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Evaluation of hemostatic biomarker abnormalities that precede platelet count decline in critically ill patients with sepsis☆,☆☆

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Abstract

Purpose: The hemostatic biomarkers for early diagnosis of sepsis-associated coagulopathy have not been identified. The purpose of this study was to evaluate hemostatic biomarker abnormalities preceding a decrease in platelet count, which is a surrogate indicator of overt coagulopathy in sepsis.

Materials and Methods: Seventy-five septic patients with a platelet count more than $80 \times 10^3/\mu L$ were retrospectively analyzed. Hemostatic biomarkers at intensive care unit admission were compared between patients with and patients without a subsequent decrease in platelet count ($\geq 30\%$ within 5 days), and the ability of biomarkers to predict a decrease in platelet count was evaluated.

Results: Forty-two patients (56.0%) developed a subsequent decrease in platelet count. Severity of illness, incidence of organ dysfunction, and 28-day mortality rate were higher in patients with a subsequent decrease in platelet count. There were significant differences between patients with and patients without a subsequent decrease in platelet count in prothrombin time–international normalized ratio, fibrinogen, thrombin-antithrombin complex, antithrombin, protein C (PC), plasminogen, and α_2-plasmin inhibitor (α_2-PI). Receiver operating characteristic curve analysis showed that PC (area under the curve, 0.869; 95% confidence interval, 0.699-0.951) and α_2-PI (area under the curve, 0.885; 95% confidence interval, 0.714-0.959) were strong predictors of a subsequent decrease in platelet count.

☆ Authors' contributions: K.K. conceived and designed the study. K.K. and S.T. prepared the data for analysis. K.K. conducted the data analysis. S.M. assisted with interpretation of the results. Y.S., J.M., and S.N. supervised the study. K.K. and S.M. drafted the article. All authors read and approved the manuscript. K.K. and S.M. take responsibility for the manuscript as a whole.

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Conclusions: Decreased PC and α_2-PI activity preceded a decrease in platelet count in intensive care unit patients with sepsis.

1. Introduction

Coagulation and fibrinolytic abnormalities are observed in most patients with sepsis [1]. Severe inflammation in sepsis is associated with tissue factor–mediated activation of coagulation, which leads to thrombin generation and results in widespread fibrin deposition. The severity of coagulopathy in sepsis ranges from subclinical abnormalities, which are detectable by a mild increase in fibrin degradation products (FDPs) and prolongation of global clotting times, to fulminant disseminated intravascular coagulation (DIC), characterized by widespread microvascular thrombosis [2]. A number of studies have reported on the association between DIC and organ failure and found that DIC is an independent risk factor for mortality in patients with sepsis [2-5]. Early diagnosis and treatment may therefore improve outcomes in septic patients with DIC [6].

The International Society on Thrombosis and Haemostasis (ISTH) criteria are currently widely used for diagnosing DIC [7] and are a strong independent predictor of mortality in patients with severe sepsis [8]. Although the ISTH criteria for overt DIC are simple and clinically useful, they have some limitations to be applied for early stage of DIC. The ISTH criteria define nonovert DIC as the stage before overt DIC, for the purpose of early diagnosis [7]. However, previous studies have shown that few patients with nonovert DIC progress to overt DIC and that mortality rates are similar between patients with nonovert DIC and patients with overt DIC [8], suggesting that septic coagulopathy diagnosed according to the ISTH criteria for nonovert DIC may not necessarily be an early stage of overt DIC.

Previous studies have evaluated a number of hemostatic biomarkers including D-dimer, antithrombin (AT), thrombin-AT complex (TAT), plasmin–α_2-plasmin inhibitor complex (PIC), and plasminogen activator inhibitor-1 (PAI-1); however, no single marker that can effectively diagnose early stage of DIC has been identified [9,10]. It is therefore important to develop clinical markers that can detect progression of septic coagulopathy in initial phase, so that early intervention can be instituted.

The objective of this study was to evaluate the ability of hemostatic biomarkers for predicting progression of coagulopathy in septic patients admitted to the intensive care unit (ICU). We used a decrease in platelet count as a marker for overt stage of septic coagulopathy. Platelet activation, consumption, and destruction may occur at the endothelial cell surface as a result of thrombin generation and fibrin meshwork formation secondary to coagulation activation. Platelet count decreases over a few days after the development of sepsis [5], which may indicate ongoing activation of coagulation [11]. Thrombocytopenia may reflect the advanced stage of DIC, which is associated with late death in patients with severe sepsis [5,12]. We therefore considered that a decreasing platelet count could be an indicator of disease progression in sepsis-induced coagulopathy.

2. Methods

2.1. Patients

The medical records of all patients admitted to the ICU at Jichi Medical University Hospital from September 2010 to December 2011 were retrospectively reviewed. Patients with a diagnosis of sepsis and a platelet count of more than $80 \times 10^3/\mu L$ on the day of ICU admission were included in the study. Sepsis was defined as fulfillment of at least 2 of the 4 criteria for systemic inflammatory response syndrome [13] and proven or suspected infection. Exclusion criteria were as follows: age younger than 18 years, prior hematologic disorder including platelet disorder, liver cirrhosis or failure, chronic renal failure with dialysis, history of chemotherapy, anticoagulation therapy with or without AT substitution, and blood transfusion during the preceding 4 weeks. This study was approved by the Institutional Research Ethics Committee of Jichi Medical University, which did not consider informed consent to be necessary because of the study design.

Our facility provides 24-hour coverage of attending ICU physicians. Management of patients followed the Surviving Sepsis Campaign Guideline [14], with the goal of initial resuscitation and infection control. Treatment for DIC was at the discretion of the responsible ICU physicians. The basic approach to treatment was anticoagulation therapy using gabexate mesilate (a serine protease inhibitor) [15,16], with or without AT substitution therapy. Some patients with a bleeding risk, or with complications, were transfused with platelet concentrate or fresh-frozen plasma at the discretion of the treating physicians.

2.2. Data collection

Descriptive data including demographic data, diagnoses, sources of infection, and clinical data were collected from the electronic medical records of all eligible patients. Acute Physiology and Chronic Health Evaluation (APACHE) II [17] and Simplified Acute Physiology (SAPS) II [18] scores were calculated to estimate the severity of disease within the

first 24 hours of ICU admission. The Sequential Organ Failure Assessment (SOFA) scoring system [19] was used to evaluate organ dysfunction during the first 7 days of ICU stay or until ICU discharge. The ISTH criteria were used to diagnose overt and nonovert DIC, with scores calculated for each of the dates tested. Prognosis was evaluated by ICU-free days during the first 28 days [20] and all-cause 28-day mortality.

2.3. Biomarker measurements

Platelet counts were measured on the day of ICU admission (day 1) and on each of days 2 to 5. Prothrombin time–international normalized ratio (PT-INR), activated partial thromboplastin time (APTT), fibrinogen, FDP, AT, protein C (PC), plasminogen, α_2-plasmin inhibitor (α_2-PI), TAT, and PIC were measured on day 1. Assays of hemostatic parameters were performed using the CS-2100i automatic coagulation analyzer (Sysmex, Hyogo, Japan). Antithrombin, PC, plasminogen, and α_2-PI were measured using Berichrom assays (Siemens Healthcare Diagnostics, Tokyo, Japan). Thrombin-AT complex and PIC were quantitated using the TAT test F and PIC test F enzyme immunoassay, respectively (Sysmex).

2.4. Data analysis

We defined a subsequent decrease in platelet count as a decrease of 30% or more within 5 days of ICU admission. The study population was grouped according to the presence or absence of a subsequent decrease in platelet count. Differences between groups were analyzed using the Student *t* test for normally distributed variables and the Mann-Whitney *U* test for nonnormally distributed variables. Categorical data were compared using the χ^2 test or Fisher exact test.

Receiver operating characteristic (ROC) curve analysis was performed to calculate the area under the curve (AUC) for coagulation and fibrinolytic biomarkers, and the AUCs were compared to evaluate their ability to predict a subsequent decrease in platelet count. Cutoff values were calculated by maximizing the sum of sensitivity and specificity.

All *P* values were 2 tailed, and $P < .05$ was considered statistically significant. Data were analyzed using JMP version 10 (SAS Institute, Tokyo, Japan).

3. Results

3.1. Characteristics of the 75 eligible patients with sepsis

Of the 1343 patients admitted to the ICU during the study period, 108 had a diagnosis of sepsis on the day of ICU admission. Thirty-three patients were excluded according to the study criteria, and the remaining 75 patients were included in the study. Forty-two patients developed a subsequent decrease in platelet count within 5 days of ICU admission (Fig. 1).

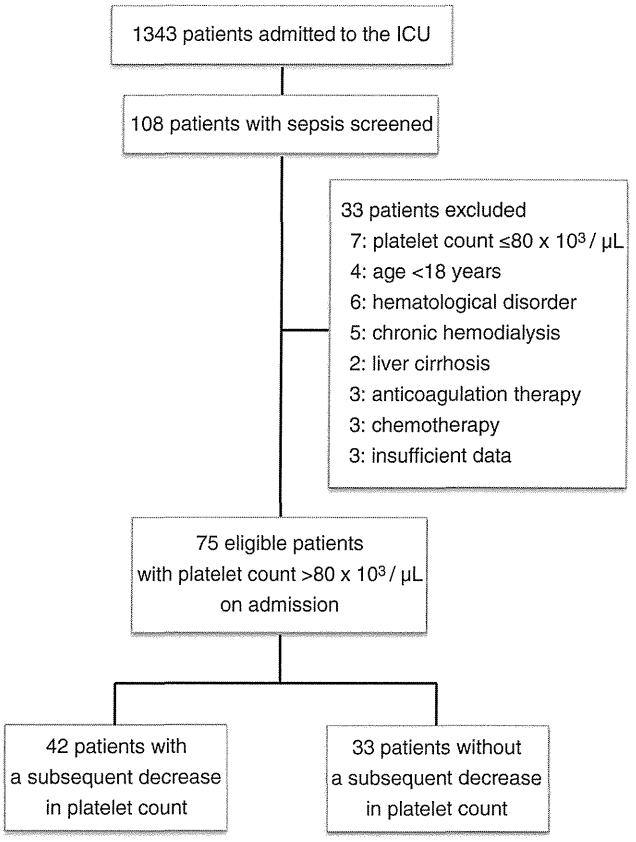

Fig. 1 Flowchart of the study patients. Sepsis was defined as fulfillment of at least 2 criteria for systemic inflammatory response syndrome and a source of infection. Eligible patients were grouped according to the presence or absence of a decrease of in platelet count 30% or more within 5 days of ICU admission.

Table 1 shows the baseline characteristics of the 75 patients. The most common cause of sepsis was abdominal infection, including 26 patients (34.7%) with lower intestinal perforation, 9 (12.0%) with acute cholangitis or cholecystitis, 5 (6.7%) with intra-abdominal abscess, and 3 (4.0%) with liver abscess (Table 1). Fifty (66.7%) of the 75 patients were surgical, and 25 (33.3%) were medical. The mean APACHE II score was 23.2 ± 7.8, and the mean SAPS II score was 49.8 ± 15.5. The all-cause 28-day mortality rate was 8.0%, with all deaths occurring during the ICU stay (Table 2).

Platelet counts over time (days 1-5) in patients with and without a subsequent decrease in platelet count are shown in Fig. 2. On the day of ICU admission, platelet counts were not significantly different between patients with and patients without a subsequent decrease in platelet count. The nadir of platelet count was on day 4 in patients with a subsequent decrease in platelet count and on day 2 in patients without a subsequent decrease in platelet count. During the first 7 days

Table 1 Baseline characteristics of the 75 patients with sepsis

	All patients (n = 75)	Subsequent decrease in platelet count (n = 42)	No subsequent decrease in platelet count (n = 33)	P [a]
Age (y)	70.0 ± 11.7	70.1 ± 11.9	70.0 ± 11.5	.98
Male	41 (54.7)	18 (42.9)	23 (69.7)	.035
Sepsis				
Pneumonia	15 (20.0)	7 (16.7)	8 (24.2)	.56
Abdominal infection	46 (61.3)	30 (71.4)	16 (48.5)	.057
Urinary tract infection	5 (6.7)	3 (7.1)	2 (6.1)	1.00
Soft tissue infection	9 (12.0)	2 (4.8)	7 (21.2)	.038
Comorbidity				
IHD	5 (6.7)	2 (4.8)	3 (9.1)	.65
CHF	5 (6.7)	2 (4.8)	3 (9.1)	.65
COPD	4 (5.3)	2 (4.8)	2 (6.1)	1.00
CVD	2 (2.7)	0 (0.0)	2 (6.1)	.19
CKD	5 (6.7)	4 (9.5)	1 (3.0)	.38
Severity of illness				
APACHE II score	23.2 ± 7.8	25.1 ± 8.2	20.8 ± 6.8	.021
SAPS II score	49.8 ± 15.5	55.5 ± 16.1	42.9 ± 11.8	.0004
DIC score on admission				
ISTH overt	2.6 ± 1.3	2.7 ± 1.5	2.5 ± 1.1	.51
ISTH nonovert	5.0 ± 1.8	5.2 ± 1.9	4.8 ± 1.5	.33

Data are expressed as mean ± SD or number (%).
IHD indicates ischemic heart disease; CHF, chronic heart failure; COPD, chronic obstructive pulmonary disease; CVD, cerebrovascular disease; CKD, chronic kidney disease.
[a] Comparison of groups with and without a subsequent decrease in platelet count.

of ICU stay, circulatory failure and renal dysfunction were more frequent in patients with than without a subsequent decrease in platelet count (Table 2). Patients with a subsequent decrease in platelet count had a higher mortality rate than did patients without a subsequent decrease in platelet count (Table 2).

Table 2 Interventions, organ dysfunction, and prognosis in 75 patients with sepsis

	All patients (n = 75)	Subsequent decrease in platelet count (n = 42)	No subsequent decrease in platelet count (n = 33)	P [a]
Transfusion (days 1-7)				
Red blood cell	18 (24.0)	11 (26.2)	7 (21.2)	.62
Platelet	9 (12.0)	9 (21.4)	0 (0.0)	.0039
Fresh-frozen plasma	16 (21.3)	15 (35.7)	1 (3.0)	.0005
Treatment for DIC (days 1-7)				
Gabexate mesilate	29 (38.7)	28 (66.7)	1 (3.0)	<.0001
Antithrombin	21 (28.0)	21 (50.0)	0 (0.0)	<.0001
Organ dysfunction (days 1-7)				
CVS (SOFA ≥ 3)	39 (52.0)	30 (71.4)	9 (27.3)	.0002
Lung (SOFA ≥ 3)	26 (48.0)	23 (54.8)	13 (39.4)	.25
Renal (SOFA ≥ 2)	16 (21.3)	14 (33.3)	2 (6.1)	.0045
Liver (SOFA ≥ 2)	28 (37.3)	18 (42.9)	10 (30.3)	.34
DIC				
ISTH overt	23 (30.7)	21 (50.0)	2 (6.1)	<.0001
ISTH nonovert	54 (72.0)	38 (90.5)	16 (48.5)	<.0001
Prognosis				
ICU-free days	19 (11.5-23)	18 (7–21)	22 (15–23.5)	.027
ICU mortality	6 (8.0)	6 (14.6)	0 (0.0)	.031
28-d mortality	6 (8.0)	6 (14.6)	0 (0.0)	.031

Data are expressed as median (interquartile range) or number (%).
CVS indicates cardiovascular system.
[a] Comparison of groups with and without a subsequent decrease in platelet count.

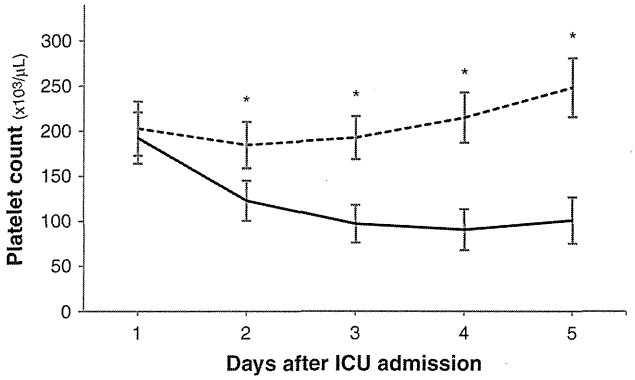

Fig. 2 Time course of platelet counts during the first 5 days of ICU stay in patients with sepsis, showing patients with a subsequent decrease in platelet count (solid line) vs patients without a subsequent decrease in platelet count (dotted line). A subsequent decrease in platelet count was defined as a decrease of 30% or more within 5 days of ICU admission. Day 1, the day of ICU admission. Data are expressed as mean and 95% CIs; $*P < .05$.

3.2. Relationships of hemostatic biomarkers with a subsequent decrease in platelet count in patients with sepsis

We evaluated coagulation and fibrinolytic markers on the day of ICU admission and examined their relationship with a subsequent decrease in platelet count. There were significant differences between patients with and patients without a subsequent decrease in platelet count in PT-INR (median, 1.33 vs 1.17; $P = .048$), fibrinogen (322 vs 526, $P = .0014$), TAT (13.8 vs 7.2, $P < .0001$), AT (51.1 vs 62.7, $P = .0099$), PC (35.1 vs 64.2, $P = .0002$), plasminogen (50.7 vs 85.3, $P = .0007$), and α_2-PI (56.3 vs 83.9, $P = .0001$) (Table 3). There were no significant differences between the 2 groups in APTT, FDP, or PIC.

3.3. Ability of hemostatic biomarkers to predict a subsequent decrease in platelet count in patients with sepsis

We conducted ROC curve analysis to evaluate the ability of hemostatic biomarkers to predict a subsequent decrease in platelet count. The AUC and best calculated cutoff values for prediction of a subsequent decrease in platelet count are shown in Table 4. The AUC for prediction of a subsequent decrease in platelet count was high for α_2-PI (0.885), PC (0.869), plasminogen (0.846), and TAT (0.846), compared with PT-INR (0.639) and fibrinogen (0.666). Interestingly, the ability to predict a subsequent decrease in platelet count was significantly higher for PC (AUC, 0.869; 95% confidence interval [CI], 0.699-0.951; $P = .0098$) and α_2-PI (AUC, 0.885; 95% CI, 0.714-0.959; $P = .029$), in pairwise comparison with PT-INR (AUC, 0.639; 95% CI, 0.432-0.799) (Fig. 3).

4. Discussion

In this retrospective study, we evaluated the usefulness of a single measurement of coagulation and fibrinolytic biomarkers on the day of ICU admission, for providing simple and prompt assessment for progression of coagulopathy in patients with sepsis. Our results show that decreased PC and α_2-PI activity were strong predictors of a subsequent decrease in platelet count, which was considered as an indicator of overt coagulopathy in sepsis.

Currently, there are no widely accepted diagnostic criteria for early stage of septic coagulopathy. Intervention to coagulopathy in initial phase might be one of the key factors in improving outcomes in patients with sepsis. Several researchers have recently established diagnostic scoring systems for the acute phase of septic coagulopathy [21-23], but these systems depend partly on changes in biomarker levels from baseline, which take at least 2 days to identify. Our results suggest that the progression of septic coagulopathy could be detected by a single measurement of PC and α_2-PI, which would help to develop early diagnostic criteria for septic coagulopathy.

Previous studies have evaluated the prognostic values of hemostatic biomarkers in patients with sepsis, but the reported results have been inconsistent [1,9,21,24]. These conflicting results may be partly caused by the multiple

Table 3 Comparison of coagulation and fibrinolytic markers on the day of ICU admission

	Reference range	Subsequent decrease in platelet count (n = 42)	No subsequent decrease in platelet count (n = 33)	*P*
Platelet count ($\times 10^3/\mu L$)	130-369	181 (123-245)	197 (162-234)	.52
PT-INR	0.9-1.2	1.33 (1.14-1.53)	1.17 (1.11-1.34)	.048
APTT (s)	23.1-36.3	44.2 (32.9-59.3)	40.6 (36.1-48.3)	.71
Fibrinogen (mg/dL)	129-271	322 (212-493)	526 (380-649)	.0014
FDP (μg/mL)	0-5.0	20.4 (12.3-30.5)	16.8 (11.2-24.3)	.21
TAT (ng/mL)	<2.4	13.8 (9.9-22.1)	7.2 (5.4-9.3)	<.0001
PIC (μg/mL)	<0.9	0.9 (0.7-1.9)	1.4 (0.8-1.8)	.19
AT (%)	88-116	51.1 (36.9-62.5)	62.7 (47.8-74.1)	.0099
PC (%)	67-129	35.1 (28.1-55.8)	64.2 (56.5-79.8)	.0002
Plasminogen (%)	85-120	50.7 (40.4-68.3)	85.3 (73.9-91.9)	.0007
α_2-PI (%)	83-115	56.5 (48.3-67.6)	83.9 (75.2-94.7)	.0001

Table 4 ROC curve analyses showing AUCs for prediction of a subsequent decrease in platelet count and cutoff values maximizing the sum of sensitivity and specificity

	AUC	*P*	Cutoff value	Sensitivity	Specificity
Platelet count	0.544	.79	$157 \times 10^3/\mu L$	0.36	0.82
PT-INR	0.639	.012	1.37	0.48	0.79
APTT	0.526	.26	48.9 s	0.45	0.76
Fibrinogen	0.666	.0019	462 mg/dL	0.71	0.66
FDP	0.587	.045	29.4 μg/mL	0.33	0.88
TAT	0.846	<.0001	13 ng/mL	0.59	1.0
PIC	0.399	.57	2.7 μg/mL	0.24	0.88
AT	0.734	.0071	55.8%	0.67	0.66
PC	0.869	<.0001	41.4%	0.67	0.94
Plasminogen	0.846	.0012	72.8%	0.91	0.77
α_2-PI	0.885	<.0001	73.3%	0.92	0.75

interactive systemic factors that are involved in the pathogenesis of organ failure and the risk of mortality, although cytokine-induced coagulopathy is considered to play a major role in the process [25]. In addition, not only the development but also subsequent progression or persistence of coagulopathy are associated with poor prognosis in patients with sepsis [9,21]. We therefore focused on the progression of coagulopathy as an outcome in patients in the acute phase of sepsis.

Because there are no established criteria for assessing the progression of septic coagulopathy, we used decreasing in platelet count as a marker of disease progression. Most diagnostic criteria for DIC, including the ISTH criteria, primarily use global coagulation tests such as PT, platelet count, fibrinogen, and FDP for scoring. These markers change differently over time as coagulopathy progresses. Production of FDP may be suppressed in severe coagulopathy because of a massive increase in PAI-1 level, and the fibrinogen level tends to increase with acute-phase behavior in septic patients [26]. In addition, appropriate thresholds for the diagnosis of septic coagulopathy have not been determined. Although coagulopathy is universal in severe sepsis, only about 30% of patients in a large multicenter trial met the ISTH criteria for overt DIC [4]. The current diagnostic criteria are not useful for the evaluation of disease progression in septic patients with coagulopathy.

Thrombocytopenia is common in critically ill patients with an incidence of 40% to 50% [27], and a 30% decrease in platelet count during ICU admission has been shown to be associated with increased mortality [28]. Thrombocytopenia can be a result of many causes such as blood loss, hemodilution, thrombotic microangiopathy, and immune and drug-induced thrombocytopenia. However, septic coagulopathy is the most common underlying cause [26]. Although a diagnosis of DIC must be supported by multiple laboratory tests and other causes of thrombocytopenia should be ruled out, thrombocytopenia is a relevant marker of DIC [5]. In addition, it has been clinically recognized that thrombocytopenia may be a warning sign of severe sepsis and that the decrease in platelet count is related to the severity of sepsis [29]. In our study, a significant decrease in platelet count after ICU admission was associated with disease severity, the incidence of organ dysfunction, and the 28-day mortality rate (Tables 1 and 2).

In our study, AT, PC, plasminogen, α_2-PI activity, and TAT on the day of ICU admission had high predictive values for a subsequent decrease in platelet count in patients with sepsis. In sepsis, physiological anticoagulation mechanisms such as the AT and PC systems are impaired because of increased consumption, impaired synthesis, extravasation from vessels, and degradation by several proteolytic enzymes such as neutrophil elastase [30,31]. We found that AT and PC activity were significantly decreased in patients with a subsequent decrease in platelet count compared with patients without a subsequent decrease in platelet count, which may have been caused by increased consumption. However, mild to moderately decreased AT and PC activity were also observed in patients without a subsequent decrease in platelet count (Table 1). Asakura et al [32] reported that AT and PC activity were related to the plasma albumin level and that decreased AT and PC activity were observed in septic patients without DIC. The mechanisms of decreased AT and PC activity in patients without a subsequent decrease in platelet count may include impaired synthesis, or degradation associated with inflammation, rather than consumption coagulopathy.

We found that PC was a stronger predictor of a subsequent decrease in platelet count than AT, which is somewhat inconsistent with the results of previous studies. Several investigators have reported that AT had a higher [33] or comparable [21] association with poor prognosis, compared with PC. However, Nilsson et al [34] showed that PC had a higher correlation with the results of global clotting tests than AT. Protein C has anticoagulant and fibrinolytic properties with neutralization of PAI-1 [35], whereas AT is an anticoagulant that mainly inhibits thrombin and other procoagulant factors.

Measurement of fibrinolytic markers showed that plasminogen and α_2-PI activity were decreased in patients with a subsequent decrease in platelet count and were near the lower

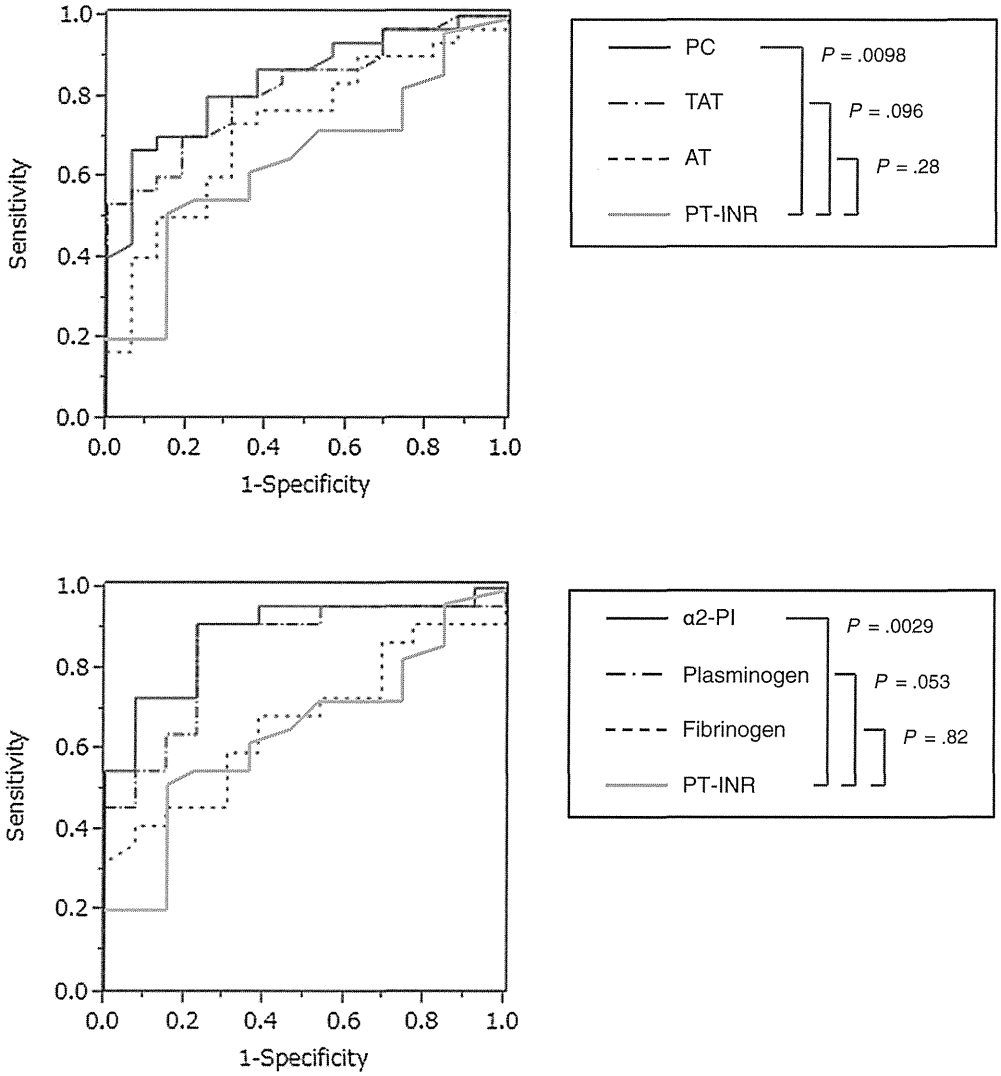

Fig. 3 Pairwise comparisons of ROC curves of biomarkers that significantly predicted a subsequent decrease in platelet count (comparison with the ROC curve of PT-INR).

limit of the reference range in patients without a subsequent decrease in platelet count. These results are consistent with those of previous study, in which only half of the 1690 patients with severe sepsis were shown to have decreased plasminogen and α_2-PI activity, although more than 80% had abnormal AT and PC activity [1]. It is known that the plasminogen activator–plasmin system is markedly depressed by PAI-1 in patients with sepsis [24]. In addition, interleukin-6 induces up-regulation of plasminogen promoter activity [36], and production of plasminogen is increased as an acute-phase protein during sepsis [37]. However, the tissue plasminogen activator level is increased in sepsis [9], and the tissue plasminogen activator/PAI-1 ratio increases with increasing severity of sepsis [33], which may explain why the levels of fibrinolytic markers were decreased under the countered increase of PAI-1.

The present study has some limitations. This was a retrospective observational study that did not promote deviation from routine clinical practices at our institution. Interventions such as treatment of DIC and blood transfusion may have influenced the levels of coagulation and fibrinolytic biomarkers and their relationships with changes in platelet count. Because our study was conducted in a single center, the relatively small size of the study population is also a limitation. A large, prospective study would be useful to validate our results.

In conclusion, decreased PC and α_2-PI activity on the day of ICU admission were strongly associated with a subsequent decrease in platelet count in patients with sepsis. A single measurement of these biomarkers may help to predict progression of septic coagulopathy and guide the decision-making process for early intervention.

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Case report

Acquired factor V inhibitor associated with life-threatening bleeding and a mixing test result that indicated coagulation factor deficiency

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A mixing test is useful for distinguishing between coagulation factor deficiency and the presence of inhibitor as the cause of coagulopathy. However, we experienced a patient with acquired factor V (FV) inhibitor whose mixing test showed a coagulation factor deficiency pattern. A 65-year-old man with a tendency for bleeding was referred to our center. The laboratory data showed remarkable prolongation of prothrombin time and activated partial thromboplastin time (APTT). FV activity was less than 3%. A mixing test showed a coagulation factor deficiency pattern. However, neither the tendency for bleeding nor the coagulation tests were corrected by transfusion of fresh frozen plasma. A few days later, a positive test for FV inhibitor of 3 Bethesda units was obtained. Therefore, we started prednisolone and plasma exchange, and the coagulation test results normalized after 6 weeks. Although an incubation period is generally not considered necessary in a mixing test for FV inhibitor, we repeated mixing tests with various incubation periods and confirmed an incubation period-dependent prolongation of the APTT. Therefore, a mixing test with an incubation period is recommended for the detection of FV inhibitor, since a mixing test without an incubation period may show a coagulation factor deficiency pattern when the titer of FV inhibitor is low.

Keywords: *Acquired factor V inhibitor, Mixing test, Coagulation factor deficiency*

Introduction

Acquired factor V (FV) inhibitor is a rare condition that is associated with clinical symptoms ranging from asymptomatic laboratory abnormalities to life-threatening bleeding.[1] Most patients with FV inhibitor have underlying diseases or risk factors, including surgical procedures, exposure to topical bovine thrombin,[2,3] antibiotic administration,[4] blood transfusions, cancers,[5] and autoimmune disorders.[6,7] A prolonged prothrombin time (PT) and activated partial thromboplastin time (APTT) are usually seen as a laboratory coagulation profile in this condition.[8] A mixing test is useful for distinguishing between coagulation factor deficiency and the presence of inhibitor as the cause of coagulopathy. In a mixing test, the patient's plasma is mixed with normal pooled plasma, and coagulation tests that include PT and APTT are repeated. Correction of any abnormalities in the coagulation test generally implies a coagulation factor deficiency. On the other hand, a failure to correct abnormalities in the coagulation test suggests the presence of an inhibitor.[9]

We report here a patient who developed a tendency for severe bleeding with a mixing test result that showed a coagulation factor deficiency pattern.

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However, the presence of FV inhibitor was confirmed by the Bethesda method.[1] The coagulopathy was not corrected by transfusion of fresh frozen plasma (FFP) and platelet concentrates (PC). Therefore, there was a discrepancy between the mixing test results and the clinical and laboratory data.

Case report

A 65-year-old man with a medical history of hypertension and gout developed bloody stool, gross hematuria, and nasal bleeding. He visited a local hospital because the bleeding symptoms persisted for 2 weeks, and was shown to have marked abnormalities on coagulation studies: PT 142.8 seconds and APTT unmeasurable prolongation. He was immediately admitted and treated with FFP and vitamin K, but there was no improvement. He was referred to our medical center for the investigation of severe coagulopathy.

On admission, gastrointestinal bleeding was observed. A physical examination revealed several ecchymoses on his extremities. Complete blood count showed mild anemia of hemoglobin 10.6 g/dl. The platelet count was 342×10^9/l and the bleeding time was 2.5 minutes (normal: 2–5 minutes). Liver and renal function tests were within the normal limits. Marked abnormalities were observed in coagulation studies: PT 55.4 seconds (normal: 10–13 seconds), APTT >200 seconds (normal: 25–40 seconds), fibrinogen 362 mg/dl (normal: 150–380 mg/dl), fibrin/fibrinogen degradation product 1.2 μg/ml (normal: <5 μg/ml), D-dimer <1.0 μg/ml (normal: <1.0 μg/ml), and antithrombin-III 95.0% (normal: 70–120%). In a mixing test, both PT and APTT were corrected by mixing with normal plasma, which implied a coagulation factor deficiency (Fig. 1). A coagulation factor assay showed a decrease in FV activity of <3% (normal: 75–135%). Other coagulation factor activities were within normal limits: factor II activity 80% (normal: 75–135%), factor VIII (FVIII) activity 127% (normal: 60–150%), factor IX activity 96% (normal: 70–130%), and factor X activity 87% (normal: 70–130%). Antiphospholipid antibodies were not detected.

The clinical course is summarized in Fig. 2. The patient was initially treated with daily transfusion of FFP and PC based on the results of a mixing study. However, neither PT nor APTT was corrected even immediately after the transfusion of FFP. Four days after admission, an FV inhibitor test was positive at 3 Bethesda units (BU). Therefore, we started prednisolone (PSL) at 1 mg/kg to suppress inhibitor production. Since there was a discrepancy between the results of the mixing test and the clinical data, we performed the mixing test again with various incubation periods (Fig. 3). Although the test showed a coagulation deficiency pattern similar to the previous test, the coagulation time tended to increase with an increase in the incubation period at the same mixing ratio. One week after admission, the patient suddenly developed severe pain in his left groin. Enhanced computed tomography (CT) showed massive hematoma of his left iliopsoas muscle with persistent active bleeding. We decided to perform plasma exchange (PE) for the immediate elimination of inhibitor. Immediately after PE, PT and APTT were corrected to the normal levels. Next, intravascular embolization of the left deep circumflex iliac artery and left lumbar artery through the right femoral artery was successfully performed by using Histoacryl® (B. Braum Aesclup, Tokyo, Japan) blue as an obstructing material. However, the next morning, PT and APTT were again markedly prolonged. Although PE provided only a temporary benefit, we decided to continue PE once daily or

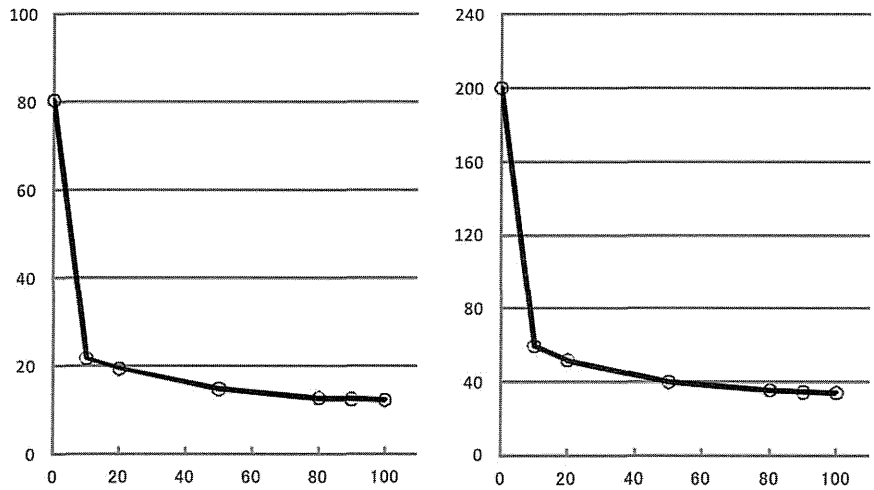

Figure 1 Results of a mixing test. Both the prothrombin time and activated partial thromboplastin time (APTT) were corrected by a small amount of normal plasma, which implied coagulation factor deficiency.

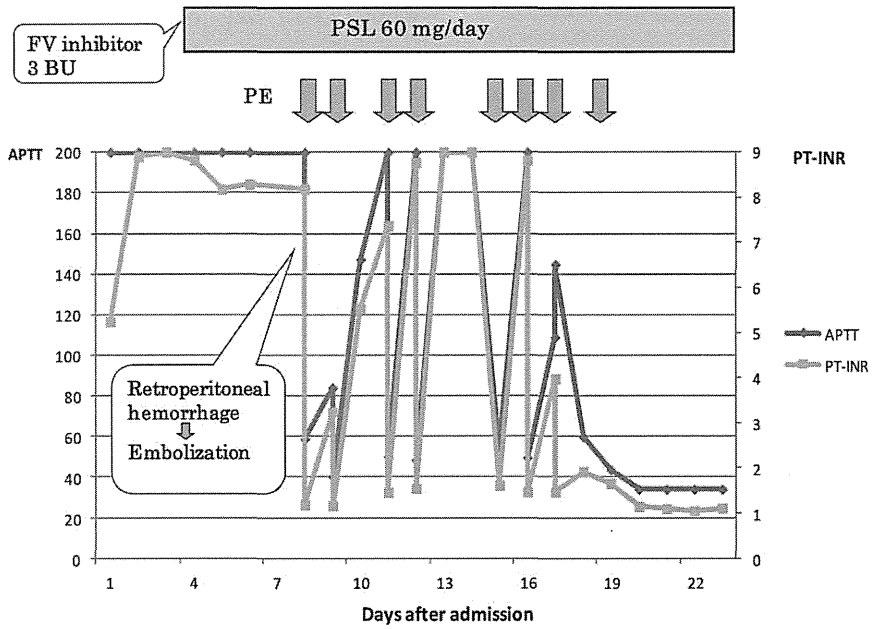

Figure 2 Clinical course after admission. Prothrombin time and activated partial thromboplastin time were extremely prolonged on admission. Prednisolone at 1 mg/kg was started 5 days after admission when tests were positive for FV inhibitor. At 8 days after admission, he developed massive retroperitoneal hemorrhage, which was successfully controlled by urgent plasma exchange (PE) and intravascular embolization. PE was repeated daily or every other day until coagulopathy was improved.

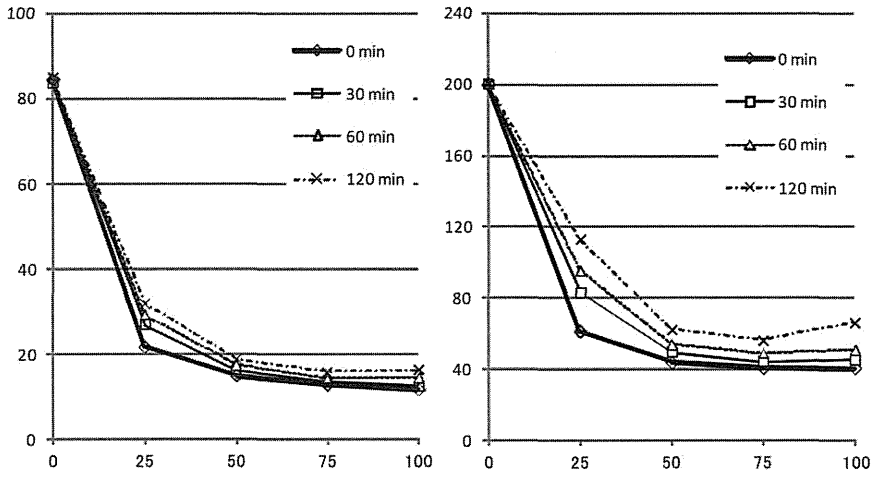

Figure 3 Results of a mixing test with various incubation periods. These tests showed a coagulation-deficiency pattern, but PT and APTT were increased with an increase in the incubation period.

every other day until the effect of immunosuppressive therapy became apparent. Approximately 2 weeks after starting PSL, improvement of the coagulation time was observed. PE was stopped after a total of eight iterations. We continued PSL at 1 mg/kg for 4 weeks, and then started to taper gradually. FV activity was 114 and 146% at 6 and 12 weeks after starting PSL, respectively. FV inhibitor was not detected at these time points. As the underlying disease of acquired FV inhibitor, rectal cancer was suspected based on CT findings. After the coagulation abnormalities were corrected, he underwent colonoscopic examination, which led to a diagnosis of rectal cancer. Elective surgical resection was performed successfully. Prednisolone was stopped 5 months after its initiation, and there has been no relapse of coagulopathy thus far.

Discussion

The clinical course and the inhibitor test of the current patient showed the presence of FV inhibitor, but the mixing test showed a coagulation factor deficiency pattern. As a possible explanation for this discrepancy, we considered that the incubation period after mixing with normal plasma might be insufficient to inactivate the coagulation factor in normal plasma. In general, FV inhibitor has been considered to bind and neutralize FV instantly, and therefore an incubation period is not routinely incorporated in the mixing test for FV inhibitor, in contrast to that for FVIII inhibitor.[10,11]

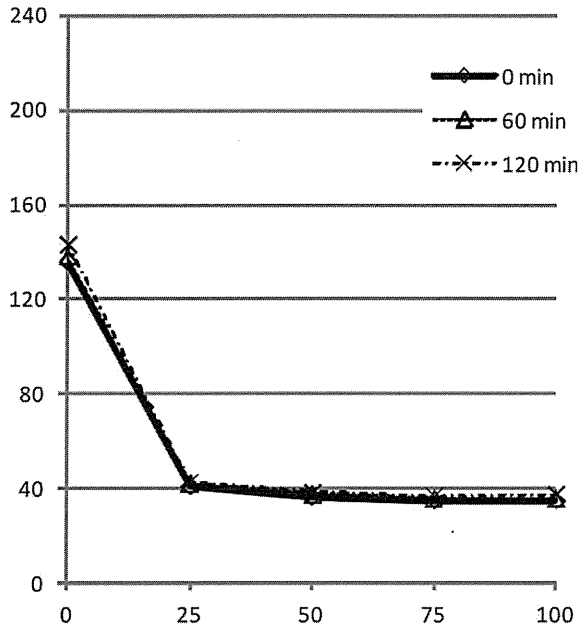

Figure 4 Results of a mixing test with FV-deficient plasma and normal plasma. A difference in incubation period did not affect the results.

Accordingly, we initially performed the mixing test without an incubation period (Fig. 1). However, the result was not consistent with the clinical course. Therefore, we repeated the mixing test with various incubation periods, such as 30, 60, and 120 minutes (Fig. 3). The curve still showed a deficiency pattern with various incubation periods, but PT and APTT tended to increase with an increase in the incubation period. To confirm that the prolongation of PT and APTT was due to the binding of FV inhibitor and FV, we carried out a mixing test using FV-deficient plasma and normal pooled plasma (Fig. 4). The different incubation periods did not affect APTT in the mixing tests with FV-deficient plasma and normal plasma. Therefore, the incubation period-dependent prolongation of APTT in the current patient resulted from the presence of FV inhibitor in his plasma. These findings are consistent with the clinical course. In this patient, PT and APTT improved immediately after PE, but then increased again several hours later. This temporary improvement and later worsening might, at least partly, be the result of slow binding between FV and FV inhibitors.

Two recent reports have suggested that the incubation period might affect the results of a mixing test for FV inhibitor (Table 1).[12,13] In Gartrell's report, while APTT was not corrected by mixture of the patient's plasma and normal plasma, APTT in the mixing test was increased from 104.7 seconds to 119.8 seconds with 1 hour of incubation. Lipshitz *et al.* also reported that APTT in a mixing test with a 1-hour incubation period was longer than that without an incubation period, but the APTT was corrected to nearly the normal value. The BU of FV inhibitor in these patients were 17 and 6, respectively. In the current patient with a FV inhibitor of 3 BU, APTT was normalized by a 1:1 mixture with normal plasma without incubation, but was prolonged after incubation for 30 minutes or longer. If we consider these findings together, a mixing test without incubation may overlook the presence of FV inhibitor at a low level (i.e. <6 BU), and therefore, we should routinely compare the APTT values of mixing tests with and without an incubation period. In the absence of FV inhibitor, there should be no change in the APTT values with various incubation periods (Fig. 4).

In general, the treatment of acquired FV inhibitor is based on supplying the coagulation factor and eradication of the autoantibody. To supply FV, FFP, and PC, which contains platelet-derived FV in the alpha granules, are administered, but in most cases their effect is insufficient. Therefore, immunosuppressive therapy to suppress autoantibodies is essential. Corticosteroids alone or in association with cyclophosphamide or other immunosuppressants have been used successfully to suppress inhibitor production. The overall probability of disappearance of the inhibitor is 88%, and the median time to disappearance is 9.7 weeks.[1] The current patient, who was treated with PSL alone as an immunosuppressive regimen and PE, achieved normal coagulation tests within 2 weeks and the disappearance of FV inhibitor was confirmed at 6 weeks. Although the effect of PE might be transient, PE can be beneficial in life-threatening hemorrhage by rapidly reducing the inhibitor titer and refilling coagulation factor.[14] We also performed intravascular embolization for the treatment of life-threatening retroperitoneal arterial bleeding. For intravascular embolization, we used Histoacryl® blue as an obstructing material. Although most obstructing materials work with coagulation factors, Histoacryl® blue can embolize a vessel in the absence of coagulation factors, and thus can be used in the setting of coagulation factor deficiency.

Table 1 Effect of an incubation period in a mixing test for Factor V inhibitor

Author	Patient's APTT (seconds)	1:1 mix APTT (immediate) (seconds)	1:1 mix APTT (60 minutes)(seconds)	Factor V inhibitor (Bethesda units)
Gartrell[12]	134.9	104.7	119.8	17
Lipshitz *et al.*[13]	151.7	49.8	55.5	6
Current report	>200	43.7	54.0	3

With regard to the possible causes for the production of inhibitors, the patient had no history of bovine thrombin and antibiotic use before the bleeding episode. As mentioned in the previous reports,[7] we considered that the inhibitor was produced associated with rectal cancer, as coagulopathy and bleeding symptom did not relapse after the resection of tumor.

In summary, when the titer of FV inhibitor is low, the results of a mixing test without an incubation period may show a coagulation factor deficiency pattern even in the presence of inhibitor. Therefore, a mixing test with an incubation period is recommended when the presence of FV inhibitor is suspected.

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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

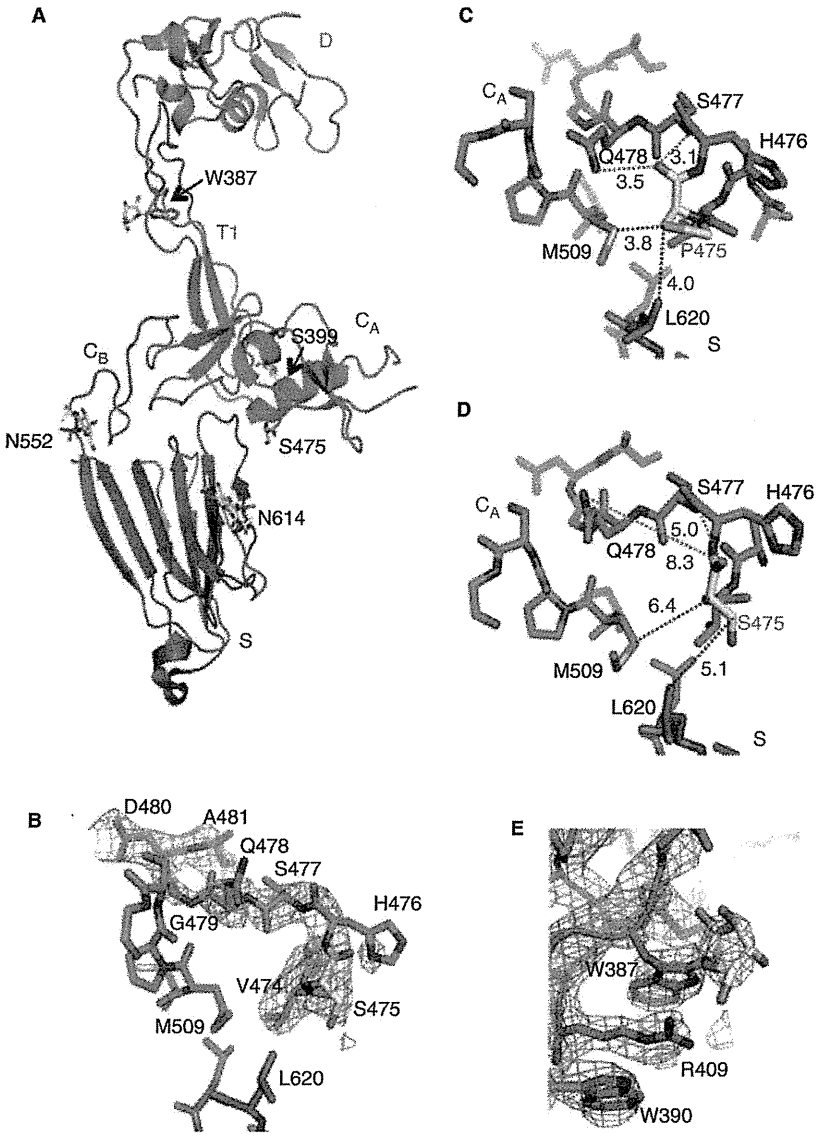

Fig. 1. Crystal structure of DTCS-P475S. (A) Overall structure of DTCS-P475S. Potential *N*-glycosylation at Asn552 (in the C_B domain) and Asn614 (in the S domain), *O*-fucosylation at Ser399 (in the T1 domain), and *C*-mannosylation at Trp387 (in the T1 domain) are shown as stick models. D, orange; 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.