sepsis-related organ failure assessment (SOFA) score (8) or the acute physiological and chronic health evaluation (APACHE) II score (9). Although several randomized controlled trials (RCTs) of severe sepsis and/or DIC investigating AT (10), activated PC (APC) (11, 12), tissue factor pathway inhibitor (TFPI) (13) and TM (14) have been conducted, few successful results have been obtained (11, 14).

Three similar diagnostic criteria have been established by the Japanese Ministry of Health and Welfare (JMHW) (15), the International Society of Thrombosis and Haemostasis (ISTH) (16) and the Japanese Association for Acute Medicine (JAAM) (17). These diagnostic criteria include global coagulation tests, such as the assessment of the prothrombin time (PT), the platelet count and the levels of fibrinogen, fibrin and fibrinogen degradation products (FDP) and D-dimer, to score hemostatic abnormalities. These diagnostic criteria tend to focus on septic DIC; however, their efficacy remains insufficient (18). Concordance in the diagnosis of DIC between the JMHW and ISTH overt-DIC diagnostic criteria is relatively high among patients with infections and low in patients with hematopoietic tumors (19).

In this study, 242 patients with infections were prospectively evaluated using the DIC diagnostic criteria established by the ISTH and JMHW, and useful markers for predicting poor outcomes were examined.

Materials and Methods

A total of 522 patients with underlying disorders known to be associated with DIC evaluated at nine institutes were registered for this prospective study of DIC diagnostic criteria between January 1, 2005 and May 31, 2008. The study protocol was approved by the Human Ethics Review Committee of the Mie University School of Medicine and signed consent forms were obtained from each subject. This study was faithfully carried out in accordance with the Declaration of Helsinki. The inclusion criteria were based on the presence of one or more of the following laboratory findings: a platelet count of less than 120×10^3 per μL, an FDP level of more than 10 μg/mL, a fibrinogen level of less than 100 mg/dL and a PT ratio of over 1.25. Patients with symptoms associated with thrombotic thrombocytopenic purpura (TTP), heparin-induced thrombocytopenia (HIT), antiphospholipid syndrome (APS) or severe liver injury were excluded. Of the 522 patients with hemostatic abnormalities, 242 with infectious diseases (85 women and 157 men, median age: 71 years; age range 62-77 years) were selected and analyzed for infectious diseases using a subclass analysis.

There were 69 septic patients without other infections, 67 patients with respiratory infections, 24 patients with hepatobiliary and pancreatic infections, 45 patients with digestive infections, 17 patients with urinary infections and three patients with other infections. DIC was diagnosed using the JMHW DIC diagnostic criteria (15). The degree of organ failure was evaluated using the SOFA score (8), and patients with a score of more than 3 points for systemic inflammatory response syndrome (SIRS) were diagnosed with SIRS (20). Treatment for DIC (7), including AT, heparin, low-molecular-weight heparin, gabexate mesilate, nafamostat mesilate and danaparoid sodium, was administered under the observation of the individual physician after blood sampling was performed at registration. Patients who were alive at 28 days after registration were considered to be survivors and those who died within 28 days were considered to be non-survivors.

The levels of PT, fibrinogen, platelets, and FDP were measured at each of the institutes based on the methods reported in numerous previous studies (21-23). The FDP assays performed at each institute correlated well with the results of LPIA FDP (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). The plasma levels of SFMC (24) and D-dimer were measured with latex immune agglutination tests using Auto LIA FM (Roche Diagnostics, Tokyo, Japan) and the LATECLE D-dimer (Kainos, Tokyo, Japan), respectively, in the central research laboratory of SRL Inc. (Tokyo, Japan). The plasma levels of thrombin-AT complex (TAT), F1+2 and TM were measured with enzyme immunoassays (EIAs) using a TAT [S] (TFB, Tokyo, Japan), Enzygnost® F1+2 monoclonal (Siemens, Tokyo, Japan) and TM Banasera (Fujirebio, Tokyo, Japan), respectively. The plasma levels of plasmin-plasmin inhibitor complex (PPIC) and PAI-I were measured with latex immune agglutination tests using LPIA-ACE PPI IIand LPIA-ACE PAI-I (Mitsubishi Chemical Medicine Corporation, Tokyo, Japan), respectively. The AT activity was measured according to the heparin cofactor activity using Testchyme S ATIII (Sekisui Medical, Tokyo, Japan).

Statistical analysis

The data are expressed as medians (95% CIs). The analysis of relationships between the underlying diseases of DIC and DIC outcomes was performed using a chi-square analysis. Differences between groups were examined for statistical significance using the Mann-Whitney U-test. A p value of less than 0.05 was considered to be statistically significant. A multiple logistic regression analysis was performed to detect factors predicting poor outcomes. A multiple linear regression analysis was performed to detect factors related to the SOFA scores. All statistical analyses were performed using the SPSS II software package (SPSS Japan, Tokyo, Japan).

Results

Seventy-seven (31.8%) of the 242 infectious patients with one or more hemostatic abnormalities, such as an abnormal platelet count, FDP level, fibrinogen level or PT ratio, while 36 (46.8%) of the 77 patients with DIC recovered from DIC (Table 1). The presence of sepsis without other infections was most frequently associated with DIC (58.0%), and patients with sepsis without other infections exhibited the highest rate of resolution of DIC (60.0%). In contrast, respi-

Table 1. Subjects

Underlying Diseases	All	DIC	Resolution of DIC	Death (Mortality)
Sepsis without Other Infection	69	40 (58.0%)***	24 (60.0%)*	16 (40%)
Respiratory Infection	67	10 (14.9%)***	3 (30.0%)	6 (60.0%)
Hepatobiliary Pancreatic Infection	24	6 (25.0%)	3 (50.0%)	1 (16.7%)
Digestive Infection	45	14 (31.1%)	2 (14.3%)*	9 (64.2%)
Urinary Infection	17	4 (23.5%)	3 (75.0%)	0 (0%)
Locomotorium Infection	17	1 (5.9%)*	0 (0%)	1 (100%)
Others	3	2 (66.7%)	1 (50.0%)	1 (50.0%)
Total	242	77 (31.8%)	36 (46.8%)	34 (44.2%)

*; p< 0.05, **; p< 0.01, ***; p< 0.001

Table 2. Scores, Factors and Parameters of the Patients with and without DIC

	Without DIC (n=165)	With DIC (n=77)
Age (years old)	72.0 (63.00-78.0)*	69.(58.0-77.0)*
CRP (g/L)	0.13 (0.06-0.22)	0.15 (0.07-0.24)
SOFA score	4.5 (2.0-8.0)***	8.0 (6.0-11.0)***
SIRS score	2.0 (2.0-3.0)***	3.0 (3.0-4.0)
WBC ($\times 10^9$/L)	10.0 (7.2-14.8)	10.8 (5.3-18.5)
PLT ($\times 10^{10}$/L)	12.1 (9.3-18.9)***	3.5 (2.1-6.6)***
PT-INR	1.20 (1.14-1.34)***	1.43 (1.22-1.84)***
FDP (mg/L)	14.5 (9.8-23.3)***	32.0 (20.5-54.4)***
Fibrinogen (g/L)	4.07 (3.02-5.67)***	3.08 (1.42-4.43)***
TAT (μg/L)	13.8 (7.3-21.8)**	19.7 (10.2-42.6)**
PPIC (mg/L)	1.60 (1.00-2.60)	1.70 (1.00-3.70)
D-dimer (mg/L)	6.4 (2.5-12.6)***	16.4 (6.4-29.7)***
SFMC (mg/L)	8.4 (4.2-23.8)***	71.7 (14.3-160.0)***
AT (%)	60.0 (49.7-72.4)***	48.3 (37.0-64.0)***
TM (FU/mL)	4.30 (3.2-5.8)***	7.3 (5.1-9.9)***
PAI-I (μg/L)	34 (24-51)***	93 (31-200)***

*; p< 0.05, **; p< 0.01, ***; p< 0.001

ratory infections were least frequently associated with DIC (14.9%) and patients with respiratory infections exhibited the lowest rate of resolution of DIC (30%). The rate of resolution of DIC was significantly lower in the patients with digestive infections (Table 1). Mortality tended to be high in the patients with respiratory and digestive infections (Table 1). The SOFA score and the levels of PT-INR, FDP, TAT, D-dimer, SFMC, TM and PAI-I were significantly higher in the patients with DIC than in those without DIC. The platelet count and the levels of fibrinogen and AT were significantly lower in the patients with DIC than in those without DIC (Table 2). The DIC score ($p<0.05$), SOFA score ($p<0.01$), PT ratio ($p<0.05$) and TAT ($p<0.05$) were significantly higher in the patients who did not recover from DIC than in those who did (Table 3). Age ($p<0.05$), the DIC score ($p<0.05$), the SOFA score ($p<0.01$), the PT ratio ($p<0.01$), the TM level ($p<0.05$) and the PAI-I level ($p<0.01$) were significantly higher in the non-survivors than in the survivors (Table 3).

A multiple logistic regression analysis was performed in all patients with infections to detect factors predicting the 28-day outcome (Table 4). Factors found to be related to the 28-day outcome included resolution of DIC on day 14 (odds ratio 5.232, p=0.001), the SOFA score (1.205, p=0.006), age (1.057, p=0.024) and the PT ratio (1.888, p=0.039), according to a stepwise regression analysis. Factors found to be related to the 28-day outcome in the 77 patients with DIC included resolution of DIC on day 14 (odds ratio 9.311, p=0.005) and age (1.102, p=0.025), according to a stepwise regression analysis (Table 4). Factors found to be related to resolution of DIC on day 14 in the DIC patients with infections included the SOFA score (1.358, p=0.003) and age (1.060, p=0.015) (Table 5). Factors found to be related to the SOFA score in the DIC patients with infections included the PAI-I level ($p<0.001$), the leukocyte count ($p<0.05$), the fibrinogen level ($p<0.05$), the D-dimer level ($p<0.05$) and the platelet count ($p<0.05$) (Table 6).

Discussion

In this study of DIC caused by infection, the frequency of DIC was 31.8%, the rate of resolution of DIC was 46.8% and the mortality rate of DIC caused by infection was 44.2%. The high mortality of DIC in the patients with infections is similar to that reported in various previous studies (25, 26). Among the patients with infections, the frequency of DIC was highest in those with sepsis without other infections and lowest in those with respiratory infections, suggesting that the SIRS scores are high and leukocytes and vascular endothelial cells are activated in patients with severe sepsis. A high SIRS score has also been reported to be a risk factor for DIC (17), as activated leukocytes highly express tissue factor (27). The rate of resolution of DIC was highest in the patients with sepsis without other infections, suggesting that this condition includes many catheter infections that do not affect organ failure in the early stage of infectious DIC. Catheter infections frequently cause sepsis and abnormalities associated with blood coagulation, such as DIC (28, 29). In this study, the patients with

Table 3. Scores, Factors and Parameters in the Patients who Did and who Did Not Exhibit Resolution of DIC and the Survivors and Non-survivors

	Resolution (n=36)	Non-resolution (n=41)	Survivor (43)	Non-survivor (34)
Age (years old)	64.5 (52.0-73.0)	69.(58.0-77.0)	66 (56.0-73.0)	73.0 (59.8-78.3)*
CRP (g/L)	0.15 (0.09-0.22)	0.13 (0.07-0.23)	0.15 (0.06-0.21)	0.13 (0.07-0.25)
DIC score	7.8 (7.0-8.0)	8.0 (7.0-10.0)*	8.0 (7.0-8.0)	8.0 (7.0-10.0)*
SOFA score	7.0 (4.3-8.8)	9.0 (7.0-12.0)***	8.0 (5.0-9.0)	9.0 (7.0-14.0)*
SIRS score	3.0 (2.0-3.0)	3.0 (3.0-4.0)	3.0 (2.0-4.0)	3.0 (3.0-4.0)
WBC ($\times 10^9$/L)	10.7 (5.5-17.9)	11.0 (5.3-18.5)	10.8 (6.7-18,4)	11.3 (2.5-18.8)
PLT ($\times 10^{10}$/L)	3.5 (2.1-6.1)	3.5 (2.0-7.0)	3.7 (2.1-6.7)	3.1 (1.6-6.2)
PT-INR	1.37 (1.17-1.51)	1.48 (1.33-2.11)*	1.36 (1.18-1.50)	1.61 (1.39-2.28)**
FDP (mg/L)	24.5 (20.0-52.7)	37.5 (21.7-60.9)	32.8 (20.4-52.7)	30.9 (21.4-60.0)
Fibrinogen (g/L)	2.57 (1.59-4.36)	3.26 (1.35-4.66)	263 (156-438)	334 (133-452)
TAT (μg/L)	14.4 (9.5-29.5)	30.5 (11.8-46.4)*	15.8 (10.2-44.4)	21.2 (10.1-41.8)
PPIC (mg/L)	1.50 (1.0-3.25)	1.90 (0.98-3.95)	1.80 (1.03-4.08)	1.50 (0.90-3.20)
D-dimer (mg/L)	19.5 (6.3-28.4)	16.2 (6.5-30.0)	19.0 (6.4-29.6)	14.1 (6.4-29.6)
SFMC (mg/L)	43.8 (12.7-155.0)	95.4 (23.6-197.5)	53.9 (12.9-155.0)	135 (21.6-240.8)
AT (%)	53.0 (34.9-61.0)	46.0 (37.1-67.7)	53.2 (37.0-67.8)	43.9 (36.3-62.1)
TM (FU/mL)	6.3 (4.3-6.8)	7.4 (5.3-10.9)	6.1 (4.1-8.3)	8.6 (6.1-11.7)*
PAI-I (μg/L)	77 (24-197)	158 (31-200)	56 (26-195)	200 (86-200)**

*; p< 0.05, **; p< 0.01, ***; p< 0.001

Table 4. Multiple Logistic Regression Analysis of the 28-day Outcome

Parameters	p value	Odds ratio	95%CI
All patients			
DIC on day 14	0.001	5.232	1.947-14.06
SOFA score	0.006	1.205	1.054-1.378
Age	0.024	1.057	1.007-1.109
PT ratio	0.039	1.888	1.032-3.452
SIRS score	0.076	0.674	0.436-1.041
Patients with DIC			
Parameters	p value	Odds ratio	95%CI
DIC on day 14	0.005	9.311	1.977-43.86
SIRS score	0.120	0.536	0.244-1.177
Age	0.025	1.102	1.013-1.198

Table 5. Multiple Logistic Regression Analysis of Non-resolution of DIC

Parameters	p value	Odds ratio	95%CI
Age	0.015	1.060	1.011-1.111
SOFA score	0.003	1.358	1.111-1.660

Table 6. Multiple Linear Regression Analysis of the SOFA Score in All Patients

Parameters	t	p
PAI-I	5.099	0.00002
Leukocyte count	2.650	0.013
Fibrinogen	2.355	0.025
SFMC	1.845	0.075
D-dimer	2.250	0.032
Platelet count	2.281	0.030
Sex	1.575	0.126

DIC caused by respiratory infections exhibited the lowest rate of resolution of DIC and the highest mortality rate. Under these conditions, acute lung injury and acute respiratory distress syndrome (ARDS) frequently occur, which result in poor outcomes (30). There were significant differences in the levels of hemostatic molecular markers, including D-dimer, SFMC, AT, TM and PAI-I, between the patients with and those without DIC. Similar results have been previously reported in patients with DIC caused by various underlying diseases (31, 32). The differences in the levels of TAT between the patients with and without DIC tended to be smaller than those of SFMC due to decreased levels of AT in the patients with sepsis (32). The SFMC levels are very sensitive for thrombosis (33) and are therefore sensitive to mild DIC.

The DIC score, SOFA score, PT ratio and TAT level were significantly high in the patients who did not recover from DIC, suggesting that resolution of DIC depends on the severity of DIC. Factors found to be related to resolution of DIC included the SOFA score and age, while factors found to be related to the SOFA score included the levels of PAI-I, leukocytes, fibrinogen, D-dimer and platelets. These findings indicate that organ failure and hemostatic abnormalities caused by DIC interact with each other.

The SOFA score and PT ratio have previously been reported to be related to the outcomes of patients with DIC (34). In this study, age, the DIC score, the SOFA score, the PT ratio and the levels of TM and PAI-I were significantly high in the non-survivors, and the factors related to poor outcomes included resolution of DIC, the SOFA score, age and the PT ratio. Although fibrin-related markers, such as FDP, D-dimer and SFMC, are sensitive for the diagnosis of DIC (34), they cannot be used to predict poor outcomes. Therefore, the outcomes of DIC depend on the persistence of DIC and the degree of organ failure. DIC worsens organ

failure, and deterioration of organ failure increases the DIC score. Treatment with APC and AT improves mortality in septic patients with DIC (25, 35), suggesting that these drugs may be effective in septic patients with organ failure.

In conclusion, the outcomes of septic patients depend on the resolution of DIC, which is related to multiple organ failure.

The authors state that they have no Conflict of Interest (COI).

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The relationships among hemostatic markers, the withdrawal of fondaparinux due to a reduction in hemoglobin and deep vein thrombosis in Japanese patients undergoing major orthopedic surgery

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ABSTRACT

Background: The relationships among the hemostatic markers, the development of deep vein thrombosis (DVT) and the withdrawal of fondaparinux due to a reduction in the hemoglobin levels were examined.
Methods: Two-hundred twenty-one Japanese patients who underwent major orthopedic surgery and were treated with 1.5 mg of fondaparinux instead of 2.5 mg of fondaparinux were studied. Forty-seven of 221 patients discontinued fondaparinux treatment (withdrawal group) and 37 patients developed DVT.
Results: The age, frequency of total knee arthroplasty (TKA), withdrawal of fondaparinux, reduction of hemoglobin and the plasma levels of soluble fibrin (SF), D-dimer and fibrinogen and fibrin degradation product (FDP) on day 1 after the operation were significantly higher in the patients with DVT. Elevated SF, D-dimer or FDP levels were associated with the risk for DVT. The age, frequency of TKA or DVT, anti-Xa activity and the creatinine, FDP and D-dimer levels were significantly higher in the withdrawal group. An anti-Xa level >0.33 mg/l and an elevated D-dimer or FDP level were associated with the risk of withdrawal.
Conclusion: The age and SF levels, TKA and withdrawal of fondaparinux were related to the risk of DVT, and the anti-Xa activity, creatinine level and DVT were related to the risk of withdrawal of fondaparinux due to a reduction in hemoglobin.

1. Introduction

The chromogenic antifactor Xa assay measures the concentration of anticoagulants that inhibit factor Xa. The assay measures the extent to which exogenous factor Xa is inhibited by complexes of unfractionated heparin (UFH)-antithrombin (AT), low- molecular weight heparin (LMWH)-AT, or fondaparinux-AT in patients being treated with UFH, LMWH, or fondaparinux, respectively [1]. The anti-Xa assay is reported to be correlated with the weight, body mass index (BMI) and renal function in patients treated with fondaparinux [2].

Preventing the development of deep vein thrombosis (DVT) is clinically important, because a pulmonary embolism (PE) caused by DVT is often fatal. Orthopedic surgery is associated with a high rate of postoperative venous thromboembolism (VTE) [3,4]. The incidence of VTE ranges from 42 to 57% after total hip arthroplasty (THA) in the absence of thromboprophylaxis, and 41 to 85% after total knee arthroplasty (TKA) [5]. Multiple studies [6–8] have established the efficacy of LMWH for VTE prophylaxis in orthopedic surgery patients.

Fondaparinux is the first selective factor Xa inhibitor approved for use in thromboprophylaxis after orthopedic surgery [9–11], and studies comparing fondaparinux to LMWH showed that it provided thromboprophylaxis in patients after orthopedic surgery [10,11]. New oral anticoagulants have recently been developed and these drugs have been compared with fondaparinux as the standard drug for prophylaxis of VTE [12,13]. However, there have been a few cases of massive bleeding in patients administered fondaparinux [14,15]. Therefore, fondaparinux is frequently administered at a dose of 1.5 mg instead of 2.5 mg in Japan to avoid serious bleeding. The administration of 1.5 mg fondaparinux instead of 2.5 mg fondaparinux is recommended for patients who have risk factors

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for bleeding in Japan. This is because about 50% of Japanese orthopedic patients weight <60 kg, so the 2.5 mg dose is relatively high. There was no significant difference in the frequency of DVT in our preliminary study. The anti-Xa activity is used, UFH or LMWH activity, to monitor the anticoagulant activity [16,17].

This study evaluated the anti-Xa activity and hemostatic markers in 221 patients who underwent major orthopedic surgery and were treated with fondaparinux for the prophylaxis of deep vein thrombosis (DVT) in order to examine the relationships among the anti-Xa activity, DVT and/or withdrawal of fondaparinux due to a reduction of hemoglobin (Hb).

2. Materials and methods

Two hundred twenty-one orthopedic patients treated with 1.5 mg of fondaparinux (GlaxoSmithKline, Tokyo, Japan) and intermittent pneumatic compression for prophylaxis of DVT from February 1, 2010, to December 31, 2011 were enrolled in this study. 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. Of these 221 patients, 47 discontinued prophylaxis (withdrawal group), 44 due to a reduction of the hemoglobin level by >2 g/dl compared with day 1 or to a hemoglobin level <7 g/dl. The other 2 patients who discontinued treatment developed DVT, and one other patient had atrial fibrillation, so all 3 patients were changed to other drugs.

The anti-Xa activity, fibrin and fibrinogen degradation products (FDP), D-dimer, soluble fibrin (SF) and AT activity were measured prospectively in the 221 patients who underwent THA or TKA and on days 1, 4, 8 and 15 of the administration of fondaparinux. The diagnosis of DVT was assessed by a whole-leg compression ultrasound examination using standardized ultrasound criteria for venous non-compressibility, and was assessed before the operation, as well as on days 4 and 14 [18]. 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 principles of the Declaration of Helsinki.

The anti-Xa activity was monitored for 3 h after the injection of fondaparinux. The anti-Xa activity of fondaparinux was measured using Testzym®Heparin S (Sekisui Medical Co. Ltd. Tokyo, Japan) and a Coagrex®800 system (Sysmex Co. Ltd. Kobe, Japan). Testzym® Heparin S contains bovine Xa (71 nkat/vial), AT (10 IU/vial), a chromogenic substrate (S-2222: Benz–Ile–Glu–Gly–Arg–pNA·HCl 25 mg), pooled lyophilized normal plasma and a buffer (pH8.4) [2,16]. A standard curve was constructed for lyophilized normal plasma using various concentrations of fondaparinux.

The reagents and objects were loaded into the Coagrex 800 (Sekisui Medical)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-Xa activity of fondaparinux was then calculated using the standard curve. The plasma levels of FDP, D-dimer and SF were measured by the latex agglutination method using Nanopia FDP, Nanopia D-dimer and Nanopia SF reagents (Sekisui Medical) [19]. The plasma levels of AT were measured using a Testzym S ATIII kit (Sekisui).

2.1. Statistical analysis

The data are expressed as the medians (25%–75% tiles). The differences between the groups were examined using the Mann–Whitney *U*-test. The differences between the groups were examined for significance using the Chi-squared test for independence. Cut-off values for the SF, D-dimer and FDP levels were analyzed by a receiver operating characteristic (ROC) curve. A multiple logistic regression analysis was performed to detect the factors predicting the development of DVT or withdrawal of fondaparinux. A *P*-value ≦ 0.05 was considered to be statistically significant. All statistical analyses were performed using Stat flex, ver 6, software package (Artec Co Ltd).

3. Results

Thirty-seven patients developed DVT, but no patients had a PE. The age was significantly higher in the patients with DVT than in the patients without DVT ($p < 0.001$, Table 1). The frequency of DVT was significantly higher in the patients who underwent TKA than in those who underwent THA ($p < 0.001$). There were no significant differences in the sex, body weight, height, body surface area, creatinine and estimated glomerular filtration rate (eGFR) between the patients with and without DVT. The frequency of withdrawal of fondaparinux and the reduction of hemoglobin were significantly higher in the patients with DVT than in those without DVT ($p < 0.01$, respectively). The plasma levels of SF, D-dimer and FDP were significantly higher in the patients with DVT than in those without DVT on day 1 after the operation (Fig. 1A, B and C). There were no significant differences in the anti-Xa activity in the patients who completed the 14 days of treatment with fondaparinux (Fig. 1D), or the AT levels in the patients with and without DVT. The results of the ROC analysis of the SF, D-dimer and FDP levels on day 1 after surgery for predicting the development of DVT are shown in Table 2. Values higher than 13.9 μg/ml of SF, 6.8 μg/ml of D-dimer or 15.7 μg/ml of FDP were associated with a low risk for DVT. In a multiple logistic regression analysis of the association with DVT, the age, TKA and plasma SF level (day 1) were found to be significant (Table 3).

The age was significantly higher in the fondaparinux withdrawal group than in complete administration (CA; 14 days of treatment) group ($p < 0.01$, Table 4). The frequency of withdrawal of fondaparinux was significantly higher in the patients who underwent TKA than in those who underwent THA ($p < 0.05$). There were no significant differences in the weight, height, body mass index (BMI) or body surface area between the withdrawal group and the CA group (Table 4). The eGFRs were significantly lower ($p < 0.01$) and the creatinine levels were significantly higher ($p < 0.01$) in the withdrawal group than in the CA group. The frequency of DVT was also significantly higher in the withdrawal group than in the CA group ($p < 0.01$).

The anti-Xa activity levels were significantly higher in withdrawal group (0.38 mg/l; 0.31–0.45 mg/l) than CA group (0.26 mg/l; 0.18–0.30 mg/l, $p < 0.001$, Fig. 2). There were no significant differences in the SF and AT between the withdrawal group and CA group. The plasma levels of D-dimer and FDP were significantly higher in the withdrawal group than in the CA group on days 1, 8 and 15 after the operation. The results of the ROC analysis of the anti-Xa activity and the SF, D-dimer and FDP levels for predicting the withdrawal of fondaparinux

Table 1
Orthopedic surgery patients with and without DVT.

	With DVT	Without DVT
Number	37	184
Age	75.0 (68.0–80.0)***	64.0 (57.0–72.0)***
Sex (F:M)	32: 5	143:41
THA:TKA	13:24***	155:29***
Withdrawal of fondaparinux	15 (40.5%)**	31 (16.8%)**
Weight (kg)	58.0 (51.9–64.3)	57.0 (49.9–66.2)
Height (cm)	149 (146–156)	153 (149–158)
Body mass index	25.8 (22.7–28.5)*	24.2 (21.2–26.8)*
Body surface area (cm^2)	1.51 (1.45–1.65)	1.53 (1.44–1.66)
Creatinine	0.68 (0.55–0.81)	0.66 (0.54–0.79)
e-GFR	73.3 (56.9–82.3)	75.4 (60.1–88.9)
Reduction of Hb (g/dl)	1.50 (0.98–2.13)**	1.20 (0.50–1.68)**

Hb; hemoglobin, THA; total hip arthroplasty, TKA; total knee arthroplasty.
* $p < 0.05$.
** $p < 0.001$.
*** $p < 0.001$.

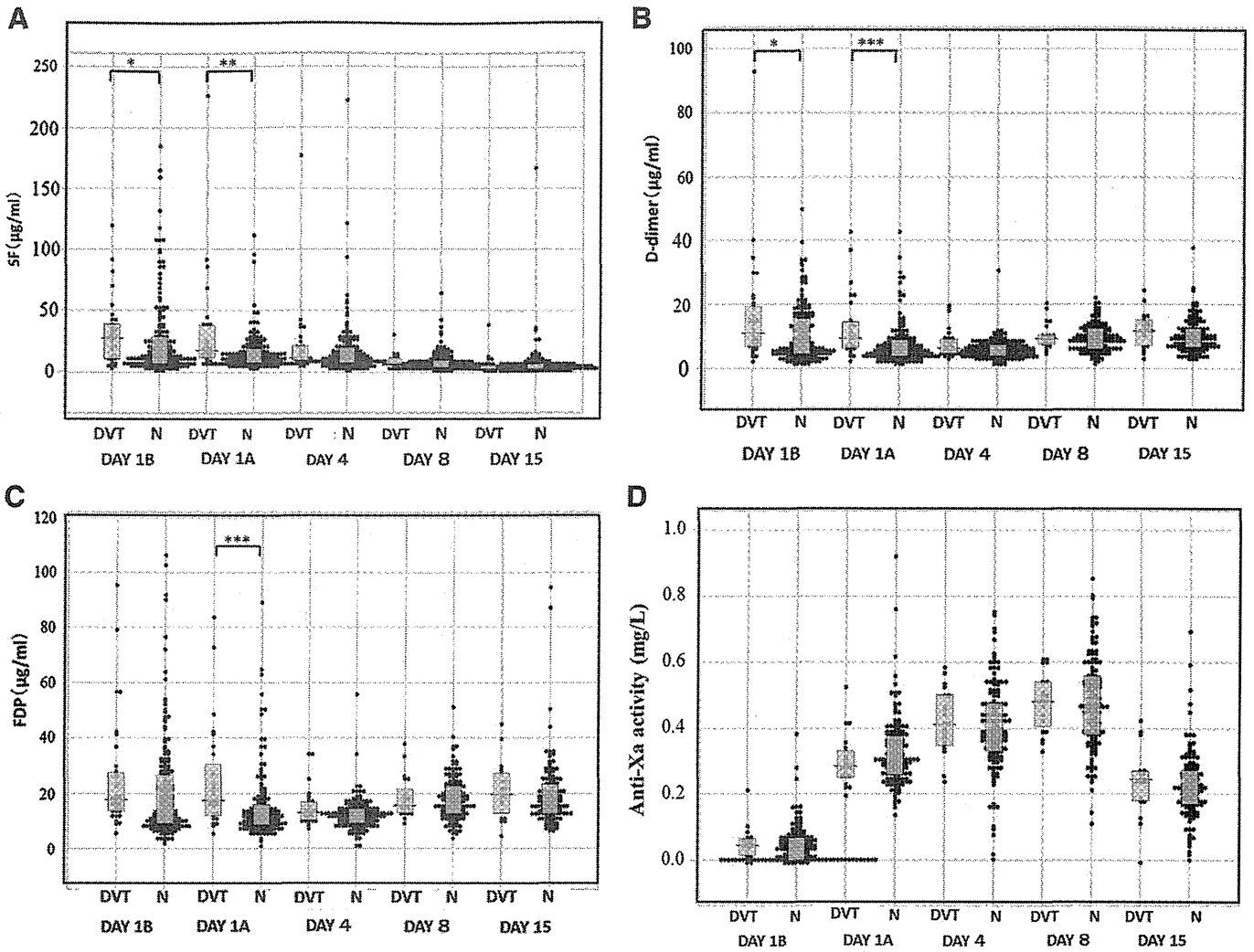

Fig. 1. The plasma levels of SF, D-dimer, FDP and anti-Xa activity in the patients who underwent major orthopedic surgery. The values were measured on days 1, 4, 8 and 15 after the operation. A. The plasma levels of SF, B. the plasma levels of D-dimer, C. the plasma levels of FDP and D. the plasma levels of anti-Xa activity in the complete administration group. For day 1, B indicates the time point before the injection of fondaparinux and A indicates the time period 3 h after the injection of fondaparinux. *; $p < 0.05$, **; $p < 0.01$ ***; $p < 0.001$.

are shown in Table 5. An anti-Xa activity higher than 0.33 mg/l or a level higher than 6.82 μg/ml of D-dimer or 13.3 μg/ml of FDP was associated with a low risk of withdrawal of fondaparinux, but high levels of SF did not show any significant association with the risk of withdrawal. In a multiple logistic regression analysis for the withdrawal of fondaparinux, the anti-Xa activity, creatinine level and the development of DVT were found to be significant predictors (Table 6).

4. Discussion

The efficacy of LMWH or fondaparinux for VTE prophylaxis in orthopedic surgery patients has been established [8,9]. However, there have been some DVT, and also withdrawal cases for both LMWH and fondaparinux due to bleeding. The rates of DVT and withdrawal of fondaparinux in this study were 16.7% and 19.9%, respectively. The frequency of DVT was significantly higher in the withdrawal group, suggesting that the use of fondaparinux for VTE prophylaxis should be continued for 14 days if possible. The age, TKA and plasma levels of SF, D-dimer and FDP were all related to the risk of DVT on day 1 after the operation. The frequency of DVT is reportedly higher for TKA than THA [5]. In the present study, we found that levels higher than 13.9 μg/ml of SF, 6.8 μg/ml of D-dimer or 15.7 μg/ml of FDP on day 1 after the operation were associated with a low risk of DVT. The elevation of these markers after the injection of fondaparinux was reported to be unrelated to the diagnosis of DVT [20]. In a multiple logistic regression analysis in the present study, the age, TKA and plasma SF level were found to be related to the onset of DVT. The plasma levels of D-dimer and FDP were not useful for the diagnosis of DVT, as these markers were still elevated in the patients with massive bleeding such as a reduction of the hemoglobin level by >2 g/dl [15]. Therefore, only SF

Table 2
Results of the ROC analysis of the SF, D-dimer and FDP levels for their prediction of DVT.

	AUC	Cut off value	Sensitivity	Specificity	Odds ratio
SF	0.67	13.9 μg/ml	67.9%	78.2%	3.07
D-dimer	0.71	6.8 μg/ml	69.0%	75.9%	3.70
FDP	0.71	15.7 μg/ml	67.9%	74.3%	6.09

Table 3
Results of the multiple logistic regression analysis for the association with DVT.

	β	SE (β)	Z	P	Odds ratio
Age	0.065	0.027	2.384	0.017	1.067 (1.011–1.126)
TKA	1.401	0.482	2.907	0.004	4.060 (1.578–10.445)
SF	0.020	0.102	0.055	0.040	1.030 (1.000–1.085)

Table 4
The groups of patients with fondaparinux withdrawal due to decreased hemoglobin and those who completed the administration.

	Withdrawal group due to decreased hemoglobin levels	Complete administration group
Number	44	175
Age	72.5 (53.0–79.0)**	64.0 (58.0–72.0)**
Sex (F:M)	28:16	145:30
THA:TKA	28:16*	139:36*
DVT	14 (31.8%)**	22 (12.6%)**
Weight (kg)	57.2 (48.0–67.6)	57.0 (50.5–64.7)
Height (cm)	155 (147–161)	152 (148–158)
Body mass index	24.2 (20.3–26.8)	24.3 (21.7–27.3)
Body surface area (cm^2)	1.51 (1.44–1.67)	1.53 (1.44–1.63)
Creatinine	0.73 (0.62–1.02)**	0.63 (0.53–0.76)**
e-GFR	66.6 (54.8–63.7)**	77.0 (63.7–68.6)**
Hemoglobin (g/dl)	12.2 (11.4–12.1)	12.2 (11.4–12.9)

Hb; hemoglobin, THA; total hip arthroplasty, TKA; total knee arthroplasty.
* $p < 0.05$.
** $p < 0.001$.
*** $p < 0.001$.

is useful for the diagnosis of DVT in orthopedic patients on day one, as the increase in the SF levels was low in these patients with massive bleeding.

One of the main causes of the withdrawal of fondaparinux is reduction of the hemoglobin level. We found that the age, TKA, eGFR, anti-Xa activity on day 1 after operation and the plasma levels of D-dimer and FDP on days 1, 8 and 15 after the operation were significantly higher in the patients who withdrew due to a reduction of the hemoglobin levels. Previous studies [2,15] could not show a relationship between the anti-Xa activity and massive bleeding or DVT. Although the previous studies were small, the present study had a sufficient number of patients for a statistical analysis. The current findings showed a broad range of anti-Xa activity on day 1 after the operation, with a peak of the first day of fondaparinux administration. Although all patients in this study were treated with 1.5 mg of fondaparinux to avoid massive bleeding, a value of 0.33 mg/l or higher of anti-Xa activity, a D-dimer level >6.82 μg/ml or a FDP level > 13.3 μg/ml showed a low risk of the withdrawal of fondaparinux. These findings suggested the usefulness of the monitoring the anti-Xa activity on day one. Our findings indicate that the anti-Xa activity should be controlled so that it remains lower than 0.3 mg/l on day one in order to reduce the risk of fondaparinux withdrawal. However, the current results showed no relationship between the anti-Xa activity and the frequency of DVT or hemoglobin reduction on days 4 and 8. The wound due to surgery is not repaired on day one, thus, the control of Xa activity might be most important

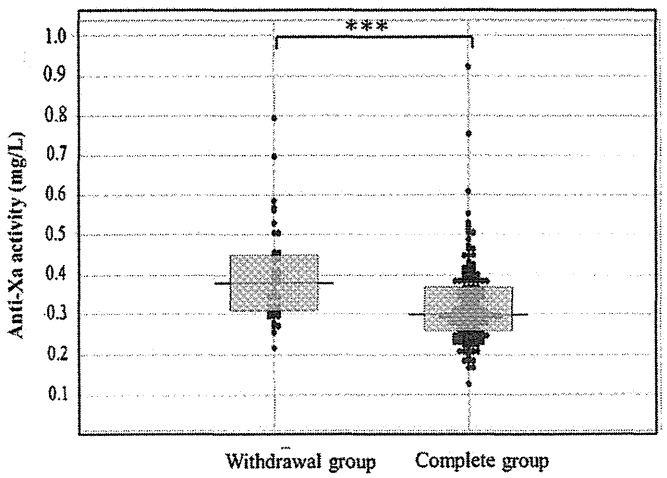

Fig. 2. The anti-Xa activity in the withdrawal and complete administration groups. G; group. ***; $p < 0.001$.

Table 5
ROC analysis of anti-Xa activity, SF, D-dimer and FDP for withdrawal due to the reduction of hemoglobin.

	AUC	Cut off value	Sensitivity	Specificity	Odds ratio
Anti-Xa	0.62	0.33 mg/L	62.2%	62.6%	2.76
SF	0.44	11.4 μg/ml	44.7%	40.6%	0.55
D-dimer	0.66	6.82 μg/ml	62.5%	63.5%	2.90
FDP	0.62	13.3 μg/ml	60.5%	61.8%	2.48

Table 6
Multiple logistic regression analysis of withdrawal of fondaparinux.

	β	SE (β)	Z	P	Odds ratio
Anti-Xa activity	6.427	1.661	3.870	0.0001	618.3 (23.9–16021)
Creatinine	3.293	0.912	3.612	0.0003	26.93 (4.511–160.8)
DVT	1.214	0.456	2.665	3.369	3.369 (1.379–8.230)

from day one to day three after the operation. In a multiple logistic regression analysis, the anti-Xa activity, creatinine level and development of DVT were significantly related to the withdrawal of fondaparinux. Therefore, the control of the anti-Xa activity may be especially important on day one after the operation.

Finally, the continuous administration of an anticoagulant is important to prevent DVT, and strict control of the anti-Xa activity on the first several days of fondaparinux administration might be necessary to reduce the frequency of VTE or a marked reduction in hemoglobin.

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A first bout of thrombotic thrombocytopenic purpura triggered by herpes simplex infection in a 45-year-old nulliparous female with Upshaw-Schulman syndrome

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Dear Sir,

Upshaw-Schulman syndrome (USS) is a congenital deficiency of ADMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motifs, 13) activity caused by gene mutations. ADAMTS13 specifically cleaves unusually large von Willebrand factor multimers produced in and released from vascular endothelial cells under high shear stress conditions in the microvasculature[1,2]. Thus, in the absence of ADAMTS13 activity, the uncleaved unusually large von Willebrand factor multimers are released into the circulation, causing a life-threatening systemic disease termed thrombotic thrombocytopenic purpura (TTP). Most cases of TTP are induced by acquired autoantibodies against this enzyme. USS is an extremely rare disease, and to date approximately 100 affected patients have been reported in the literature, of whom 43 are in Japan[3].

According to our experience in Japan, bouts of TTP in USS patients are triggered by various stimuli, including pregnancy, severe infection, administration of 1-deamino-8-D-arginine vasopressin (DDAVP) and drinking large amounts of alcohol. Pregnancy is the single most common trigger in female patients. In fact, in an analysis of the natural history of our 43 USS patients in Japan, we found that 26 (60%) were diagnosed during childhood (early-onset phenotype), and the remaining 17 (40%) were diagnosed after 15 years of age (late-onset phenotype). In the early-onset group, the female:male ratio was 13:13, while it was 14:3 in the late-onset group. These 14 female patients were aged between 15 and 45 years, and nine were diagnosed during pregnancy. In contrast, all three male patients had their first bouts after 45 years of age. With regards to ADAMTS13 activity, 35 patients had extremely low levels (<0.5% of normal), seven had trace amounts (0.5-0.8% of normal), and one male patient (USS-GG2) who had his first bout of TTP at 63 years of age had some activity (2.4-3.6%). Thus, one important determinant of the late-onset phenotype in USS patients is the level of ADAMTS13 activity. However, here we present the late-onset phenotype found in a middle-aged nulliparous USS female with severe deficiency of ADAMTS13 activity (<0.5% of the normal), whose first bout of TTP was triggered by an oral herpes simplex infection at the age of 45.

The proband (USS-Y3), born in Sapporo in 1960, was the first of three siblings born to non-consanguineous parents. Her parents and two brothers have had no episodes of thrombosis or excessive bleeding. Her perinatal medical history was unclear, but she did not have any exchange blood transfusions as a neonate. By the age of 3, she suffered from repeated episodes of thrombocytopenia and was diagnosed with idiopathic thrombocytopenic purpura, for which she received transfusions of fresh whole blood on a few occasions. Further details of her medical history during childhood were unavailable. Since the age of 38, her platelet count has been occasionally evaluated at a nearby hospital. The counts were almost normal (104-175×10^9/L). However, when she has a cold, her platelet count temporarily drops to less than 50×10^9/L (minimum 19×10^9/L), but normalises without any specific medical therapy. At the age of 45, she suffered from an oral herpes simplex infection complicated by thrombocytopenia (11×10^9/L), for which the antiviral aciclovir (1,000 mg/day) was prescribed. Subsequently, she has had repeated episodes of oral herpes simplex infection; therefore, she received a prescription of acyclovir for 5 months but has not had an appreciable clinical improvement. She was referred to our hospital for analysis of the cause of her thrombocytopenia. Laboratory findings on admission were as follows: thrombocytopenia (9×10^9/L), haemolytic anaemia (red cell count 1.84×10^9/L, haemoglobin 6.7 g/dL, reticulocyte 168‰, schistocytes on a peripheral smear [2+], total bilirubin 2.8 mg/dL, lactate dehydrogenase 872 IU/L, and haptoglobin <10 mg/dL), near-normal renal function (blood urea nitrogen 19 mg/dL, creatinine 1.07 mg/dL, and positive occult blood in urine), C-reactive protein 0.1 mg/dL, negative direct and indirect Coombs' tests, and normal haemostatic

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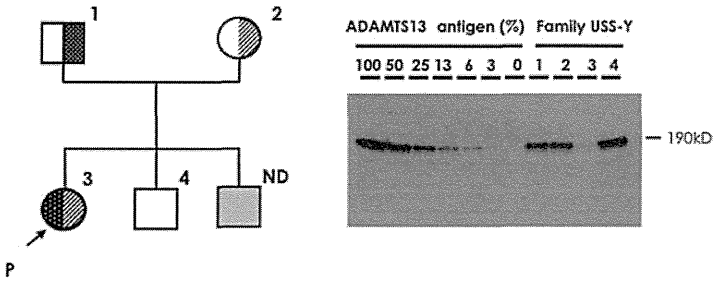
tests. She was initially treated with oral prednisolone (50 mg/day) for a diagnosis of Coombs-negative Evans syndrome, but soon thereafter her general condition worsened, and ADAMTS13 analyses were performed for diagnostic purposes.

The family pedigree of this patient is shown in Figure 1A (left). The patient had very low ADAMTS13 activity (<0.5% of normal) and an absence of ADAMTS13 inhibitor (<0.5 BU/mL). Both of her parents had mild deficiencies of ADAMTS13 activity (both 34%) without its inhibitor. Plasma levels of ADAMTS13 activity in one of her younger brothers were normal (74%). Plasma levels of ADAMTS13 antigen as analysed by enzyme-linked immunosorbent assay (Figure 1B) were 2.1% of the normal control in the patient, and 25%, 22%, and 104% of normal in her father, mother, and

A



B

ADAMTS13 analysis in USS –Y family

	ADAMTS13 activity (%)	ADAMTS13 antigen (%)		*ADAMTS13 gene*		
	ELISA	ELISA	WB	Gly385	Gln448	Arg1206
Y-1	34	25	52	G/E	Q/E	R/R
Y-2	34	22	58	G/G	Q/Q	R/*
Y-3	<0.5	2.1	4	G/E	Q/Q	R/*
Y-4	74	104	102	G/G	Q/E	R/R

C

***ADAMTS13* mutations found in family Y**

Exon	Family member	Nucleotide	Amino acid	Remarks
5	Y1, Y4	c. 420 T>C	silent	common SNP
10	Y1, Y3	c. 1154 G>A	p.G385E	disintegrin-like domain
12	Y1, Y4	c. 1342 C>G	p.Q448E	common SNP
15	Y1, Y4	c. 1716 G>A	silent	common SNP
19	Y1, Y2, Y3, Y4	c. 2280 C>T	silent	common SNP
26	Y2, Y3	c. 3616 C>T	p.R1206*	CUB-1 domain
29	Y1, Y4	c. 4221 C>A	silent	common SNP

Figure 1 - The family pedigree of USS-Y is shown in Figure 1A (left). Squares and circles indicate males and females, respectively, and the arrow with P indicates the proposita. Filled symbols represent a patient of USS-Y3. The half-filled symbols represent asymptomatic carriers. Figure 1A (right) shows western blot (WB) analysis of ADAMTS13 antigen followed by anti-ADAMTS13 monoclonal antibody detection using plasma samples, according to the previous method. Note a trace amount of ADAMTS13 antigen in USS-Y3 (patient) in lane 3 of Figure 1A (right). ND indicates not determined. ADAMTS13 activity was measured by chromogenic act-enzyme linked immunosorbent assay (ELISA), and the ADAMTS13 antigen was determined by both WB and antigen-ELISA (Figure 1B). The ADAMTS13 gene mutations found in this family are shown as one-letter amino acid abbreviations (Figure 1B). The ADAMTS13 single nucleotide polymorphisms (SNP) are also shown in Figure 1C.

younger brother, respectively. Furthermore, as analysed by western blot, plasma levels of ADAMTS13 antigen (Figure 1A right and B) were 4% of the normal control in the patient, and 52%, 58%, and 102% of normal in the father, mother, and younger brother, respectively. *ADAMTS13* gene analysis revealed that the patient was a compound heterozygote for two mutations in *ADAMTS13*: p.G385E (c.1154G>A, exon 10) from her father and p.R1206* (c.3616 C>T, exon 26) from her mother. Her parents were heterozygous carriers of each of the two mutations (Figure 1B). These two mutations were not found in her younger brother. p.Q448E was reported as a single nucleotide polymorphism causing a missense mutation[4]. All mutations found in this family are shown in Figure 1C, including common single nucleotide polymorphism without amino acid substitutions. We previously reported the p.R1206X nonsense mutation in a USS-I4 patient[5]. The p.G385E missense mutation presented here is novel. Our experience indicates that the clinical phenotype of females with USS who have never been pregnant is almost indistinguishable from that of males.

Acknowledgements

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Conflict of interest disclosure

Yoshihiro Fujimura is a member of clinical advisory boards for Baxter BioScience.

All other Authors declare no conflicts of interest.

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ORIGINAL ARTICLE

Crystal structure and enzymatic activity of an ADAMTS-13 mutant with the East Asian-specific P475S polymorphism

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Summary. *Background:* An East Asian-specific P475S polymorphism in the gene encoding ADAMTS-13 causes an approximately 16% reduction in plasma ADAMTS-13 activity. *Objectives:* To demonstrate the impact of this dysfunctional polymorphism by characterizing the structure and activity of the P475S mutant protein. *Methods:* We determined the crystal structure of the P475S mutant of ADAMTS-13-DTCS (DTCS-P475S, residues 287–685) and compared it with the wild-type structure. We determined the enzymatic parameters of ADAMTS-13-MDTCS (residues 75–685) and MDTCS-P475S, and further examined the effects of denaturants and reaction temperature on their activity. We also examined the cleavage of shear-treated von Willebrand factor (VWF) by MDTCS-P475S. *Results:* MDTCS-P475S showed a reaction rate similar to that of wild-type MDTCS, but showed two-fold lower affinity for the peptidyl substrate, indicating that the Pro475-containing V-loop (residues 474–481) in the C_A domain is a substrate-binding exosite. Structural analysis showed that the conformation of the V-loop was significantly different in DTCS-P475S and the wild type, where no obvious interactions of Ser475 with other residues were observed. This explains the higher susceptibility of the enzymatic activity of MDTCS-P475S to reaction environments such as denaturants and high temperature. MDTCS-P475S can moderately cleave shear-treated VWF. *Conclusions:* We have provided structural evidence that the P475S polymorphism in ADAMTS-13 leads to increased local structural instability, resulting in lowered affinity for the substrate without changing the reaction rate. The moderate activity of ADAMTS-13-P475S for shear-treated VWF is sufficient to prevent thrombotic thrombocytopenic purpura (TTP) onset.

Keywords: ADAMTS-13, crystallography, genetic polymorphism, human, proteins, thrombotic thrombocytopenic purpura, von Willebrand factor.

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Introduction

von Willebrand factor (VWF) is a plasma glycoprotein synthesized primarily in vascular endothelial cells and megakaryocytes [1]. VWF is released into plasma as ultralarge multimeric forms (ultralarge VWF [UL-VWF]) that are highly active in platelet aggregation. ADAMTS-13 specifically cleaves the Tyr1605–Met1606 bond within the A2 domain of VWF in a fluid shear stress-dependent manner, and controls platelet thrombus formation [2,3]. Severe deficiency in ADAMTS-13 activity, caused by either genetic mutations or acquired autoantibodies against ADAMTS-13, results in the accumulation of UL-VWF in plasma, which leads to the hyperaggregation of platelets. This prothrombotic condition can cause thrombotic thrombocytopenic purpura (TTP) [4].

The human *ADAMTS13* gene encodes a precursor protein of 1427 amino acids with a modular structure consisting of a signal peptide, a propeptide, a metalloprotease (M) domain, a disintegrin-like (D) domain, a thrombospondin type 1 repeat (TSR) (T1), a cysteine-rich (C) domain, a spacer (S) domain, seven TSRs (T2–T8), and two CUB domains [5–7]. In addition to the causative mutations for TTP, a number of missense mutations and polymorphisms have been identified in *ADAMTS13* [6,8,9]. Among them, P475S (c.1423C>T) is a dysfunctional missense polymorphism with a minor allele frequency of 5.0% [8,10]. Subjects carrying the minor

allele residue (serine) showed ~ 16% lower ADAMTS-13 activity than those without the polymorphism. The P475S polymorphism has also been identified in Koreans (allele frequency of 4.0%) [11] and Chinese (1.5%) [12], but is absent in Caucasians [13], suggesting that ADAMTS-13-P475S is an East Asian-specific natural dysfunctional mutant [14]. An in vitro study demonstrated that ADAMTS-13-P475S is normally secreted from cultured cells. However, the culture medium containing ADAMTS-13-P475S showed greatly reduced enzymatic activity (~ 10%) in the VWF multimer assay [8]. On the other hand, partially purified ADAMTS-13-P475S showed ~ 70% of wild-type activity in an assay with a synthetic peptidyl fluorogenic substrate, FRETS-VWF73 [15]. The difference in enzymatic activity of ADAMTS-13-P475S between the two assays was probably attributable to the presence and absence of urea in the reaction mixture [15]. These experiments were performed with ADAMTS-13-containing culture medium or partially purified ADAMTS-13; therefore, analysis of enzyme kinetics with the purified protein remains to be performed.

Several studies have indicated that ADAMTS-13-MDTCS has VWF-cleaving activity that is nearly identical to that of full-length ADAMTS-13 in vitro [16,17]. We recently determined the crystal structures of ADAMTS-13-DTCS [18]. The C domain was further divided into the globular C_A domain and elongated C_B domain. Extensive structure-based mutagenesis indicated that ADAMTS-13 can bind to VWF through at least three VWF-binding exosites on the linearly aligned discontinuous surfaces of the D, C_A and S domains [18], and this substrate-binding mode with multiple binding sites is supported by other studies [19–22]. The Pro475 in question is located in the V-loop (residues 474–481) of the C_A domain. Mutations in the V-loop of the C_A domain resulted in significantly reduced enzymatic activity, suggesting that the V-loop creates a VWF-binding exosite [18].

In this study, we determined the crystal structure of DTCS-P475S, and characterized the enzymatic activity of MDTCS-P475S. The present study provides evidence that the P475S substitution in ADAMTS-13 destabilizes the local conformation of the V-loop in the C_A domain, resulting in lowered substrate affinity without changing the reaction rate. Furthermore, the moderate cleavage of shear-treated VWF by ADAMTS-13-P475S suggests that the VWF-cleaving activity of the mutant is sufficient to prevent TTP onset.

Materials and methods

Preparation, crystallization and structural analysis of DTCS-P475S

Production of DTCS-P475S was performed with a previously described method [23], with some modifications. Briefly, a stable cell line (HEK293S GnTI$^-$ cells) [24] secreting DTCS-P475S (residues 287–685) with a C-terminal tobacco etch virus (TEV) proteinase cleavage site followed by tandem His-tag sequences was selected and cultured. The culture medium was first concentrated with 50% (w/v) ammonium sulfate, and DTCS-P475S was purified by Ni^{2+}–nitrilotriacetic acid (NTA) agarose column chromatography (Sigma-Aldrich, St Louis, MO, USA). After digestion with TEV proteinase, DTCS-P475S was further purified with a Resource S cation-exchange column (GE Healthcare, Hatfield, UK), concentrated to 10 mg mL^{-1}, and crystallized in 20% (w/v) poly(ethylene glycol) 1500 and 100 mM Mes (pH 6.0), with the same method as described for wild-type DTCS [23]. The diffraction data were collected at the SPring-8 beamline BL38B1 by use of a Rayonix MX225HE CCD detector with a wavelength of 1.0 Å at 100 K. The structure of DTCS-P475S was solved with the molecular replacement method, with the MOLREP program of the CCP4 suite [25], and the structure of wild-type DTCS (Protein Data Bank [PDB] ID: 3GHM) as a starting model. After manual rebuilding with COOT [26], the model was refined with the REFMAC program in CCP4 [25] and CNS [27]. Data collection and refinement statistics are summarized in Table S1. Figures were generated with the PYMOL Molecular Graphics System (Version 1.5; Schrödinger, LLC, Boston, MA, USA). The atomic coordinates of DTCS-P475S have been deposited in the PDB (ID: 3VN4).

Expression and purification of MDTCS and MDTCS-P475S

Recombinant MDTCS and MDTCS-P475S (residues 75–685) were expressed in CHO Lec 3.2.8.1 cells [23] with a BelloCell Cell Culture System (CESCO Bioengineering, Taichung, Taiwan). After 50% (w/v) ammonium sulfate precipitation of the culture medium, MDTCS and MDTCS-P475S were each purified with an Ni^{2+}–NTA column followed by a Resource S cation-exchange column. Both recombinant proteins, when resolved on an SDS–polyacrylamide gel and stained with Coomassie Brilliant Blue, showed a single band of molecular mass 74 kDa, which coincided well with the molecular mass of 75 kDa estimated from their sequences. The protein concentration was determined by use of the 660-nm Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as a protein concentration standard, and adjusted to 1.8 nM in 10 mM Hepes (pH 7.4), 150 mM NaCl, 0.005% Tween-20, and 50% glycerol.

Kinetic analysis of FRETS-VWF73 cleavage by MDTCS and MDTCS-P475S

The kinetic parameters of MDTCS and MDTCS-P475S were determined with FRETS-VWF73 (Peptide Institute, Minoh, Japan), as described previously [28,29]. FRETS-VWF73 (0–4 μM) was incubated with 0.18 nM MDTCS or MDTCS-P475S in 10 mM Hepes (pH 7.4), 150 mM NaCl

and 0.005% Tween-20 at 37 °C. Fluorescence intensities were measured with the M × 3000p QPCR System (Agilent Technologies, Santa Clara, CA, USA) equipped with 340-nm excitation and 450-nm emission filters. The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 to 60 min with PRISM 5 software (GraphPad Software, La Jolla, CA, USA). To obtain the reaction rate, various amounts of FRETS-VWF73 (5, 10, 20 and 50 μmol) were completely cleaved with 10 nM ADAMTS-13-MDTCS for 90 min, and their fluorescence intensities were used to estimate the amount of cleaved product (Fig. S1). The reaction rates as a function of substrate concentration were fitted to the Michaelis–Menten equation, and the maximum velocity (V_{max}), the rate constant (k_{cat}) and the affinity (K_m) were calculated with PRISM 5.

Cleavage of shear-treated VWF by MDTCS and MDTCS-P475S

The cleavage of shear-treated VWF by ADAMTS-13 was performed as described previously [30], with some modifications. Briefly, purified plasma VWF (25 μg mL^{-1}, 100 nM VWF monomers) [31] was vortexed at a rotation rate of 2500 r.p.m. for the indicated times in 10 mM Hepes (pH 7.4), 150 mM NaCl and 0.005% Tween-20 at 24 °C on a digital vortex mixer (Scientific Industries, Bohemia, NY, USA). MDTCS or MDTCS-P475S (1 nM each) was added to the VWF solution and incubated for the indicated times at 37 °C. The digested samples were separated by SDS-PAGE under reducing conditions, and transferred to a poly(vinylidene difluoride) membrane. The cleavage products were detected by western blotting with horseradish peroxidase-conjugated anti-VWF polyclonal antibody (Dako, Carpinteria, CA, USA) and the Luminata Forte Chemiluminescent Reagent (Millipore, Billerica, MA, USA). The band intensities of the cleavage products (150 kDa) were quantified with MULTI GAUGE software (Fuji Film, Tokyo, Japan).

Effects of denaturants and temperature on FRETS-VWF73 cleavage by MDTCS and MDTCS-P475S

MDTCS or MDTCS-P475S (1 nM each) was mixed with 1 μM FRETS-VWF73 in 10 mM Hepes (pH 7.4), 150 mM NaCl and 0.005% Tween-20 containing urea (0–2.5 M) or guanidine-HCl (0–0.5 M), and incubated for 2 min at 37 °C; the fluorescence intensities were then measured at 37 °C for 60 min. To investigate the effects of reaction temperature, the cleavage reaction mixtures were incubated for 2 min at 37, 40, 45 and 50 °C, and the fluorescence intensities were then measured for 60 min at the respective temperatures. The reaction rate was calculated by performing linear regression analysis of the plot of fluorescence against time from 0 to 60 min with PRISM 5.

Results

Crystal structure of DTCS-P475S

The overall structure of DTCS-P475S refined at 2.8-Å resolution is shown in Fig. 1A. The structure includes ADAMTS-13 residues 298–324, 328–458, and 466–682. The backbone structure of DTCS was very similar to the previously solved wild-type DTCS structure [18], with an overall root mean square deviation of 0.421 Å for 369 Cα atoms (Fig. 1B). The electron densities associated with the V-loop (Val474–Ala481) were clearly observed in the current DTCS-P475S structure (Fig. 1C), enabling a detailed structural comparison between the wild type and the mutant. The conformation of the V-loop in the C_A domain of DTCS-P475S was significantly different from that in two previously determined DTCS structures. In DTCS, an oxygen atom in the main chain of Pro475 formed hydrogen bonds with the side chains of Ser477 and Gln478, the distances of which were 3.5 Å/3.8 Å and 3.1 Å/3.5 Å, respectively (calculated from two DTCS structures) (PDB ID: 3GHM/3GHN) (Fig. 1D). On the other hand, these distances in DTCS-P475S were 5.0 Å and 8.3 Å, respectively (Fig. 1E). In the DTCS structure, Pro475 also formed van der Waals contacts with Met509 in the C_A domain (3.8 Å/3.5 Å) and with Leu620 in the β6–β7 loop of the S domain (3.5 Å/4.0 Å), and stabilized the structure (Fig. 1D). The distances between Ser475 and Met509 (7.1 Å), and between Ser475 and Leu620 (5.1Å), were longer in DTCS-P475S than in DTCS, where the interactions no longer occurred (Fig. 1E). The lack of obvious interactions of Ser475 with other residues in the C_A and S domains in DTCS-P475S suggests that the V-loop structure is less stable in DTCS-P475S than in DTCS. The structures of the other loops in the C_A domain of DTCS-P475S did not differ significantly from those of DTCS.

Electron densities for the carbohydrate moieties of two potential *N*-linked sites (Asn552 and Asn614) and a potential *O*-linked site (Ser399) were present in both DTCS-P475S and DTCS [18]. An electron density linked to the side chain of Trp387 in the T1 domain was detected in DTCS-P475S (Fig. 1F). Trp387 is a conserved *C*-mannosylation site (WXXW, where the first tryptophan would be glycosylated), and *C*-mannosylation has been observed on conserved tryptophan residues in a number of TSRs [32], including ADAMTS-5 [33], suggesting that Trp387 is possibly *C*-mannosylated, although this modification was not clear in the electron densities of wild-type DTCS structures. In *C*-mannosylation, a mannose group is added to the C2 atom of the tryptophan.

Kinetic parameters of MDTCS and MDTCS-P475S

We measured the ADAMTS-13 activities of MDTCS and MDTCS-P475S with FRETS-VWF73, and determined their kinetic parameters. The cleavage reaction was monitored as

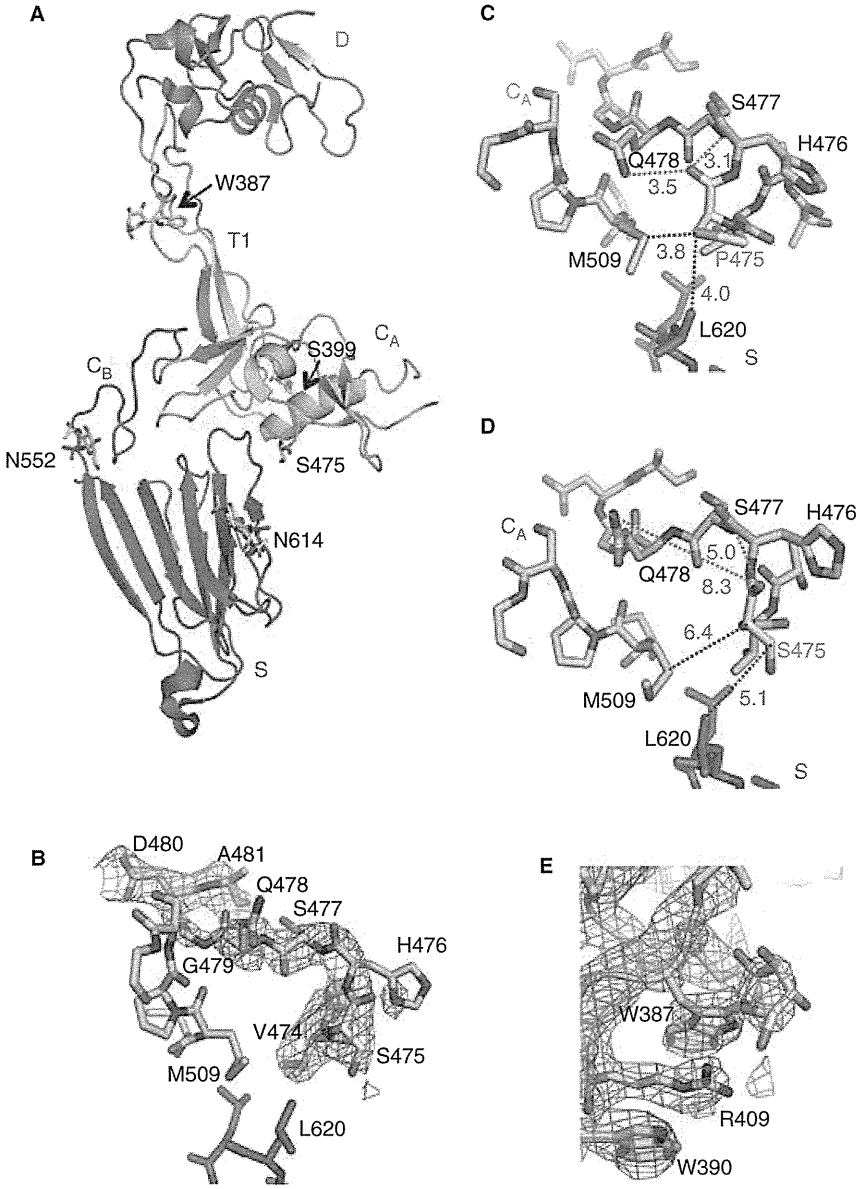

Fig. 1. Crystal structure of DTCS-P475S. (A) Overall structure of DTCS-P475S. Potential *N*-glycosylation at Asn552 (in the C_B domain) and Asn614 (in the S domain), *O*-fucosylation at Ser399 (in the T1 domain), and *C*-mannosylation at Trp387 (in the T1 domain) are shown as stick models. D, orange; T1, cyan; C_A, green; C_B, red; S, magenta. (B) A $2F_o - F_c$ electron density map (contoured at 1.2σ) associated with the V-loop in the C_A domain of DTCS-P475S. (C, D) Close-up view around the V-loop of the C_A domain in DTCS (C) and DTCS-P475S (D). The magenta and blue dotted lines show the hydrogen bonds and van der Waals contacts, respectively, of Pro475 in DTCS (C, PDB ID: 3GHN). For comparison, the corresponding lines are also shown in DTCS-P475S (D). The blue numbers show the distances between the atoms (Å). (E) A $2F_o - F_c$ electron density map (contoured at 1.2σ) is associated with the carbohydrate moiety linked to Trp387, most likely mannose.

an increase in the fluorescence of cleaved FRETS-VWF73, and converted to the reaction rate. The reaction showed typical Michaelis–Menten kinetics (Fig. 2). The V_{max} values of MDTCS and MDTCS-P475S were the same (0.35 nM s^{-1}), and their k_{cat} values were similar (MDTCS, 1.94 ± 0.08 s^{-1}; MDTCS-P475S, 1.90 ± 0.11 s^{-1}; Table 1). On the other hand, the K_m of MDTCS-P475S (0.82 ± 0.12 μM) was two-fold higher than that of MDTCS (0.37 ± 0.06 μM). These results indicated that the P475S substitution in MDTCS resulted in a two-fold reduction in catalytic efficiency (k_{cat}/K_m), mainly because of its lower affinity for FRETS-VWF73. We performed a thermal shift assay involving MDTCS and MDTCS-P475S by using SYPRO Orange (Fig. S2). The T_m values of MDTCS and MDTCS-P475S were identical in the absence (50 °C) and presence (46 °C) of 1.5 M urea.

Shear-treated VWF cleavage by MDTCS and MDTCS-P475S

As the scissile Tyr1605–Met1606 bond of VWF is buried within the core of the globular A2 domain [34], VWF under static conditions is not a good substrate for ADAMTS-13.

Table 1 Michaelis–Menten kinetic parameters of MDTCS and MDTCS-P475S

	MDTCS	MDTCS-P475S
K_m (μM)	0.37 ± 0.06	0.82 ± 0.12
k_{cat} (s^{-1})	1.94 ± 0.08	1.90 ± 0.11
k_{cat}/K_m (μM^{-1} s^{-1})	5.26 ± 0.52	2.32 ± 0.67
V_{max} (nM s^{-1})	0.35 ± 0.02	0.35 ± 0.02

Values are means ± standard deviation.

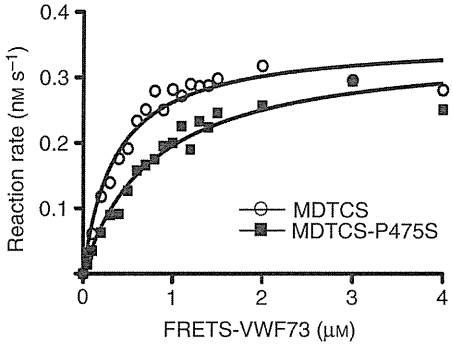

Fig. 2. Kinetic analysis of the cleavage of FRETS-VWF73 by MDTCS and MDTCS-P475S. FRETS-VWF73 (0.05–4 μM) was incubated with MDTCS (open circles, 0.18 nM) or MDTCS-P475S (closed squares, 0.18 nM) at 37 °C. The reaction rate was obtained from the increase in fluorescence over time. The line represents the non-linear fit to the Michaelis–Menten equation. VWF, von Willebrand factor.

When VWF is subjected to shear stress in circulation in vivo or denaturants in vitro, the A2 domain unfolds and adopts a partially extended conformation that makes its scissile peptide bond accessible for cleavage by ADAMTS-13 [19,35]. VWF treated with mechanistic-induced flow on a vortex mixer can be easily cleaved by ADAMTS-13 [30], so this fluid shear-treated VWF-cleaving assay is useful for the investigation of ADAMTS-13 function and regulation. We examined the activities of MDTCS and MDTCS-P475S against shear-treated VWF. VWF cleavage was monitored by the appearance of the 150-kDa band on western blotting with the anti-VWF antibody. Although both MDTCS and MDTCS-P475S produced the 150-kDa fragment band after a 10-min incubation, the band intensity was weaker for MDTCS-P475S (63% ± 8.2% at 120 min) than for MDTCS (Fig. 3A). Shearing treatment of MDTCS-P475S did not affect VWF-cleaving activity (Fig. 3B). These results suggest that MDTCS-P475S moderately cleaves VWF, once the scissile bond in VWF is exposed by shear stress. Shear treatment-induced cleavage of VWF by MDTCS-P475S was more severely inhibited than that by MDTCS upon the addition of urea. The VWF-cleaving activities of MDTCS and MDTCS-P475S in the presence of 1.5 M urea were 47.0% ± 6.5% and 8.9% ± 1.9%, respectively (Fig. 3C).

Effects of denaturants and temperature on the enzymatic activities of MDTCS and MDTCS-P475S

As the addition of urea severely inhibited the shear-dependent cleavage of VWF by MDTCS-P475S, we examined the effects of denaturants on the activity of MDTCS and MDTCS-P475S by using FRETS-VWF73. The reaction rates of MDTCS and MDTCS-P475S were

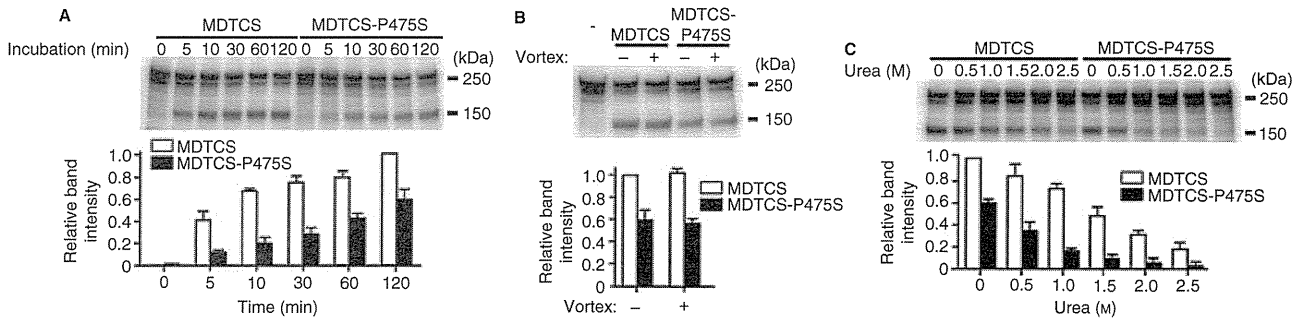

Fig. 3. Cleavage of shear-treated von Willebrand factor (VWF) by MDTCS and MDTCS-P475S. (A) Top: western blotting image showing VWF cleavage at each time point. The VWF multimer (25 μg mL^{-1}; 100 nM VWF monomers) was treated with shear with a 3-min vortex at 2500 r.p.m. at 24 °C, and MDTCS or MDTCS-P475S (1 nM) was then added to the reaction mixture. After incubation for the indicated times (0–120 min) at 37 °C, VWF cleavage was detected by the appearance of the 150-kDa band on western blotting with the anti-VWF antibody. Bottom: densitometric analysis of VWF cleavage. The band intensities of the cleavage products (150 kDa) were quantified as described in Materials and Methods. Band intensities relative to that of MDTCS at 120 min (set as 1) were plotted as means ± standard deviation (n = 3). (B) Top: western blotting image showing VWF cleavage by shear-treated or untreated MDTCS or MDTCS-P475S. The VWF multimer (25 μg mL^{-1}) was treated by shearing with vortexing for 3 min, and MDTCS or MDTCS-P475S (1 nM each), treated with shearing (2500 r.p.m. for 10 min), was then added to the reaction mixture. After incubation for 120 min at 37 °C, the cleavage of shear-treated VWF was detected as described in (A). Bottom: densitometric analysis of VWF cleavage. Band intensities relative to that of MDTCS without vortex (set as 1) were plotted as means ± standard deviation (n = 3). (C) Top: western blotting image showing VWF cleavage at various concentrations of urea. The VWF multimer (25 μg mL^{-1}) was treated with shear with a 3-min vortex, and MDTCS or MDTCS-P475S (1 nM each) was then added to the reaction mixture containing 0–2.5 M urea. After incubation for 120 min at 37 °C, the cleavage of shear-treated VWF was detected as described in (A). Bottom: densitometric analysis of VWF cleavage. Band intensities relative to that of MDTCS at 0 M urea (set as 1) were plotted as means ± standard deviation (n = 3).

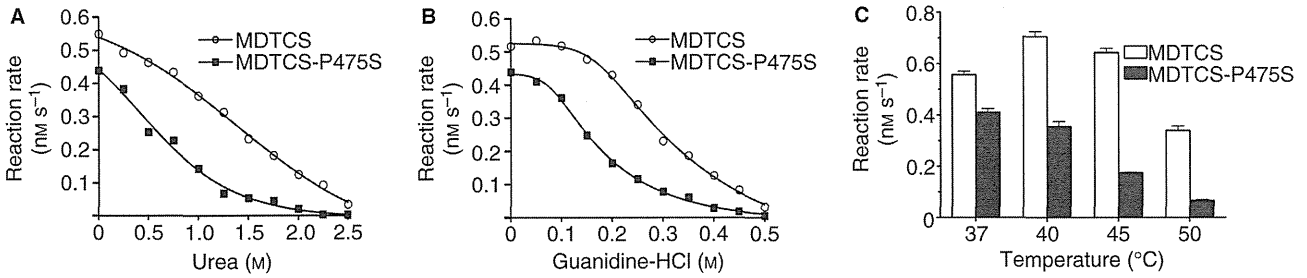

Fig. 4. Effects of denaturants and temperature on the FRETS-VWF73-cleaving activity of MDTCS and MDTCS-P475S. The enzymatic activities of MDTCS and MDTCS-P475S (0.8 nM) were measured for 60 min by the use of FRETS-VWF73 (1 μM) in the presence of urea (A) or guanidine-HCl (B) at 37 °C, or at different reaction temperatures (C). Values are means ± standard deviation (C: n = 3). VWF, von Willebrand factor.

reduced according to the concentrations of urea (Fig. 4A) or guanidine-HCl (Fig. 4B). The half-maximal inhibitory concentration (IC_{50}) for urea was 1.5 M for MDTCS and 0.8 M for MDTCS-P475S. The IC_{50} for guanidine-HCl was 0.3 M for MDTCS and 0.15 M for MDTCS-P475S. Furthermore, the activities of MDTCS and MDTCS-P475S were measured at 37, 40, 45 and 50 °C (Fig. 4C). A higher reaction temperature caused a more severe decrease in the activity of MDTCS-P475S than of MDTCS.

Discussion

Racial or ethnic group-specific genetic factors are now recognized to be important factors in the pathogenesis of thrombosis [36,37]. We previously identified the dysfunctional P475S polymorphism in ADAMTS-13 [8], and showed that it is East Asian-specific [14]. In the present study, we demonstrated that MDTCS-P475S showed moderate cleavage activity against shear-treated VWF, suggesting that the P475S polymorphism is not a causative factor in the development of TTP. However, this does not exclude the possibility that the P475S polymorphism could increase the risk for acquired TTP caused by inhibitory autoantibodies against ADAMTS-13. The relationship of the P475S polymorphism with acquired TTP remains to be addressed.

Recent studies have indicated the important function of the D and S domains as substrate-binding exosites [19–21]. We have identified at least three putative VWF-binding exosites, within the D, C_A and S domains, linearly aligned in the three-dimensional structure [18]. In the present study, kinetic analysis with FRETS-VWF73 showed that the lower catalytic efficiency (k_{cat}/K_m) of MDTCS-P475S was caused by lower affinity (higher K_m) but not by lower catalytic activity (lower k_{cat}). This also supports the idea that the V-loop in the C_A domain is a VWF-binding exosite.

The thermal shift assay showed that the thermostabilities of MDTCS and MDTCS-P475S were almost the same. Although the interaction between Ser475 in the V-loop of the C_A domain and Leu620 in the β6–β7 loop of the S domain was disrupted in the MDTCS-P475S structure, van der Waals contacts between Leu621 in the S domain and a hydrophobic pocket in the C_A domain formed by Gln442, Leu443, Met446, Val474, Arg507, Cys508 and Met509 [18] were retained. These interactions may minimize the adverse effect of P475S substitution on the stability of the S domain. These observations suggest that the reduction in the activity of MDTCS-P475S is not a consequence of global protein instability, but rather of a reduction in the enzyme–substrate interaction owing to the local conformational change in the V-loop (especially interatomic interactions with Ser475) (Fig. 1D). The greatly reduced enzymatic activity of MDTCS-P475S in the presence of urea and at a higher reaction temperature suggests that the P475S substitution induces a more severe local conformational change in the V-loop under these conditions and reduces the enzyme–substrate interaction.

Pro475 is conserved in all primates examined but not in other species (Fig. S3). Mouse ADAMTS-13 shows significantly reduced enzymatic activity against human VWF [38], indicating the species specificity of the ADAMTS-13–VWF interaction. Several amino acids in the core binding region for ADAMTS-13 in the A2 domain of VWF (VWF73) [39] differ among species (Fig. S4). Non-conserved amino acids in the exosites of ADAMTS-13 and in the VWF73 region of VWF may have coevolved for species-specific ADAMTS-13–VWF interface recognition.

Post-translational protein *C*-mannosylation is the attachment of an α-mannopyranosyl residue to the indole C-2 of tryptophan via a C–C linkage [32]. Proteins known to be *C*-mannosylated are RNase, interleukin-12, the mucins MUC5AC and MUC5B, and several proteins containing TSRs, such as thrombospondin-1, F-spondin, C6, C7, properdin, ADAMTS-L1, and ADAMTS-5 [33]. In the present study, potential *C*-mannosylation of Trp387 in the T1 domain of ADAMTS-13 has been suggested for the first time. *C*-mannosylation typically occurs in TSRs within the sequence motif W*XX*W, which is highly conserved among ADAMTS family members (ADAMTSs and ADAMTS-Ls) [33]. The functional role of *C*-mannosylation in ADAMTS-13 is unknown; however, a *C*-

mannosylation defect in ADAMTS-L1 decreases its secretion, suggesting a role in the regulation of protein secretion [33].

Addendum

M. Akiyama, D. Nakayama, and S. Takeda: performed research, analyzed data, and wrote the manuscript; K. Kokame: designed the experiments; J. Takagi: provided laboratory reagents; T. Miyata: wrote the manuscript and provided funding. All authors have approved the final draft.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Calibration curves for FRETS-VWF73 cleavage.
Figure S2. Thermal shift assay profiles of MDTCS and MDTCS-P475S.
Figure S3. Amino acid sequence alignment of the C_A domain of ADAMTS-13 from different species.
Figure S4. Amino acid sequence alignment of the core binding region for ADAMTS-13 in the A2 domain of VWF from different species.
Table S1. Data collection and refinement statistics.

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