis, termed thrombotic thrombocytopenic purpura. Thus, our experimental device mimics *in vitro* blood flow in thrombotic thrombocytopenic purpura patients by using the murine anti-ADAMTS13:AC-neutralizing monoclonal antibody (mAb) A10 (Uemura et al., 2005).

Here, we demonstrate that cilostazol down-regulates the height of mural platelet thrombi formed on a collagen surface under a high-shear rate flow in the absence of ADAMTS13:AC without affecting the surface coverage. Our results potentially explain why cilostazol does not prolong the bleeding time, even though it has potent anti-platelet activity.

2. Materials and methods

2.1. Blood

A total volume of 5 ml of whole blood was collected from 10 normal volunteers (5 females and 5 males, aged 20–40 years), mixed with the anti-coagulant compound argatroban (200 μM, final), and then incubated for 5 min at room temperature. This anti-coagulated normal whole blood was then incubated for 15 min with an ADAMTS13:AC-neutralizing monoclonal antibody (mAb), termed A10, at a final concentration of 50 μg IgG-$(Fab')_2$/ml, to decrease the plasma ADAMTS13:AC levels to less than 0.5% of normal levels, a plasma condition that is equivalent to inhibitory antibody-induced acquired thrombotic thrombocytopenic purpura. The A10-IgG-$(Fab')_2$ was prepared by digesting with pepsin and then purifying the protein according to the methods of Hamaguchi et al. (1979).

2.2. Anti-platelet drugs

Cilostazol powder was kindly provided by Ohtsuka Pharmaceutical Co. (Japan), and then dissolved in dimethylsulfoxide (DMSO) at a concentration of 120 mM. This stock solution was stable for 6 months at 4 °C. Just before each experiment, this stock was diluted with 20 mM hepes–saline buffer (HSB, pH 7.4). Tirofiban, an anti-GP IIb/IIIa agent (Aggrastat; Merck & Co., Inc., West Point, PA), was also used in this study.

2.3. Flow chamber experiments and confocal laser scanning microscopy

Thrombus generation on a type I collagen-coated (Sigma-Aldrich, Tokyo, Japan) glass surface was studied under a high-shear rate in a parallel plate flow chamber system as previously described (Tsuji et al., 1999). Briefly, glass coverslips coated with type I collagen were prepared and placed in a parallel plate flow chamber (rectangular type; flow path of 1.9 mm in width, 31 mm in length, and 0.1 mm in height). The chamber was assembled and mounted on a confocal laser scanning microscope (FV300; Olympus Co., Tokyo). Whole blood that was anti-coagulated with argatroban was incubated with the fluorescent dye DiOC6 (1 μM; Molecular Probes Inc., Eugene, Oregon) for 10 min at 37 °C to label the platelets, allowing the platelet–surface interactions to be visualized by confocal laser scanning microscopy (CLSM). The whole blood containing DiOC6-labeled platelets was aspirated through the chamber and across the collagen-coated coverslip using a syringe pump (Model CFV-3200, Nihon Kohden Co., Ltd., Tokyo) at a constant flow rate producing a wall shear rate of $1500\ s^{-1}$. The DiOC6 fluorescence corresponding to the platelets was examined at an excitation wavelength of 488 nm with a barrier filter at 500 nm. The fluorescence images were viewed at 1-μm intervals from the collagen surface to a height of 60 μm from the surface. The percentage of the area covered by adhering platelets (surface coverage) and each thrombus volume in a defined area ($211 \times 317\ mm^2$) were evaluated at the indicated time points during perfusion (3, 5, and 7 min) based on the CLSM fluorescence images.

The surface coverage of thrombi was calculated based on the confocal images at a 2-μm height from the collagen surface, and three-dimensional (3D) images were constructed using the CLSM image analyzing system based on successive horizontal slices.

2.4. Statistical analysis

Data are shown as mean values ± standard deviations (S.D.). The effects of increasing amount of thrombus surface coverage and volume on fluorescence intensities were analyzed using the Mann–Whitney *U*-test. The significance level of *P*-values was set at 5%. Calculations were performed using the ystat2004 software.

3. Results

3.1. Visualization of heightened mural platelet thrombi formed under a high-shear rate flow in the absence of ADAMTS13:AC and the reversal by cilostazol

Normal whole blood, anti-coagulated with argatroban, was perfused under a high-shear rate flow at $1500\ s^{-1}$ for 7 min in the presence or absence of the anti-ADAMTS13 mAb A10. In the absence of A10 (control), the 3D images of mural platelet thrombus formation on the collagen surface at 3, 5, and 7 min after perfusion showed small and low-height platelet thrombi (Fig. 1, top). However, in the presence of A10, large and high-height platelet thrombi were observed (Fig. 1, 2nd). Furthermore, the height phenomenon in the latter conditions was apparently reversed by pre-incubating whole blood with A10 and cilostazol (120 μM, final; Fig. 1, 3rd). As controls, we performed the same experiments using whole blood with A10 and tirofiban (1 μM, final), anti-GPIIb/IIIa agent. Tirofiban reduced both surface coverage and thrombus volume significantly (Fig. 1, bottom).

3.2. Time- and dose-dependent abilities of cilostazol to inhibit the height of platelet thrombi formed under a high-shear rate flow without affecting the surface coverage

The above-mentioned flow experiments were repeated 10 times per experiment using 10 different normal donors. These results are shown in Fig. 2, where the *x*-axis shows the perfusion time (min) and the *y*-axis indicates the mean value ± standard deviation (S.D.). Fig. 2 (top) depicts a time-dependent increase in the surface coverage in the absence of ADAMTS13:AC. It is noteworthy that the percentages of surface coverage with a fixed perfusion time did not significantly differ at any of the examined cilostazol concentrations.

However, unlike the surface coverage, the thrombus volume that was measured simultaneously in the above experiments was inhibited by cilostazol in a dose- and time-dependent manner, as shown in Fig. 2 (bottom). There were statistically significant differences at both 5 and 7 min, but not at 3 min, after perfusion. More precisely, the thrombus volume was significantly reduced at 5 min after perfusion by adding 120 μM cilostazol (final). Furthermore, there was a profound dose-dependent inhibition at 7 min

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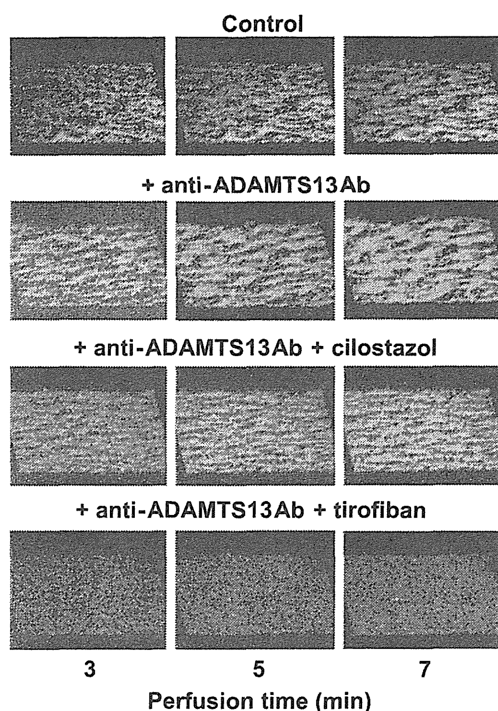

Fig. 1. Effects of cilostazol on mural thrombus generation on a collagen surface when whole blood from healthy volunteers that had been pre-incubated with the anti-ADAMTS13 mAb A10 was perfused under high shear rate flow conditions. Whole blood from healthy volunteers that contained DiOC6 (1 μM)-labeled platelets and had been anti-coagulated with argatroban was perfused over a type I collagen-coated glass surface under a high shear rate (1500 s^{-1}) with control mouse IgG (top column), the anti-ADAMTS13 antibody A10 (2nd column), A10 and cilostazol (120 μM; 3rd column), or A10 and tirofiban (1 μM; bottom column). Kinetic changes in the 3D images of thrombi (original magnification × 600) were constructed using the image analyzing system of the confocal laser scanning microscope. These images are based on successive horizontal slices at identical portions and are representative of 10-pair flow experiments using blood from 10 independent donors. As controls, we performed the same experiments using whole blood with A10 and tirofiban, anti-GPIIb/IIIa agent. Tirofiban reduced both surface coverage and thrombus volume significantly.

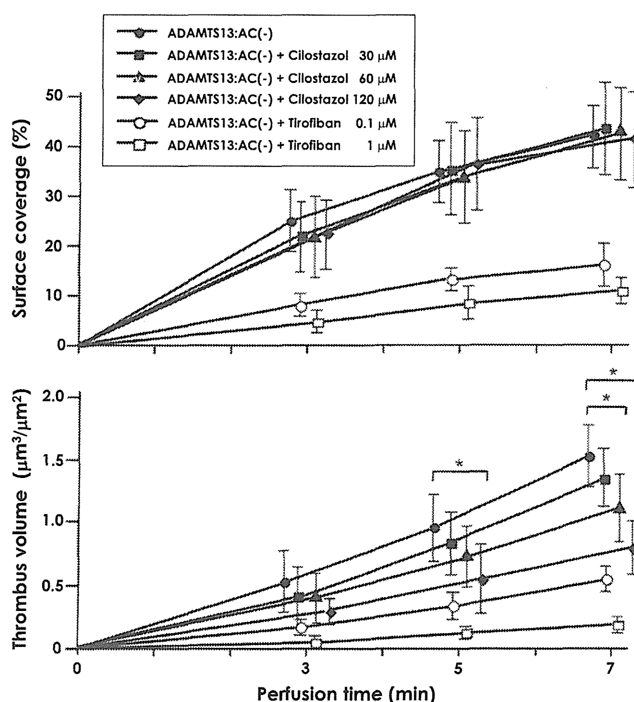

Fig. 2. Effects of cilostazol on kinetic changes in the thrombus surface coverage and volume generated when whole blood from healthy volunteers that was pre-incubated with the anti-ADAMTS13 mAb A10 was perfused under high shear rate conditions. Whole blood from a healthy volunteer was pre-incubated with the anti-ADAMTS13 mAb A10 (IgG-(Fab')$_2$; 50 μg/ml, f.c.) (ADAMTS13:AC < 0.5% of normal; ●) for 5 min at room temperature. After adding serial dilutions of cilostazol (30, 60, and 120 μM), these samples containing DiOC6 (1 μM)-labeled platelets were anti-coagulated with argatroban and then perfused for 7 min over a type I collagen-coated glass surface under a high shear rate (1500 s^{-1}). Each data point represents the mean (± S.D.) value of 10 areas (211 × 317 mm^2 each) that were randomly selected from each single perfusion of blood from 10 individual donors. The time-dependent increase in the surface coverage was not changed by adding the serial dilutions of cilostazol (top). However, the thrombus volume was significantly reduced by adding cilostazol at a final concentration of 60 μM for 7 min of perfusion and 120 μM for 5 and 7 min of perfusion in a dose-dependent manner (bottom). We performed the same experiment using tirofiban, an anti-GPIIb/IIIa agent, for comparison. Tirofiban significantly reduced not only surface coverage but also thrombus volume of thrombi at each time point in a dose-dependent manner. *: $P < 0.05$.

after adding cilostazol at final concentrations of 60 and 120 μM, but not 30 μM (Fig. 2, bottom). We performed the same experiment using tirofiban for comparison. In sharp contrast to cilostazol, tirofiban significantly reduced not only surface coverage but also thrombus volume at each time point in a dose-dependent manner. These results indicate that cilostazol does not affect the initial formation of platelet thrombi on the collagen surface, or more precisely the 2D attachment of platelet thrombi, but does inhibit the 3D growth and height of thrombi.

3.3. Comparison of surface coverage and thrombus volume at 7 min after perfusion

Figs. 3 and 4 show a quantitative measurement of the surface coverage and thrombus volume at 7 min after perfusion. As shown in Fig. 3, the surface coverage was virtually unchanged, regardless of the cilostazol concentrations (Fig. 3, middle). However, it was significantly reduced in a dose-dependent manner with tirofiban. The percent surface coverage at tirofiban concentrations 0.1 and 1 μM was 41.0% and 27.0% of the control, respectively (Fig. 3, right). On the other hand, the thrombus volume was clearly reduced in a dose-dependent manner with cilostazol. In fact, the percent thrombus volume that was formed at cilostazol concentrations of 30, 60, and 120 μM was 69.4%, 60.9%, and 47.4% of the control, respectively (Fig. 4, middle). The IC50 value of cilostazol for inhibition on thrombus volume was 83.3 ± 25.9 μM. Tirofiban also significantly reduced thrombus volume of thrombi at concentrations 0.1 and 1 μM and it was 37.7% and 10.9% of the control, respectively (Fig. 4, right).

4. Discussion

To address why the bleeding time is not prolonged in patients who receive cilostazol, we used a recently established *in vitro* perfusion chamber system that measures the mural platelet thrombi that form on a collagen surface under a high-shear rate flow in the absence of ADAMTS13:AC, which was neutralized using the unique anti-ADAMTS13 mAb A10. This experimental design is thought to partially mimic the blood flow in thrombotic thrombocytopenia purpura patients (Furlan and Lammle, 2001).

Using this assay, we clearly showed that cilostazol decreases the height of mural platelet thrombi formed on a collagen surface without affecting the surface coverage, which reflects the *in vitro* platelet adhesion process. These data indicate that cilostazol does not inhibit thrombus growth in the 2D horizontal direction, but inhibits growth in the 3D vertical direction. The fact that

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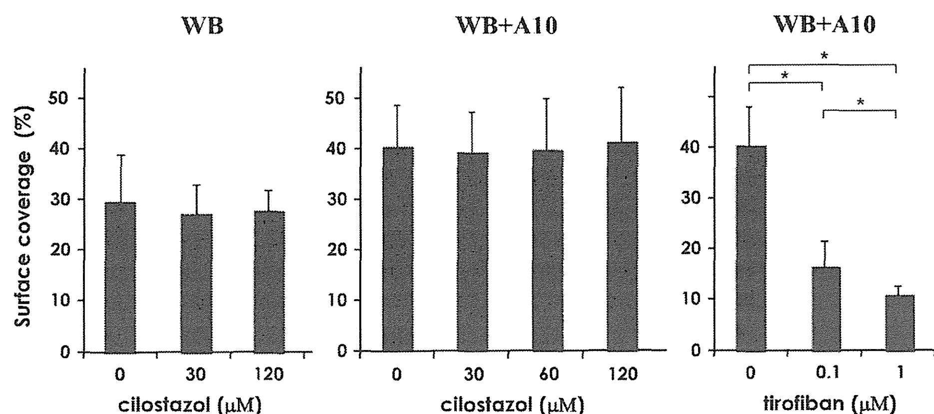

Fig. 3. Effects of cilostazol on the thrombus surface coverage at 7 min of perfusion under high shear stress in the absence of ADAMTS13:AC. Whole blood (WB) from healthy volunteers was pre-incubated with the anti-ADAMTS13 mAb A10 (ADAMTS13:AC < 0.5%) and then examined for changes in thrombi generated under high shear rate conditions (1500 s^{-1}) at 7 min of perfusion after adding serial dilutions of cilostazol (30, 60, and 120 μM). Each data point represents the mean (±S.D.) value in 50 areas (5 areas randomly selected from each single perfusion of blood from 10 individual donors). The surface coverage of thrombi was increased by addition of A10 (middle) compared to control group (left). However, the surface coverage of thrombi was not changed by cilostazol in each group, regardless of its concentrations. In sharp contrast to cilostazol, tirofiban significantly reduced surface coverage of thrombi in a dose-dependent manner. The percent surface coverage at tirofiban concentrations of 0.1 and 1 μM was 41.0% and 27.0% of the control, respectively (right). *: $P < 0.05$.

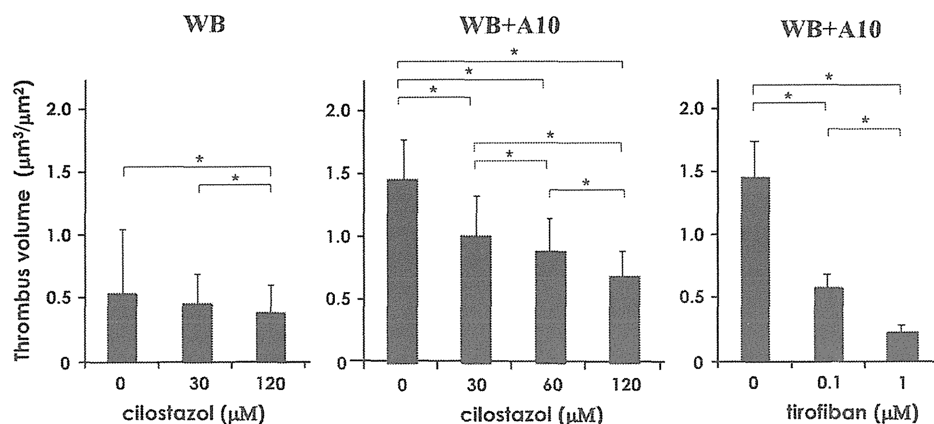

Fig. 4. Effects of cilostazol on the thrombus volume at 7 min of perfusion under high shear stress in the absence of ADAMTS13:AC. Quantitative measurements of the thrombus volume 7 min after perfusion were performed. In marked contrast to surface coverage, thrombus volume was remarkably increased by addition of A10, and this enhancement was significantly reduced by cilostazol in a dose dependent manner using whole blood (WB). The percentages of the formed thrombus volume after adding cilostazol (30, 60, and 120 μM) compared to the untreated sample were 69.4%, 60.9% and 47.4%, respectively (middle). On the other hand, this reducing effect induced by cilostazol in thrombus formation was only observed at a concentration of 120 μM, and to a lesser extent in the control group (left). Tirofiban also significantly reduced thrombus volume of thrombi at concentrations of 0.1 and 1 μM and it was 37.7% and 10.9% of the control, respectively (right). *: $P < 0.05$.

cilostazol did not disrupt the platelet surface coverage in our assay may partially indicate that the initial adhesion of platelets to exposed subendothelial matrices on an injured vessel wall *in vivo* is not significantly impacted by cilostazol treatment. On the other hand, tirofiban is a strong anti-platelet drug which prolongs the bleeding time, and one of its major adverse events is bleeding (The RESTORE Investigators, 1997). In 2004, Goto et al. (2004) clearly demonstrated that anti-GPIIb/IIIa agents, including tirofiban, significantly reduced both the volume and surface coverage of thrombi under high-shear rate flow condition using the same experimental equipment of this study. Tirofiban significantly reduced not only thrombus volume but also surface coverage of thrombi, suggesting that reduced surface coverage might closely relate to bleeding tendency *in vivo*. Furthermore, this might explain why *in vivo* the bleeding time is not prolonged in cilostazol-treated patients. However, it is noteworthy that the concentration of cilostazol used in this study was significantly higher (60–120 μM) than therapeutically relevant concentrations (1–3 μM). PDE-III is a ubiquitous enzyme that is expressed in platelets, vascular endothelial cells and smooth muscle cells and apparently up-regulates the anti-thrombogenic network consisting of platelets and vessel walls. The experimental device used in this study does not include vessel wall components except for collagen. Indeed, it has been noted that cilostazol simulates prostacyclin release from vascular endothelial cells, which accelerates the conversion of adenosine triphosphate (ATP) to cAMP. Thus, it is assumed that both the *in vivo* up-regulation of cAMP by prostacyclin stimulation and the down-regulation of 5'-AMP by inhibiting PDE-III synergistically increase cAMP and potently inhibit platelet thrombus formation. Consistent with our results, Minami et al. (1997) reported that cilostazol had to be used at much higher concentrations than clinical concentrations in order to inhibit high-shear stress-induced platelet aggregation in an *in vitro* study with a cone-plate type aggregometer.

On the other hand, plasma exchange (PE) with a high dose of prednisolone is the first-line therapeutic approach for acquired autoantibody-induced thrombotic thrombocytopenia purpura. The aim of this therapy is to reduce the plasma levels of anti-ADAMTS13 autoantibodies and UL-VWFM and to replenish ADAMTS13. Both ticlopidine and clopidogrel have been shown to be useful anti-platelet drugs when administered as an adjunct

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to PE and high-dose prednisolone therapy in acquired thrombotic thrombocytopenia purpura patients, and both drugs increased the remission rate. However, ticlopidine has been shown to potentially induce thrombotic thrombocytopenia purpura via the production of anti-ADAMTS13 auto-antibodies, although the incidence was quite low (1/1600–5000). Thus, the British Society of Hematology guidelines do not recommend these two antiplatelet drugs for thrombotic thrombocytopenia purpura patients (Bobbio-Pallavicini et al., 1997). Subsequently, two other antiplatelet drugs, aspirin and dipyridamole, have been used for this purpose. Patients receiving a set of these drugs had a good remission rate in an initial phase study, but a subsequent small retrospective study reported serious bleeding complications. Thus, the same guidelines as before state the rationale for administering aspirin therapy when platelet counts increase over 50×10^9 /L because a rapid rise in platelet counts during recovery has been anecdotally associated with thromboembolic events. However, the benefits of aspirin have not been proven. In fact, low-dose aspirin therapy may reduce adverse bleeding complications, but has little effect on high-shear stress-induced platelet aggregation.

Our results suggest that cilostazol may compensate for the role of ADAMTS13 by inhibiting the excessive formation of platelet thrombi in the vertical direction against peripheral microvascular cell walls in thrombotic thrombocytopenia purpura patients without causing excess bleeding.

Acknowledgments

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ORIGINAL ARTICLE

Increased fibrinolysis increases bleeding in orthopedic patients receiving prophylactic fondaparinux

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Abstract We evaluated hemostatic markers in patients who underwent major orthopedic surgery, including total hip and total knee arthroplasty, and were treated for the prophylaxis of deep vein thrombosis (DVT) with or without fondaparinux (anti-Xa group, $n = 98$ and without anti-Xa group, $n = 20$). The frequency of DVT was significantly higher in the without anti-Xa group than in the anti-Xa group, but the reduction of hemoglobin and fibrinolytic marker levels was significantly lower in the without anti-Xa group than in the anti-Xa group. Eighteen patients in the anti-Xa group showed a reduction in hemoglobin of more than 2 g/dl, and those individuals were considered to be the increased bleeding (IB) group. The concentration of fibrinolytic markers in the anti-Xa group was significantly higher in the IB group than in the non-IB group. There were also no significant differences in the levels of anti-Xa activity, plasminogen activator inhibitor-I, soluble fibrin and antithrombin between the IB and non-IB groups. In conclusion, elevated fibrinolysis induced by increased bleeding may lead to further increases in bleeding in patients receiving thromboprophylaxis with fondaparinux following major orthopedic surgery.

Keywords Deep vein thrombosis (DVT) · Fibrinolysis · Fondaparinux · Orthopedic surgery · Bleeding

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Introduction

Fibrin-related markers such as D-dimer, fibrin and fibrinogen degradation products (FDP) and soluble fibrin (SF) are useful for the diagnosis of thrombosis, and are elevated in deep vein thrombosis (DVT)/pulmonary embolism (PE) and disseminated intravascular coagulation (DIC) [1–5]. PE is a common, frequently undiagnosed and potentially fatal cause of several common symptoms: dyspnea and chest pain [6–8]. Preventing the development of DVT is clinically important, because PE is often a fatal disease caused by DVT. Orthopedic surgery is associated with a very high rate of postoperative venous thromboembolism (VTE) [9, 10]. The incidence of venographically proven VTE ranges from 42 to 57% after total hip arthroplasty (THA) surgery in the absence of thromboprophylaxis, and 41 to 85% after total knee arthroplasty (TKA) surgery [11]. Multiple studies [12–14] have established the usefulness of low molecular weight heparin (LMWH) for VTE prophylaxis in orthopedic surgery patients.

Fondaparinux is the first selective factor Xa inhibitor approved for use in thromboprophylaxis after orthopedic surgery [15–17], and studies comparing fondaparinux to LMWH showed very useful thromboprophylaxis in

patients after orthopedic surgery [16, 17]. There are a few cases with massive bleeding in patients administered fondaparinux. Therefore, fondaparinux is frequently administered at a dose of 1.5 mg instead of 2.5 mg in Japan to avoid serious bleeding. Anti-Xa activity has been measured as UFH or LMWH activity to monitor the anticoagulant activity [18, 19].

This study evaluated the hemostatic markers in patients who underwent major orthopedic surgery and were treated for the prophylaxis of DVT with or without fondaparinux ($n = 98$ and $n = 20$) to examine the fibrinolytic activity in patients with major bleeding.

Materials and methods

From 1 January 2007 to 30 March 2007, 20 patients (median age 67.5 years of age, 25–75% range, 63.5–75.0 years of age; 16 females and 4 males) underwent orthopedic surgery (14 THA and 6 TKA) and were treated with only intermittent pneumatic compression for prophylaxis of DVT (the without anti-Xa group). Ninety-eight orthopedic patients treated with 1.5 mg of fondaparinux (GlaxoSmithKine, Tokyo, Japan) and intermittent pneumatic compression for prophylaxis of DVT from 1 February 2010 to 31 December 2010 were enrolled in this study (the with anti-Xa group). These patients received 1.5 mg of Fondaparinux by hypodermic injection once a day for 14 days beginning 24 h after extubation of lumbar anesthesia. There were no significant differences in the age, sex and type of operation between the without and with anti-Xa groups (Table 1). A reduction of more than 2 g/dl of hemoglobin from day 1 to 14 was observed in 18 patients (increased bleeding group; IB group; Table 2). No patients with IB received blood transfusion and none had associated DIC. There was no significant difference in the age, weight, height, body mass index (BMI), body surface area, estimated glomerular filtration rate (eGFR), hemoglobin and antithrombin (AT) before surgery between patients with and without IB. Only the creatinine concentration and number of males were higher in patients with IB than in those without IB. Anti-Xa activity, fibrin and FDP, D-dimer, soluble fibrin (SF) and AT activity were measured in 73 patients after THA and 23 patients after TKA before and on days 1, 4, 8 and 15 of the administration of fondaparinux. The study protocol was approved by the Human Ethics Review Committee of the Mie University School of Medicine and a signed consent form was obtained from each subject. This study was faithfully carried out in accordance with the Declaration of Helsinki.

The anti-Xa activity was monitored 3 h after injection of fondaparinux. The anti-FXa activity of fondaparinux was

Table 1 Subjects with and without anti-Xa treatment

	Without anti-Xa	With anti-Xa
Age (years)	67.5 (63.5–75.0)	68.0 (61.0–75.0)
Female:male	16:4	75:23
THA:TKA	14:6	73:25
DVT	10/20 (50%)**	16/98 (14.3%)**
Reduction of hemoglobin (g/dl)	0.55 (0.00–1.10)***	2.20 (1.60–3.28)***
Weight (kg)	56.8 (49.7–65.2)	57.1 (50.1–66.9)
Creatinine (mg/dl)	0.68 (0.54–0.79)	0.67 (0.56–0.80)
Underlying diseases	Osteoarthritis[a]	Osteoarthritis[a]

THA total hip arthroplasty, *TKA* total knee arthroplasty

[a] Patients with rheumatoid arthritis were excluded from this study

** $p < 0.01$, *** $p < 0.001$

Table 2 Subjects treated with anti-Xa therapy

	Reduction by >2.0 g/dl of Hb	Reduction by <2.0 g/dl of Hb
Age (years)	71.5 (63.0–79.0)	68.0 (61.0–73.5)
Weight (kg)	53.9 (48.2–69.3)	57.4 (50.6–66.9)
High (cm)	155.9 (146.1–164.0)	151.5 (147.7–158.0)
BMI (kg/m^2)	22.5 (20.4–25.2)	24.5 (22.6–27.3)
Body surface area (cm^2)	1.50 (1.41–1.71)	1.53 (1.44–1.68)
Creatinine (mg/dl)	0.82 (0.60–1.03)*	0.64 (0.56–0.77)*
eGFR	68.9 (51.1–83.0)	76.5 (59.6–86.5)
Hb (g/dl)	12.1 (10.9–13.4)	12.2 (11.4–12.8)
AT (%)	79.5 (68.5–84.1)	81.6 (73.4–89.8)
Female:male	10:8*	65:15*
THA:TKA	14:4	59:21

THA total hip arthroplasty, *TKA* total knee arthroplasty

* $p < 0.05$

measured using Testzym® Heparin S (Sekisui Medical Co. Ltd., Tokyo, Japan) and a Coagrex® 800 (Sysmex Co. Ltd., Kobe, Japan).Testzym® Heparin S contains bovine FXa (71 nkat/vial), AT (10 IU/vial), chromogenic substrate (S-2222: Benz-Ile-Glu-Gly-Arg-pNA HCl 25 mg), pooled lyophilized normal plasma and buffer (pH 8.4) [18, 19]. A standard curve was constructed for lyophilized normal plasma using various concentrations of fondaparinux.

The reagents and objects were loaded into the Coagrex 800, and the anti-FXa activity of fondaparinux was measured automatically. A 135-μl aliquot of FXa was added to 8 μl of plasma (with diluent solution added in advance) and 75 μl of substrate was added. The released p-NA was measured photometrically at 405 nm. The anti-FXa activity of fondaparinux was then calculated using the standard curve.

Plasma levels of FDP, D-dimer, SF, plasmin plasmin inhibitor complex (PPIC) and plasminogen activator inhibitor-I (PAI-I) were measured by the latex agglutination method using Nanopia FDP, Nanopia D-dimer, Nanopia SF (Sekisui Medical), LPIAACE PPIII (Mitsubishi Chemical Medience, Tokyo, Japan) and LPIA-tPAI test (Mitsubishi Chemical Medience), respectively [20]. The plasma levels of AT were measured using a Testzym S AT III kit (Sekisui Medical). Diagnosis of DVT was assessed by whole-leg compression ultrasound examination using a standardized ultrasound criterion of venous noncompressibility before the operation, as well as on days 4 and 14 [21].

Statistical analysis

The data are expressed as the medians (25–75 percentile). Any differences between the groups were examined using the Mann–Whitney U test. The correlations between the reduction of hemoglobin and hemostatic markers were examined using the Spearman's rank correlation coefficient. A p value of less than 0.05 was considered to be statistically significant. All statistical analyses were performed using the SPSS II software package (SPSS Japan, Tokyo).

Results

DVT was observed in 10 patients (50%) including 3 patients with proximal DVT in the without anti-Xa group and 16 patients (14.3%) including one patient with proximal DVT in the with Xa group, indicating that the frequency of DVT was significantly higher in the without anti-Xa group than in the with anti-Xa group ($p < 0.01$). Symptomatic PE was not observed in either group. As these DVT were not observed before the operations, they were therefore considered to be fresh DVT. The reduction of hemoglobin from day 1 to 14 was significantly higher in the with anti-Xa group than in the without anti-Xa group (Table 1, $p < 0.001$).

The plasma levels of FDP were significantly higher in the with anti-Xa group than in the without anti-Xa group on day 4 (12.0 μg/ml: 9.6–14.1 μg/ml vs. 10.0 μg/ml: 9.1–11.0 μg/ml, $p < 0.05$), day 8 (14.9 μg/ml: 11.2–19.7 μg/ml vs. 10.8 μg/ml: 8.4–13.6 μg/ml, $p < 0.001$) and day 15 (15.1 μg/ml: 11.0–20.5 μg/ml vs. 10.2 μg/ml: 6.9–12.5 μg/ml, $p < 0.001$, Fig. 1a). The plasma levels of D-dimer were significantly higher in the with anti-Xa group than in the without anti-Xa group on day 8 (8.2 μg/ml: 5.6–10.4 μg/ml vs. 6.3 μg/ml: 4.9–7.5 μg/ml, $p < 0.05$) and day 15 (7.7 μg/ml: 5.6–11.7 μg/ml vs. 5.7 μg/ml: 4.0–12.5 μg/ml, $p < 0.01$, Fig. 1b). The plasma levels of PPIC were significantly higher in the with anti-Xa group than in the without anti-Xa group on day 4 (2.68 μg/ml: 2.23–3.16 μg/ml vs. 1.13 μg/ml: 0.96–1.25 μg/ml, $p < 0.001$) and day 8 (1.61 μg/ml: 1.50–2.23 μg/ml vs. 1.13 μg/ml: 0.92–1.24 μg/ml, $p < 0.01$, Fig. 1c). The plasma levels of SF were significantly higher in the with anti-Xa group than in the without anti-Xa group on day 4 (11.8 μg/ml: 8.2–17.1 μg/ml vs. 7.2 μg/ml: 5.6–8.7 μg/ml, $p < 0.001$), day 8 (7.0 μg/ml: 4.6–11.2 μg/ml vs. 4.6 μg/ml: 3.5–6.3 μg/ml, $p < 0.05$) and day 15 (4.6 μg/ml: 3.0–8.3 μg/ml vs. 3.3 μg/ml: 2.5–5.0 μg/ml, $p < 0.05$, Fig. 1d).

The concentration of hemoglobin in the anti-Xa group was significantly lower in the IB group than in the non-IB group on day 4 (8.6 g/dl: 7.9–9.0 g/dl vs. 10.3 g/dl: 9.2–11.4 g/dl, $p < 0.001$) and day 8 (9.0 g/dl: 8.4–9.7 g/dl vs. 10.3 g/dl: 9.4–11.2 g/dl, $p < 0.001$). There was no significant difference in the anti-Xa activity between the IB and non-IB groups before and on days 1, 4 and 8 of administration (Table 3; Fig. 2). The concentration of hemoglobin was significantly lower in the IB group than in the non-IB group on days 4 and 8 ($p < 0.001$).

The concentration of FDP in the anti-Xa group was significantly higher in the IB group than in the non-IB group before (31.3 μg/ml: 14.6–46.7 μg/ml vs. 15.8 μg/ml: 9.7–27.3 μg/ml, $p < 0.01$) and on day 1 (17.2 μg/ml: 11.2–27.1 μg/ml vs. 12.3 μg/ml: 9.0–17.6 μg/ml, $p < 0.05$) and day 8 (21.1 μg/ml: 17.4–25.0 μg/ml vs. 14.5 μg/ml: 11.2–18.9 μg/ml, $p < 0.05$; Fig. 3a). The concentration of D-dimer in the anti-Xa group was significantly higher in the IB group than in the non-IB group on day 0 (17.3 μg/ml: 6.5–27.4 μg/ml vs. 7.8 μg/ml: 4.6–14.4 μg/ml, $p < 0.01$), day 1 (8.2 μg/ml: 6.7–8.2 μg/ml vs. 5.8 μg/ml: 3.8–9.1 μg/ml, $p < 0.05$) and day 8 (11.4 μg/ml: 9.5–13.7 μg/ml vs. 8.1 μg/ml: 5.5–10.3 μg/ml, $p < 0.05$; Fig. 3b). No significant difference was observed regarding PAI-I, SF and AT between the IB and the non-IB groups before and on days 1, 4 and 8 of administration (Table 4). The concentration of PPIC was significantly higher in the IB group than in the non-IB

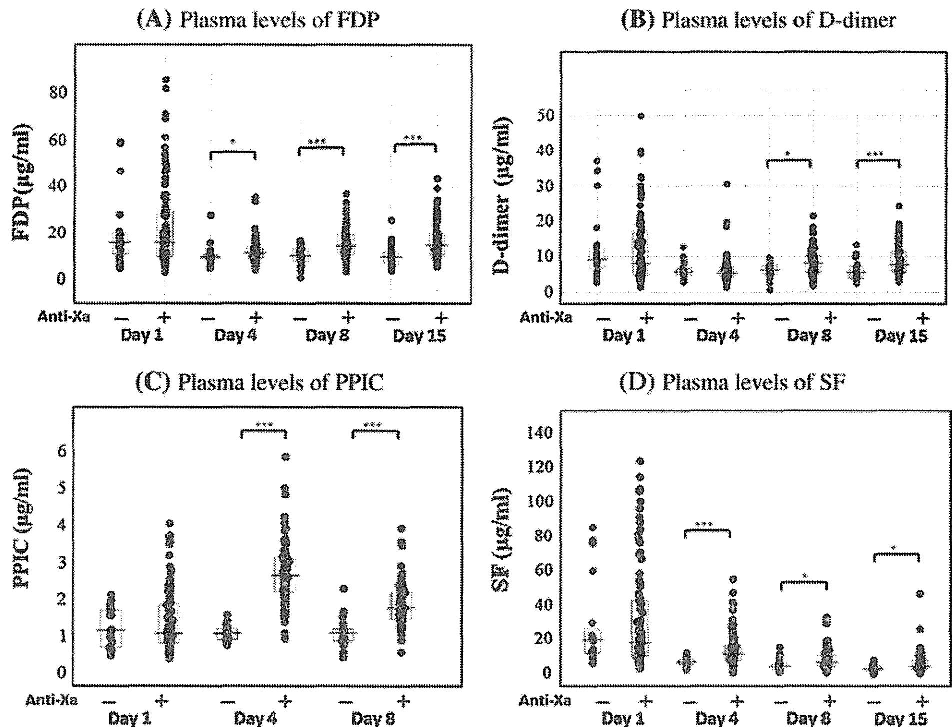

Fig. 1 The plasma levels of FDP, D-dimer, PPIC and SF in the orthopedic patients treated with and without fondaparinux. The plasma levels of **a** FDP, **b** D-dimer, **c** PPIC, and **d** SF *FDP* fibrinogen and fibrin degradation products, *PPIC* plasmin plasmin inhibitor complex, *SF* soluble fibrin; − without fondaparinux, + with fondaparinux. **p* < 0.05, ****p* < 0.001

Table 3 The anti-Xa activity between the patients with and without a reduction in Hb by >2.0 g/dl

Fondaparinux (mg/l)	Reduction of Hb >2.0 g/dl	Reduction of Hb <2.0 g/dl
Before	0.02 (0.00–0.06)	0.03 (0.00–0.07)
Day 1	0.31 (0.27–0.36)	0.30 (0.25–0.38)
Day 4	0.41 (0.35–0.51)	0.39 (0.34–0.50)
Day 8	0.52 (0.44–0.60)	0.46 (0.38–0.54)
Day 15	0.24 (0.10–0.54)	0.22 (0.17–0.26)

group on day 0 (1.5 μg/ml: 1.2–2.5 μg/ml vs. 1.1 μg/ml: 0.8–1.6 μg/ml, $p < 0.05$). The concentration of PPIC was highest at day 4, but there was no significant difference between the IB and non-IB groups (2.7 μg/ml: 2.3–3.1 μg/ml vs. 2.7 μg/ml: 2.2–3.2 μg/ml; Fig. 3c). The correlation with reduction of hemoglobin was more significant in PPIC, FDP and D-dimer levels than in SF levels (Table 5).

Discussion

The frequency of DVT was significantly higher in the without anti-Xa group than in the anti-Xa group, but the reduction of hemoglobin was significantly lower in the without anti-Xa group than in the anti-Xa group, indicating that fondaparinux is useful for the prophylaxis of DVT, but may increase bleeding. In the anti-Xa group, the

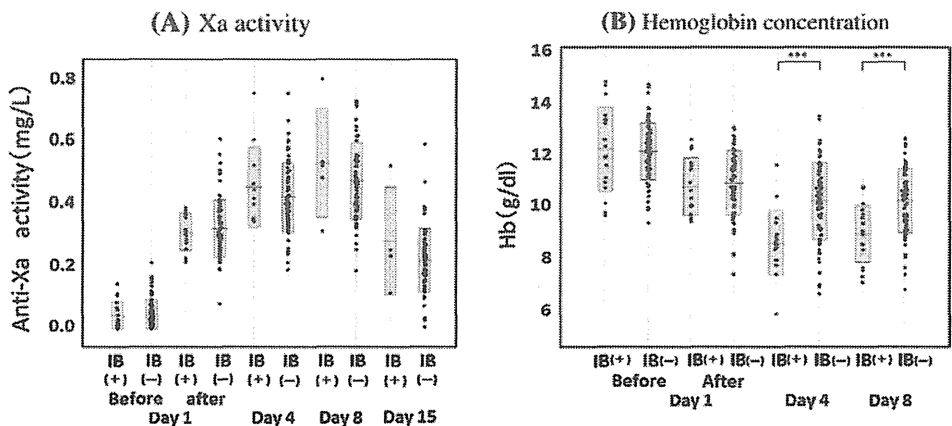

Fig. 2 Anti-Xa activities and hemoglobin concentrations in orthopedic patients treated with fondaparinux. **a** The anti-Xa activity and **b** hemoglobin concentration. *IB* increased bleeding: a reduction of more than 2 g/dl of hemoglobin, *Before* before the administration of fondaparinux, *After* after the administration of fondaparinux. ****p* < 0.001

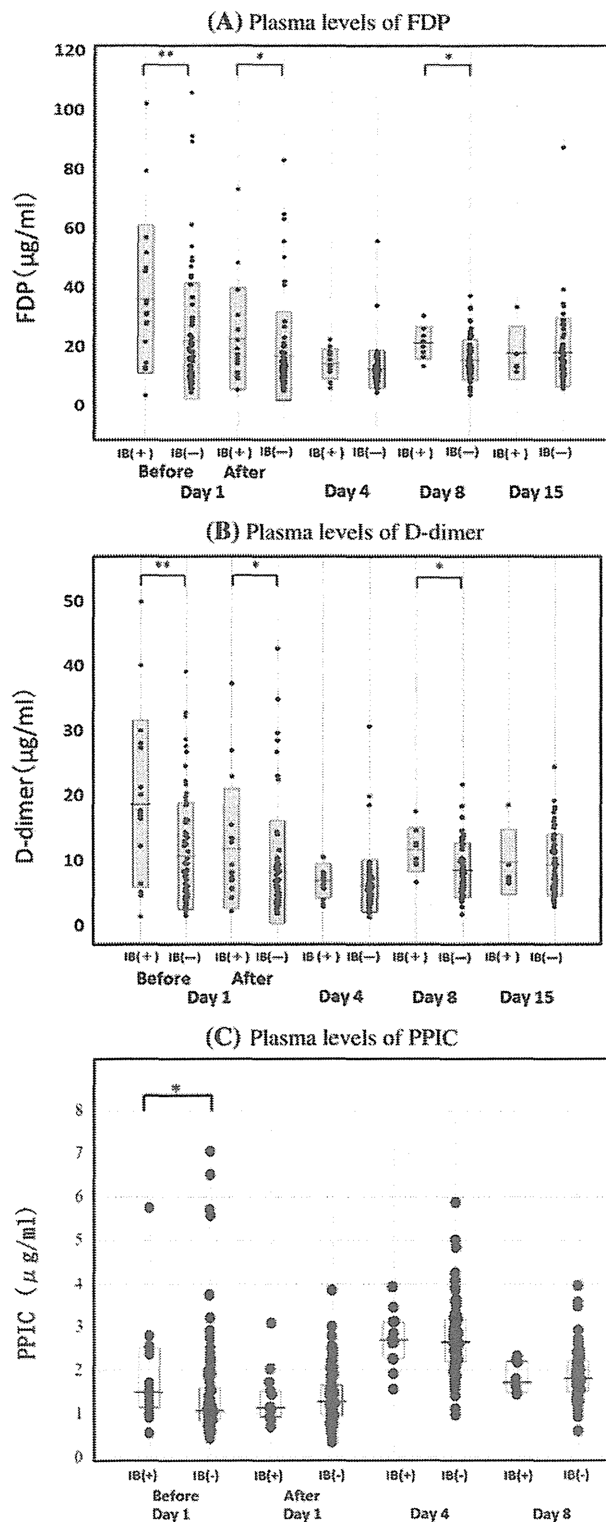

Fig. 3 The concentration of FDP, D-dimer and PPIC in orthopedic patients treated with fondaparinux. The plasma levels of **a** FDP, **b** D-dimer, **c** PPIC *FDP* fibrinogen and fibrin degradation products, *PPIC* plasmin plasmin inhibitor complex, *IB* increased bleeding: a reduction of hemoglobin by more than 2 g/dl, *Before* before the administration of fondaparinux, *After* after the administration of fondaparinux. $^{**}p < 0.01$, $^{*}p < 0.05$

hemoglobin concentrations significantly decreased on days 4 and 8, thus suggesting that the decrease in hemoglobin was not due to the surgical procedure. The FDP, D-dimer, PPIC and SF levels were significantly higher in the anti-Xa group than in the without anti-Xa group, suggesting that a hyperfibrinolysis and hypercoagulable state exists after administration of fondaparinux. Fondaparinux cannot directly activate the fibrinolytic system, but it may increase secondary fibrinolysis, thus leading to increased bleeding. The increased bleeding causes a fibrin clot formation to activate fibrinolysis, followed by increases in the SF, FDP, D-dimer and PPIC levels, which all increase after treatment with fondaparinux. Therefore, fondaparinux may be useful for the treatment of DVT.

Eighteen of the 98 orthopedic patients who were treated with fondaparinux showed a reduction of more than 2 g/dl of hemoglobin (IB group). Total joint arthroplasty is associated with significant perioperative blood loss. The average range is from 761 to 1549 ml in a single TKA without antifibrinolytics [22–26]. There were no patients who stopped the fondaparinux treatment, and none of the patients required a blood transfusion. As the drainage tube was extracted before the injection of fondaparinux, the blood loss could not be measured. While there was no massive bleeding at the site of the operation after the administration of the anti-Xa inhibitor, there might have been some minor blood loss at the site. There are several causes of IB, such as surgery, hyperfibrinolysis, hemostasis in the host and anti-Xa agents. The current reports [22–26] suggest that as first suspected in this study, IB may be caused by elevated anti-Xa activity by fondaparinux. There was a wide range of anti-Xa activity in patients treated with fondaparinux, suggesting that high-dose administration of fondaparinux should be monitored by the anti-Xa activity. However, no significant difference was seen in the anti-Xa activity between the IB and non-IB groups before treatment and on days 1, 4 and 8 of fondaparinux administration. In addition, the anti-Xa activity was not markedly high in either group, thus suggesting that such increased bleeding may occur independent of the anti-Xa activity of fondaparinux. It might not be necessary to monitor anti-Xa activity following the injection of 1.5 mg fondaparinux.

The concentrations of FDP and D-dimer were significantly higher in the IB group than in the non-IB group before and on days 1 and 8, but there was no significant difference in the SF between these groups during that period. These findings suggest that clot formation and fibrinolysis before and on day 1 were higher in the IB than

Table 4 The plasma levels of PAI-I, SF and AT in the patients with and without a reduction in Hb >2.0 g/dl

	PAI-I (ng/ml)		SF (μg/ml)		AT (%)	
	IB+	IB−	IB+	IB−	IB+	IB−
Before	23.4 (21.2–26.3)	23.7 (19.2–35.0)	26.4 (19.7–71.0)	16.6 (10.7–38.3)	79.5 (68.5–84.1)	81.6 (73.4–89.8)
Day 1	19.5 (13.4–27.1)	16.7 (13.3–23.8)	10.6 (7.4–15.4)	13.3 (8.2–20.2)	78.5 (75.1–87.9)	84.1 (75.2–91.0)
Day 4	18.8 (13.0–23.1)	17.2 (12.8–23.1)	9.5 (7.0–17.3)	12.0 (8.7–17.3)	102.3 (86.8–104.8)	92.2 (84.0–103.1)
Day 8	15.0 (9.2–24.5)	15.7 (11.5–20.5)	5.2 (3.8–8.7)	7.1 (4.6–11.9)	104.9 (98.2–112.2)	99.9 (91.5–113.5)
Day 15			2.2 (1.9–4.5)	4.6 (3.2–8.6)	94.9 (91.3–105.9)	94.5 (87.8–104.8)

PAI-I plasminogen activator inhibitor-I, *SF* soluble fibrin, *AT* antithrombin, *IB* increased bleeding

Table 5 The correlation between the reduction of the hemoglobin concentration and hemostatic markers

	rS			
	Day 1	Day 5	Day 8	Day 15
SF	0.157 (NS)	0.199 ($p < 0.05$)	0.144 (NS)	0.052 (NS)
D-dimer	0.082 (NS)	0.051 (NS)	0.405 ($p < 0.001$)	0.336 ($p < 0.001$)
FDP	0.115 (NS)	0.195 ($p < 0.05$)	0.390 ($p < 0.001$)	0.284 ($p < 0.01$)
PPIC	0.020 (NS)	0.388 ($p < 0.001$)	0.328 ($p < 0.001$)	

rS Sperman's correlation coefficient

in the non-IB group. Therefore, bleeding due to surgery may affect the bleeding level from day 1 to 14.

D-dimer and FDP levels were again elevated at day 8 and those were significantly higher in the IB group in comparison to the non-IB group. Plasma levels of D-dimer, FDP and SF are elevated in patients with thrombosis [2–4], but D-dimer levels remain elevated long after the onset of DVT, but the levels of SF decrease rapidly [2], thus suggesting that the elevation of FDP and D-dimer indicates not only a thrombotic state, but also secondary fibrinolysis. These findings suggest that a hyperfibrinolysis instead of anti-coagulation may cause IB. The concentration of PPIC was significantly higher in the IB group in comparison to the non-IB group at day 0. The concentration of PPIC was highest on day 4, and the PAI-I level reduced at this point. There are many retrospective studies showing that tranexamic acid is useful for controlling blood loss after orthopedic surgery [22, 24]. Several prospective randomized double-blind studies [23, 27] also report that tranexamic acid significantly reduces blood loss after TKA.

In conclusion, the administration of 1.5 mg of fondaparinux was useful for the prevention of fatal PE and increased bleeding and fibrinolysis. Increased fibrinolysis and the use of an anti-Xa drug may, therefore, be associated with the IB group receiving thromboprophylaxis with fondaparinux following major orthopedic surgery. Future studies should therefore investigate the efficacy of 1.5 mg fondaparinux and anti-fibrinolytic therapy in patients with hyperfibrinolysis after orthopedic surgery.

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ORIGINAL ARTICLE

Frequent association of thrombophilia in cerebral venous sinus thrombosis

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Abstract Thrombophilia is frequently associated with venous thromboembolism (VTE) including cerebral venous sinus thrombosis (CVST). The possibility of thrombophilia was examined in 12 patients with CSVT diagnosed in the past 9 years. Thrombophilia due to abnormalities in antithrombin (AT), protein C (PC), or protein S (PS) or antiphospholipid syndrome (APS) was evaluated. Nine patients with abnormally decreased AT, PC or PS and one patient with APS were examined. Of the nine patients examined by a gene analysis of AT, PC, or PS, one had a congenital AT deficiency, one had a congenital PC deficiency, and two had congenital PS deficiencies including a novel mutant (Gly189Ala). AT, PC and PS levels were all decreased in one patient, PS level was decreased in three patients, and AT level was decreased in one patient at the onset of CVST, but these concentrations improved after treatment. CVST is frequently associated with thrombophilia and a transient decrease in AT, PC or PS may be a causal factor.

Keywords Cerebral venous sinus thrombosis (CVST) · Thrombophilia · Antithrombin · Protein C · Protein S

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Introduction

There are approximately 170,000 new cases of clinically recognized venous thromboembolism (VTE) in patients treated in short-stay hospitals in the United States each year [1]. While cerebral venous sinus thrombosis (CVST) is a rare disease with an estimated annual incidence of 3–4 cases per 2 million adults, and 7 cases per 1 million neonates, its precise incidence is not known because there have been only a few epidemiological studies [2–4]. The risk factors for CVST are tumors [5], cerebral infections or trauma [5, 6], oral contraceptive use [7], pregnancy and the peripartum period [8] and thrombophilia [4, 7, 9]. Thrombophilia is defined as patients having a high risk for thrombosis, which can be either inherited or acquired. The main acquired thrombophilia is due to the presence of antiphospholipid antibodies [10, 11], and congenital thrombophilia includes antithrombin (AT), protein C (PC) or protein S (PS) abnormalities [12–14] and Factor V {G1691A} and Factor II (G20210A) abnormalities [15, 16]. However, Factor V {G1691A} and Factor II (G20210A) abnormalities have never been reported in Japan [17, 18].

The overall annual incidence of VTE was 1.53% in patients with deficiencies of AT, PC and PS [19] and thrombophilia has also been reported to be a risk factor for CVST [20].

In this study, we examined in 12 patients with CVST for thrombophilia due to AT, PC, and PS abnormalities and anti-phospholipid antibodies, and the expression of novel PS mutant was determined.

Materials and methods

Twelve patients with CVST were diagnosed at Mie University Hospital from 1 January 2003 to 28 February 2011 (Table 1). The CVST was diagnosed by magnetic resonance imaging (MRI), magnetic resonance venography (MRV) or cerebral angiography (CAG). The study protocol was approved by the Human Ethics Review Committee of the Mie University School of Medicine and a signed consent form was obtained from each subject. This study has been faithfully carried out in accordance with the Declaration of Helsinki. Case 1 was pregnant, case 3 had severe inflammation, case 5 had iron deficient anemia, and case 6 had been taking a contraceptive drug. Case 1 was complicated with deep vein thrombosis (DVT) and case 11 was associated with mesenteric venous thrombosis (MVT). No patients suffered from recurrent either CVTS or any other types of VTE after the 1st episode of CSVT.

Measurement of AT, PC, PS and antiphospholipid antibody concentrations

Peripheral blood samples were collected in a 1/10 volume of 3.13% sodium citrate. The plasma free PS antigen concentration was measured by a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) using an Asserachrom free PS kit (Diagnostica Stago, Asnières, France). The plasma PS and PC activity levels were measured by a clotting time method using a STA®-Staclot® Protein S and a STA®-Staclot® Protein C kit (Diagnostica Stago, respectively). The plasma PC antigen concentration was measured by a latex agglutination test using a LPIA-ACE PC (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). The plasma AT activity was measured by a synthetic substrate assay using a Chromorate ATIII (C) kit (Mitsubishi Chemical Medience Corporation). An activity or antigen level of less than 70% in AT, PC and PS of the patients who did not receive warfarin treatment, was considered to be an abnormal decrease in AT, PC or PS. An abnormal decrease in PC or PS was defined as a >2-fold ratio of PS/PC or PC/PS activity in the patients who received warfarin treatment.

The dilute Russell's viper venom time (DRVVT) was measured by a clotting time method using a Gradipore LA test (Gradipore, Sydney, Australia). Anti-caldiolipin-β2 glycoprotein I (ACL-β2GPI) antibodies were measured with an ELISA kit (Yamasa Co, Tokyo, Japan).

Gene analysis of AT, PC and PS

Genomic DNA was prepared from peripheral blood leukocytes using a QIAamp DNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions. Each exon and exon/intron boundary of the gene was amplified from genomic DNA using the polymerase chain reaction (PCR) as described previously. The PCR products were directly sequenced using a Big-Dye Terminator Cycle Sequencing Kit and a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The transient and stable expression levels of recombinant Protein S and quantification of the expression level of PS were carried out as in a previous report [21]. The initiating Methionine was set to +1 and the amino acid residue of PS was numbered.

Statistical analysis

The differences in PS expression between mutant and wild type patients were determined using the unpaired t test. A value of $p < 0.05$ was considered to be significant.

Results

Decreased activity of AT was observed in 3 of the 12 patients (cases 1, 3, and 12) with CVST, decreased activity or antigen of PC was observed in 5 of the 12 patients (cases 3, 6, 7, 9, and 11), and decreased activity or antigen of PS was observed in 7 of the 12 patients (cases 2, 3, 4, 5, 6, 7, and 11), but 2 patients (cases 7 and 11) were already being treated with warfarin (Table 1). Case 8 was positive for both DRVVT and the ACL-β2GPI antibody, and was diagnosed as having antiphospholipid syndrome (APS). A gene analysis for AT, PC and PS was carried out, and 4 patients were diagnosed as having congenital thrombophilia: two with PS genetic abnormalities (cases 2 and 11), one with a PC genetic abnormality (case 9) and one with a AT genetic abnormality (case 1) (Table 2). Case 1 had a heterozygous Pro429Leu (AT Budapest) mutation of the AT gene. The pedigree of the family under investigation is shown in Fig. 1a. The proband developed CVST and DVT when she was pregnant. Her mother had the same mutation of AT and also had CVST. Her brother had no mutation, but the proband's child had same mutation. Case 2 had two heterozygous PS mutations (Asp79Tyr and Thr630Ile). Case 9 had a heterozygous Glu153 (458-460delAGG) mutation of PC. The pedigree of the family under investigation is shown Fig. 1b. The proband developed CVST at the age of 47 years, and her daughter was detected to have the same PC mutation. Therefore, prophylaxis for VTE could be performed for the daughter during her pregnancy.

Table 1 Subjects

Case	Age	Sex	Onset	CSVT	Additional factor	Other VTE	AT (%)	PC (%)		PS (%)		DRVVT (0.0–1.2)	ACL-β2GPI antibody
							Act (70–130)	Act (70–140)	Ag (70–130)	Ag (70–150)	Free Ag (70–150)		
1	27	F	2003	Straight sinus	Pregnancy	DVT	**55**	107	108	127	120	1.1	Negative
2	60	M	2010	Superior sagittal sinus	None	(–)	**88**	ND	96	**43**	**50**	ND	Negative
3	81	M	2010	Left sigmoid sinus	Inflammation, MOF	(–)	**64**	**44**	**31**	**34**	**69**	1.0	Negative
4	52	M	2007	Superior sagittal sinus Transverse sinus	None	(–)	109	115	112	**61**	**59**	ND	Negative
5	54	F	2005	Superior sagittal sinus	IDA	(–)	117	125	110	**65**	ND	0.9	Negative
6	40	F	2010	Superior sagittal sinus	Contraceptive use	(–)	88	**57**	76	**30**	**105**	1.2	Negative
7	57	M	2009, 2010	Superior sagittal sinus Transverse sinus	None	(–)	88	ND	65[a]	ND	52[a]	1.0	Negative
8	18	F	2006	Right transverse sinus	None	(–)	115	133	ND	73	ND	**1.3**	Positive
9	47	F	2008	Left transverse sinus	None	(–)	85	**59**	**60**	79	ND	1.0	Negative
10	49	M	2008	Superior sagittal sinus	None	(–)	98	96	99	103	97	ND	Negative
11	71	F	2009	Superior sagittal sinus	None	MVT	114	49[a]	55[a]	**18**[a]	45[a]	1.1	Negative
12	43	F	2011	Superior sagittal sinus	None	(–)	**65**	105	91	80	94	1.2	Negative

Values in parentheses show a reference. The bold values indicate no abnormal value

DVT deep vein thrombosis, *MVT* mesenteric venous thrombosis, *MOF* multiple organ failure, *F* female, *M* male, *IDA* iron deficient anemia, *Act* activity, *Ag* antigen, *ND* not done, *DRVVT* dilute Russell's viper venom time

[a] Treated with warfarin