Table 2. Clinical Characteristics and the Activity of Plasma ADAMTS13 on Admission in Patients with Severe Alcoholic Hepatitis

Case	Age/Sex	Encephalopathy	Ascites	Renal failure	DIC	Pneumonia	Heart failure	Maddrey score[a]	Treatment	Outcome	ADAMTS13 (%)
1	61/M	II	+	+	+	+	+	53	FFP,HD	dead (61 days)	4.5
2	35/M	III	+	+	+	+	+	67	FFP,Pred.	dead (2 days)	5.0
3	38/M	III	+	+	-	+	+	71	FFP,Pred.	dead (6 days)	16
4	42/F	-	+	-	-	-	-	56	FFP	alive (90 days)[b]	44

Case	T.Bil (mg/dl)	AST (IU/L)	ALT (IU/L)	γ-GTP (IU/L)	Alb (g/dl)	PT (%)	WBC (/mm^3)	Platelet (x10^4/mm^3)	PMN (/mm^3)	CRP (mg/dl)	Cr (mg/dl)
1	8.0	368	78	955	2.7	38	3500	2.8	3220	12.2	1.7
2	13.8	709	165	1000	1.8	27	9900	6.3	9306	2.7	3.5
3	24.3	292	97	225	2.5	36	26600	13.5	25004	2.7	4.7
4	11.4	340	123	181	3.0	38	8300	16.4	5976	1.7	0.4

Abbreviations: DIC=disseminated intravascular coagulation, ADAMTS13=A Disintegrin-like And Metalloproteinase domain, with ThromboSpondin type-1 motif 13, T.Bil=total bilirubin, AST=aspartate aminotransferase, ALT=alanine aminotransferase, γ-GTP=γ-Glutamyl transpeptidase, Alb=albumin, PT=prothrombin time, WBC=white blood cell count, PMN=polymorphonuclear neutrophil, CRP=C-reactive protein, Cr=creatinine, FFP=fresh frozen plasma, HD=hemodialysis, Pred=prednisolone.

[a]According to the formula as described by Carithers, et al. (1989).

[b]Duration until serum total bilirubin has been normalized.

and 21 days at the recovery stage, and in a patients with SAH, blood was sequentially collected every 2 week for 2 months until the terminal stage. Citrated platelet-poor plasma was prepared by centrifugation of the plasma at 3000 × *g* at 4°C for 15 min and was stored in aliquots at − 80°C until analysis. The activity of ADAMTS13 in the plasma was assayed according to the method of Furlan et al., (1998) with slight modification, as described in a recent publication (Mori et al., 2002). Briefly, the pooled normal human plasma was serially diluted with 0.05 M Tris-HCL (pH 7.4) containing 0.15 M NaCL (Tris-buffered saline), and 10 μl of each dilution were mixed with 1 μl of 100 mM PMSF (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Then, 90 μl of the purified VWF (20μg/ml) dissolved in a urea buffer (1.5 M urea, 0.05% NaN3, 10 mM BaCL2 and 5mM Tris-HCL) (pH 8.0) were added to each mixture and incubated at 37°C for 24 hr. After that, 10 μl of 100 mM ethylenediaminetetraacetic acid (pH 8.0) were added to each mixture to quench the enzyme activity. A portion of each reaction mixture was separated by SDS-1.2-% agarose gel electrophoresis, and then the VWF multimers were visualized by Western blotting and luminography. Quantification of ADAMTS13 was performed by scanning densitometry. A value of 100% activity was defined as the amount of the enzyme contained in 1 ml of the pooled normal human plasma. The detection limit of ADAMTS13 activity in this assay was approximately 3%, and the results obtained for normal subjects (n = 60; 30 females and 30 males, 20–39 years old) were 102 ± 23% (mean ± SD) (Mori et al., 2002). We, therefore, considered it low when the activity was less than 50% of the healthy subjects (mean − 2SD).

Statistics

The differences between the paired and unpaired groups were analyzed using the Mann- Whitney *U* test. The correlations were calculated by the Spearman rank test. To determine which parameters of laboratory data were independently correlated with the plasma activity of ADAMTS13 in the patients with AH, we applied the stepwise selection procedure by multiple regression analysis. The variables were selected by forward elimination. The analysis was carried out using the statistical software Statview (version 5.0; SAS Institute, Cary, NC, USA). The data are expressed as the mean ± SD. A two-tailed P value less than 0.05 was considered significant.

RESULTS

Clinical Characteristics and Laboratory Values

The clinical features and laboratory data of our patients were shown in Table 1 and Table 2. Six of 14 patients with AH and all of SAH were complicated by LC (Table 1). Maddrey score of SAH showed 53 to 71 (mean 62) on admission (Table 2). All patients with AH were alive, and 3 of 4 patients with SAH died because of hepatic failure within 2 to 61 days. Patients with AH and SAH were younger than in LC. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-Glutamyl transpeptidase (γ-GTP), white blood cell count (WBC) and peripheral polymorphonuclear neutrophil (PMN) count were higher in patients with AH and SAH than in LC. The values of serum total bilirubin, AST, lactate dehydrogenase (LDH), PMN count and C-reactive protein (CRP) were the highest, and those of hemoglobin, albumin and prothrombin time were the lowest in patients with SAH (Table 1). In 3 patients with SAH (case 1 to 3), hepatic encephalopathy of Grade II to III, ascites, renal failure, pneumonia, heart failure, and disseminated intravascular coagulation were noted on admission, indicating the occurrence of multiorgan failure (Table 2). These nonsurvivors were treated with the administration of fresh frozen plasma and/or prednisolone, without plasma exchange, because of systemic circulatory disturbance on admission. Case 4 had moderate ascites, but without the complication of multiorgan failure, and her serum total bilirubin has been normalized on the 90th hospitalization day (Table 2).

Plasma Activity of ADAMTS13

The activity of the plasma ADAMTS13 was significantly lower in patients with AH (59 ± 13%, $p < 0.001$), SAH (17 ± 19%, $p < 0.001$) and LC (76 ± 33%, $p < 0.02$) than in the healthy subjects (Fig. 1). The activity further decreased in patients with SAH as compared with those with AH ($p < 0.02$) and LC ($p < 0.02$) (Fig. 1). The activity did not differ between patients with AH and LC.

In 14 patients with AH, it tended to be lower in 6 cirrhotics with superimposed AH than in 8 patients without

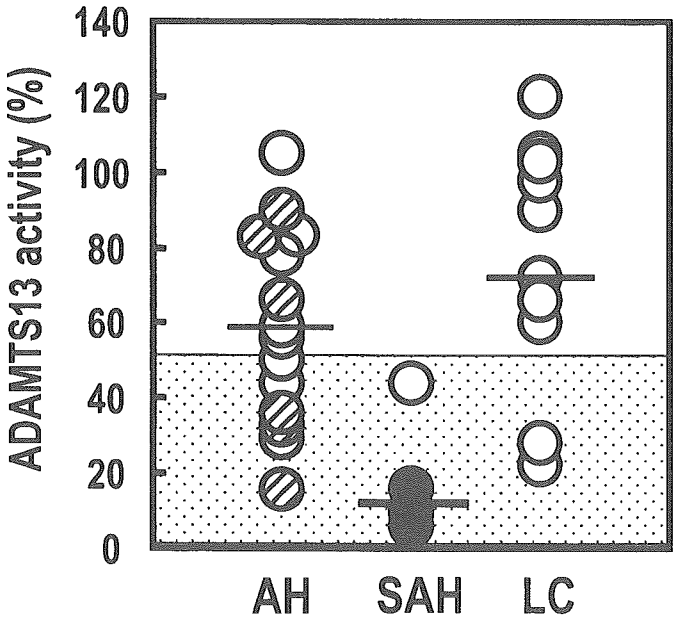

Fig. 1. The activity of the plasma ADAMTS13 in the patients with alcoholic hepatitis, severe alcoholic hepatitis and liver cirrhosis on admission. The shaded area shows below 50% of the healthy subjects (mean - 2SD). Open and shaded circles indicate survivors and closed circles indicate nonsurvivors, respectively. Shaded circles show liver cirrhotics with superimposed alcoholic hepatitis. The protease activity was significantly lower in patients with alcoholic hepatitis ($p < 0.001$), severe alcoholic hepatitis ($p < 0.001$) and liver cirrhosis ($p < 0.02$) than in normal subjects, although each value was widely distributed. The activity further decreased in patients with severe alcoholic hepatitis, as compared with alcoholic hepatitis ($p < 0.02$) and liver cirrhosis ($p < 0.02$). In three nonsurvivors with severe alcoholic hepatitis, the activity was extremely low. AH = alcoholic hepatitis, SAH = severe alcoholic hepatitis, LC = liver cirrhosis.

LC (55 ± 29% vs. 62 ± 25%), but the difference did not reach statistical significance. The activity tended to be lower in cirrhotics with superimposed AH (55 ± 29%) and patients with AH without LC (62 ± 25%) as compared with LC (76 ± 33%), but no significant differences were found among these three groups. In 10 patients with LC, it tended to be lower as cirrhotic stage progressed (Child A 96 ± 23%, Child B 62 ± 38%, Child C 50 ± 31%, respectively). The protease activity of nonsurvivors with multiorgan failure (case 1 to 3) markedly decreased, and especially in case 1 and 2, the each value was extremely low, which was consistent with that of the patient with typical thrombotic thrombocytopenic purpura (Table 2, Fig. 1 & Fig. 2). On the other hand, in a survivor with SAH (case 4), it remained mild decrease (44%) (Table 2, Fig. 1 & Fig. 2). In a sequential study, in patients with AH, the activity significantly increased at the recovery stage (42 ± 20% → 75 ± 34%, $p < 0.05$), but in a nonsurvivor with SAH (case 1), it remained extremely low (4.5% → 12.0% → 4.5%)(Fig. 3).

Relationships Between the Plasma ADAMTS13 Activity and Clinical Variables

In the univariate analysis of patients with AH and SAH, the activity of plasma ADAMTS13 correlated negatively with CRP, serum total bilirubin, LDH, peripheral PMN, serum creatinine, and WBC, and positively with total protein, serum albumin, prothrombin time and platelet count (Table 3). As the variables were mutually correlated, multivariate analysis was performed using the stepwise selection procedure. As a result, serum total bilirubin and CRP independently correlated with the activity of the plasma ADAMTS13 (Fig. 4).

This Model Resulted:

y (ADAMTS13 activity)(%) = 72.603 – 2.257 × serum total bilirubin (mg/dl) – 4.918 × CRP (mg/dl)

with r = 0.797 and p value of less than 0.001.

DISCUSSION

We demonstrated that the ADAMTS13 activity markedly decreased in SAH in addition to AH. Especially in nonsurvivors with SAH who showed multiorgan failure, it was extremely low, but in a survivor with SAH it showed mild decrease (Table 2, Fig. 1 & Fig. 2). In a sequential study, the activity increased at the recovery stage in AH, but it remained extremely low in a nonsurvivor with SAH (Fig. 3). These results suggest that plasma ADAMTS 13 activity is closely related to the severity of liver disturbance in AH, and is useful marker for predicting the outcome in SAH with multiorgan failure. Regarding the relationship of the ADAMTS13 activity to each clinical parameter in the univariate analysis, it significantly correlated with 10 clinical variables including functional liver capacity, inflammation signs, renal function, and platelet count (Table 3). Among these, the multivariate analysis showed that serum total bilirubin and CRP independently correlated with the protease activity (Fig. 4), indicating that the activity is strongly associated with hyperbilirubinemia and inflammation signs in AH and SAH. It is of particular interest that these two parameters were selected in the multivariate analysis, because serum total bilirubin is one of important indicators for estimating the severity of liver disturbance as used in the formula of Maddrey's score in SAH (Carithers et al., 1989), and CRP one of characteristic factors associated with the occurrence of AH together with prognostic factors (Fujimoto et al., 1999). Alternatively, in our study, the activity of ADAMTS13 did not differ between patients with AH and LC. In 14 patients with AH, it tended to be lower in cirrhotics with superimposed AH than in patients without LC, but the difference did not reach statistical significance. In addition, in cirrhotic patients, the activity tended to be lower as cirrhotic stage progressed, indicating that the decrease in the activity in cirrhotics with superimposed AH would be, therefore, attributable to the severity of both functional liver capacity in cirrhotics and superimposed alcoholic hepatitis.

With respect to the pathogenesis of SAH in addition to AH, endotoxemia due to hepatic reticuloendothelial dysfunction and increased intestinal permeability may trigger the enhancement of pro-inflammatory cytokine including

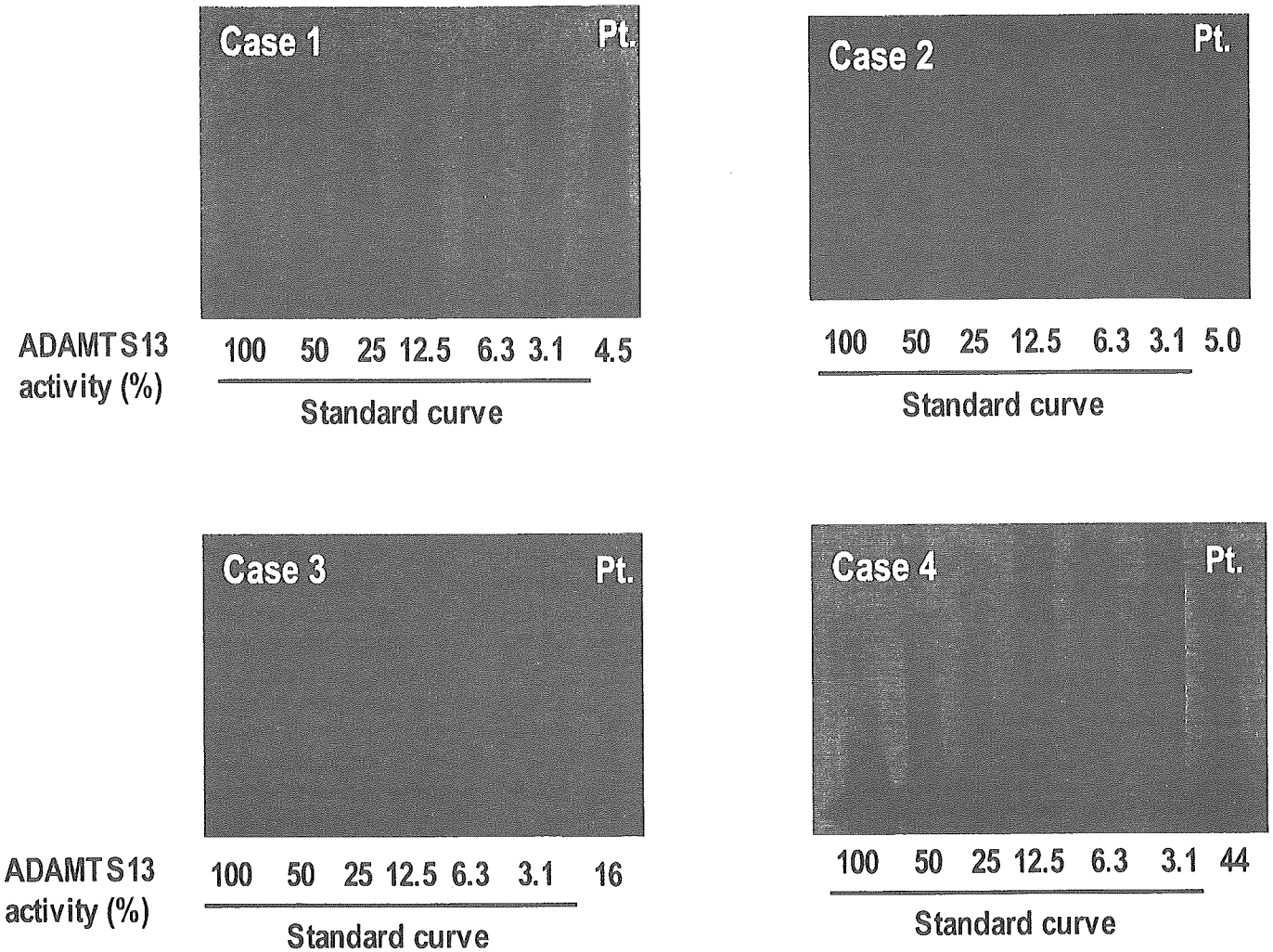

Fig. 2. Standard curve of ADAMTS13 activity and its individual value in plasma from 4 patients (case 1 to 4) with severe alcoholic hepatitis. Quantification of ADAMTS13 was estimated by the length of VWF multimers using scanning densitometry, and the detection limit of the protease activity was approximately 3%. The activity of patients with severe alcoholic hepatitis was 4.5% in case 1, 5.0% in case 2, 16% in case 3, and 44% in case 4, respectively. The protease activity of case 1 to 3 markedly decreased, and especially in case 1 and 2, the each value was extremely low, which was consistent with that of the patient with typical thrombotic thrombocytopenic purpura.

tumor necrosis factor-α (TNF-α), which may lead to systemic inflammatory response syndrome together with microcirculatory disturbance and systemic hemodynamic derangements, resulting in the occurrence of multiorgan failure (Fukui et al., 1991; Ishii et al.,1993, Mookerjee et al., 2003). Alternatively, the deficiency of plasma ADAMTS13 activity causes an increase in UL-VWFM, which interacts with the circulating platelets and leads to platelet clumping, resulting in microcirculatory disturbance and multiorgan failure as seen in TTP (Furlan et al., 1997; Tsai and Lian, 1998; Fujimura et al., 2002). Interestingly, in our nonsurvivors with SAH and multiorgan failure, the activity of ADAMTS13 was extremely low value, which is consistent with that of typical TTP, indicating that the deficiency of ADAMTS13 may, in part, contribute to the development of multiorgan failure through microcirculatory disturbance in patients with SAH. Indeed, acquired "secondary" TTP different from patients of "idiopathic" TTP who suddenly develop TTP without any underlying disease, is in a significant number of patients with a variety of clinical conditions, including autoimmune disease, stem cell transplantation, malignancies, drug, pregnancy, and infections (Matsumoto et al., 2004).

The reason why the protease activity decreased in patients with AH and SAH remains unclear, but we could speculate as follows; the direct inhibition of the protease activity by ethanol itself, the consumption of the protease due to a large amount of UL-VWFM, the presence of inhibitors against the protease, which is detected in the majority of patients diagnosed as having "idiopathic," pregnancy- or drug-associated with TTP (Matsumoto et al., 2004), and unknown factors such as pro-inflammatory cytokines, which could be involved in the hemostatic abnormalities including thrombotic conditions (Grignani G and Maiolo A, 2000). Recently, it was demonstrated that inflammatory cytokines, IL-8 and TNFα, may stimulate the

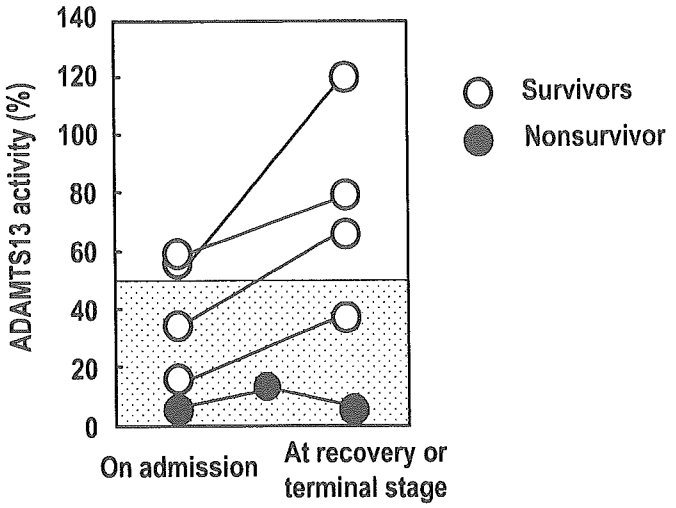

Fig. 3. Changes in the activity of the plasma ADAMTS13 in the patients with alcoholic hepatitis and severe alcoholic hepatitis during admission. The shaded area shows below 50% of the healthy subjects (mean - 2SD). Open circles indicate survivors and closed circle indicates a nonsurvivor, respectively. The protease activity significantly increased at the recovery stage in survivors with alcoholic hepatitis ($p < 0.05$), but it remained extremely low in a nonsurvivor with severe alcoholic hepatitis.

Table 3. Correlation Coefficients Between Plasma ADAMTS13 Activity and Clinical Variables in Patients With Alcoholic Hepatitis

Variables	Coefficient correlation	*p*-values
C-reactive protein (mg/dl)	−0.658	0.0023
Serum total bilirubin (mg/dl)	−0.616	0.0054
Lactate dehydrogenase (IU/L)	−0.606	0.0065
Total protein (g/dl)	0.539	0.0197
Serum albumin (g/dl)	0.535	0.0208
Polymorphonuclear neutrophil (/mm^3)	−0.523	0.0245
Prothrombin time (%)	0.518	0.0317
Serum creatinine (mg/dl)	−0.500	0.0355
White blood cell count (/mm^3)	−0.488	0.0390
Platelet count (×10^4/mm^3)	0.472	0.0471
Aspartate aminotransferase (IU/L)	−0.452	0.0594
Blood urea nitrogen (mg/dl)	−0.340	0.1707
Age (yr)	0.326	0.1894
Alanine aminotransferase (IU/L)	−0.176	0.4913
γ-Glutamyl transpeptidase (IU/L)	0.043	0.8711

UL-VWFM release and IL-6 may inhibit the action of ADAMTS13 using human umbilical vein endothelial cells (Bernardo et al., 2004). The plasma levels of these cytokines have been reported to be remarkably high especially in SAH (Ishii et al., 1993; Fujimoto et al., 2000; Mookerjee et al., 2003), indicating that extremely low activity of plasma ADAMTS13 in SAH may be caused by enhanced inflammatory cytokinemia.

Microcirculatory disturbance in the liver is considered to be one of important pathogenetic roles of alcoholic hepatitis. It includes the narrowing of sinusoidal space due to ballooned hepatocyte and perisinusoidal fibrosis (French et al., 1984), the imbalance of endothelin and nitric oxide (Oshita et al., 1993), and the contraction of hepatic stellate cell (Itatsu et al., 1998). Considering that ADAMTS13 is presumably produced in the perisinusoidal cells (Lee et al., 2002), and its substrate, UL-VWFM, is produced in the vascular endothelial cells transformed into capillaries in the liver, the deficiency of plasma ADAMTS13 activity may play an important role on a sinusoidal microcirculatory disturbance and subsequent liver disturbance in patients with AH. Indeed, positive VWF antigen staining was observed along the sinusoidal wall and perivenular region in the liver of rats with the initial stage of alcoholic liver diseases and high plasma values of hyaluronic acid, a marker of hepatic sinusoidal endothelial cell function (Nanji et al., 1996). It will be necessary to be clarified the relationship of VWF and UL-VWFM together with the activity of plasma ADAMTS13 to the development of multiorgan failure in SAH as well as the progression of liver disturbance in AH. The determination of ADAMTS13 and its substrate would give us new insights on understanding the pathophysiology and its therapeutic approach in acute alcoholic liver injury.

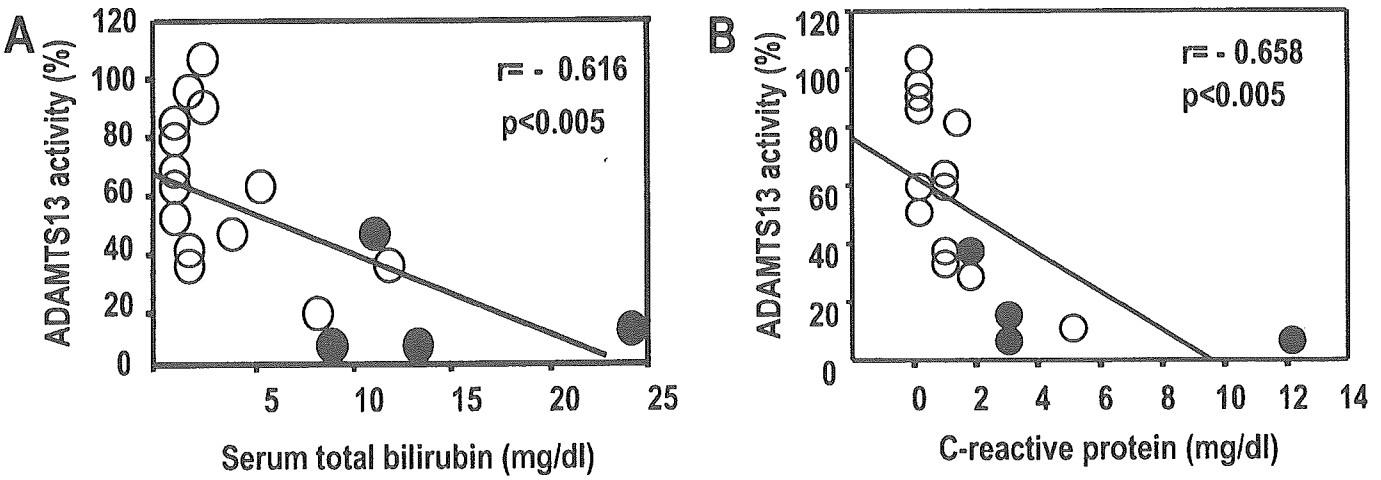

Fig. 4. Relationship of the activity of the plasma ADAMTS13 to serum total bilirubin and C-reactive protein in patients with alcoholic hepatitis and severe alcoholic hepatitis on admission. The open circles indicate the patients with alcoholic hepatitis and the closed circles indicate the patients with severe alcoholic hepatitis. The protease activity negatively correlated with serum total bilirubin (A) and C-reactive protein (B).

ACKNOWLEDGMENTS

The authors sincerely thank Ayami Isonishi and Hitomi Nishimura for their great help in the assay of the plasma ADAMTS13 activity throughout this work.

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Original Paper

Pathophysiology of Haemostasis and Thrombosis

Pathophysiol Haemost Thromb 2005;34:35–40
DOI: 10.1159/000088546

Received: January 6, 2005
Accepted after revision: March 31, 2005

Platelets Treated with Ticlopidine Are Less Reactive to Unusually Large von Willebrand Factor Multimers than Are Those Treated with Aspirin under High Shear Stress

M. Matsumoto[a] S. Kawaguchi[b] H. Ishizashi[c] H. Yagi[a] J. Iida[b] T. Sakaki[b]
Y. Fujimura[a]

Departments of [a]Blood Transfusion Medicine, [b]Neurosurgery and [c]Health Science, Nara Medical University, Kashihara, Japan

Key Words
Ticlopidine · Aspirin · von Willebrand factor · Cerebral ischemia

Abstract
Much attention has recently been focused on the interaction between unusually large von Willebrand factor multimers (UL-VWFM) and platelets under high shear stress in pathological thrombus formation. The antiplatelet drugs acetylsalicylic acid (aspirin) and a thienopyridine derivative (ticlopidine) are commonly used to treat cerebral ischemia but exert different effects on high-shear-stress-induced platelet aggregation (H-SIPA) in the plasma. To examine the effects of these drugs in the absence of plasma factors, we studied H-SIPA using washed platelets (WPs) and purified UL-VWFM. WPs were prepared from the blood of 9 aspirin-treated and 11 ticlopidine-treated patients with cerebral ischemia, and H-SIPA in the presence of UL-VWFM was measured using a cone plate aggregometer. Plasma levels of VWF antigen with its multimer analysis, ristocetin cofactor and VWF-cleaving protease (ADAMTS13) activity were also measured. Forty-six healthy volunteers from 2 age groups, 20–40 years (n = 20) and 41–60 years old (n = 26), were also tested as controls. H-SIPA was significantly inhibited for ticlopidine-treated platelets, but it was observed to a lesser extent for aspirin-treated platelets. For both groups, no difference in the plasma levels of VWF antigen, ristocetin cofactor and ADAMTS13 activity was noted. All patients possessed UL-VWFM, and it was detected in healthy volunteers with increasing frequency with increasing age. Under plasma-free conditions, platelets from aspirin-treated patients exhibit marginal but significant inhibition of H-SIPA. Furthermore, the presence of UL-VWFM in the plasma of patients and normal volunteers is directly related to their age rather than being a consequence of underlying disease.

Introduction

Antiplatelet drugs such as aspirin and thienopyridine derivatives are commonly used to treat patients with cerebral ischemia to prevent the occlusion of brain arteries. Aspirin inhibits thromboxane A_2 synthesis [1], and the thyenopyridine derivatives ticlopidine and clopidogrel block the ADP receptor $P2Y_{12}$ [2–4].

In 1994, Uchiyama et al. [5] reported that high-shear-stress-induced platelet aggregation (H-SIPA) using platelet-rich plasma (PRP) is enhanced in patients with cere-

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Yoshihiro Fujimura, MD
Department of Blood Transfusion Medicine, Shijyo-Cho 840
Kashihara City, Nara 634-8522 (Japan)
Tel. +81 744 22 3051, ext. 3289, Fax +81 744 29 0771
E-Mail yfujimur@naramed-u.ac.jp

Table 1. Patient characteristics

	Age years	Sex	Underlying disease	Complication
Ticlopidine				
1	67	M	TIA	DM
2	64	M	TIA	HT
3	66	F	TIA	–
4	53	M	TIA	DM, HT
5	54	M	RIND	–
6	65	M	RIND	DM, HL
7	60	M	TIA	–
8	31	F	TIA	–
9	56	M	TIA	HT
10	68	M	TIA	DM
11	77	M	TIA	HT
Aspirin				
1	65	F	TIA	HT
2	67	M	TIA	HT
3	65	M	TIA	–
4	52	M	TIA	–
5	63	M	TIA	DM, HL
6	41	M	TIA	–
7	52	F	TIA	HT
8	36	M	TIA	AP
9	67	M	TIA	DM

TIA = Transient ischemic attack; RIND = reversible ischemic neurological deficit; DM = diabetes mellitus; HT = hypertension; HL = hyperlipidemia; AP = angina pectoris.

bral ischemia due to the increase in large von Willebrand factor (VWF) multimers and that the enhancement of H-SIPA can be corrected by taking ticlopidine but not low-dose aspirin. H-SIPA is mediated by the interaction of the platelet receptors glycoprotein (GP) Ibα and GP IIb/IIIa with VWF, which is a plasma GP exclusively synthesized in vascular endothelial cells and secreted into the circulation as unusually large VWF multimer (UL-VWFM). UL-VWFM most actively interacts with platelets and induces the formation of platelet thrombi under high shear stress conditions [6]. In the normal circulation, however, UL-VWFM is rapidly cleaved and degraded into smaller VWFM by the plasma protease VWF-cleaving protease (ADAMTS13), which attacks the Tyr842-Met843 bond [7].

In this study, we analyzed the effect of two antiplatelet drugs on H-SIPA using a washed platelet (WP) system and examined the UL-VWFM of patients with cerebral ischemia using SDS-0.9% agarose gel electrophoresis.

Materials and Methods

Subjects

Twenty patients with cerebral ischemia who were being treated with antiplatelet drugs were enrolled in this study as listed in table 1 after informed consent had been obtained. Diagnoses were made based on neurological examinations, routine laboratory data, brain computed tomography, brain magnetic resonance imaging and cerebral angiography. None of the patients had findings of major vessel occlusions and neurological symptoms at the time of registration. They were not given any antiplatelet drugs for 2 weeks and any anticoagulants or fibrinolytic drugs for 24 h prior to the study. Then the patients were randomly allocated to receive 200 mg ticlopidine (Panaldin®, Daiichi Parmaceutial Corp.) or 81 mg aspirin (Bufferin®, Lion Corp.) by choosing the envelope including a card with a drug name, ticlopidine or aspirin. There was no statistically significant difference between these two groups concerning the demographics such as age, gender and clinical symptoms.

For each patient, 18 ml of blood was withdrawn by venipuncture from an antecubical vein using a 21-gauge needle before and days 7 after oral administration of ticlopidine or aspirin; the patient was then anticoagulated with a 1/10th volume of 3.8% Na_3-citrate. We chose day 7 after oral administration as the 2nd examination point because ticlopidine inhibits platelet aggregation after 3–5 days of use [8]. Five milliliters of the citrated blood were subsequently centrifuged at 3,000 *g* for 15 min at 4°C, and the platelet-poor plasma was separated and stored in aliquots at –80°C until use. The remaining 15 ml was used to prepare WPs as described below.

For the control experiments, citrated platelet-poor plasma was also prepared from two groups of healthy volunteers composed of 13 males and 7 females aged 20–40 years and 25 male and 1 female volunteers aged 40–61 years.

Preparation of UL-VWFM

Purification of VWF from cryoprecipitate was performed as described elsewhere [9]. Briefly, cryoprecipitate was prepared from 3 liters of outdated fresh frozen plasma, provided from the Japan Red Cross Blood Center by freezing at –80°C and thawing overnight at 4°C. After centrifugation at 7,500 *g* for 30 min at 4°C, the cryoprecipitate was then collected and dissolved in 300 ml of 25 mmol/l Tris-HCl buffer (pH 7.3) containing 0.5 mmol/l EDTA·4Na, 150 mmol/l NaCl and 1 mmol/l phenylmethylsulfonyl fluoride. Next, it was centrifuged at 7,500 *g* for 15 min at room temperature, and the supernatant was applied to a gelatin-sepharose 4B (Amersham Bioscience) column (Vt = 200 ml) at room temperature to remove fibronectin, after which the fall-through fractions were pooled. Following precipitation with 40% saturated $(NH_4)_2SO_4$ and centrifugation, the precipitate was separated and dissolved in 50 ml of 20 mmol/l imidazole-HCl buffer (pH 6.5) containing 20 mmol/l ε-aminocapronic acid, 1 mol/l NaCl and 10 mmol/l sodium citrate. It was then centrifuged again at 7,500 *g* for 15 min, and the resulting supernatant was applied to a sepharose 4B gel filtration column (5 × 100 cm, Amersham Bioscience). The eluate was collected in 8-ml volumes in separate tubes and dialyzed against phosphate-buffered saline (pH 7.3) at 4°C overnight, and the fractions containing UL-VWFM (fig. 1 and Results) were pooled and kept frozen in aliquots at –80°C. This purified material was used subsequently throughout this study.

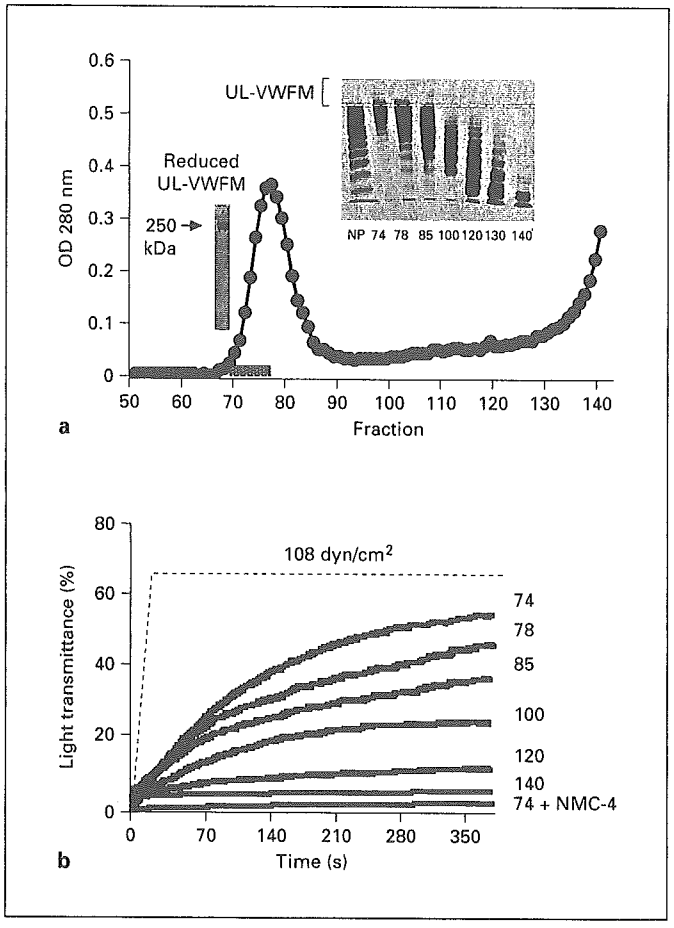

Fig. 1. Preparation of UL-VWFM. **a** Chromatographic separation of VWF on a Sepharose 4B column, where the black bar indicates the fractions which contain UL-VWFM. The right inset shows the results of the multimeric analysis performed by SDS-1.2% agarose gel electorophoresis. NP = Normal human plasma. To check the purity of VWF, the purified UL-VWF fraction was subjected to SDS-5% polyacrylamide gel electrophoresis under reducing conditions as shown in the left inset. **b** H-SIPA using normal WPs and the purified VWF fraction. H-SIPA using a mixture of normal WPs at a final concentration of 30 × 10^8/ml and each purified VWF fraction at a final concentration of 5 μg/ml was dependent on its multimeric size.

Measurement of H-SIPA Using WPs

WPs were prepared under room temperature as follows [10]. First, PRP was produced by centrifugation at 200 *g* for 10 min using 15 ml citrated blood from patients as mentioned in the Subjects section and from a normal volunteer (blood type 0, Rho(D)+), and then acidified to pH 6.5 with acid citrate dextrose. The resulting platelets were separated from the PRP by centrifugation at 800 *g* for 10 min in the presence of 1 U/ml apyrase (Sigma-Aldrich) and 1 mmol/l prostaglandin E_1 (PGE_1; Sigma-Aldrich), and were then washed twice in platelet-washing buffer (113 mmol/l NaCl, 4.3 mmol/l K_2HPO_4, 4.2 mmol/l Na_2HPO_4, 24.4 mmol/l NaH_2PO_4 and 5.5 mmol/l glucose, pH 6.5) that contained 1 U/ml apyrase and 1 mmol/l PGE_1. The platelet pellets were next resuspended in HEPES-Tyrode's buffer (138 mmol/l NaCl, 2.8 mmol/l KCl, 2 mmol/l $CaCl_2$ and 10 mmol/l HEPES, pH 7.4) at a concentration of 3 × 10^8/ml and used within 2.5 h.

H-SIPA was measured using an argon-laser-assisted cone platelet aggregometer (Torey Medical Inc., Tokyo, Japan) [11] at room temperature. Before applying constant high shear stress at 108 dyn/cm^2, the purified UL-VWFM was added to the platelet suspensions at a final concentration of 5 μg/ml in a total volume of 400 μl. For some experiments, H-SIPA was measured in the presence of anti-VWF monoclonal antibody (NMC-4), for which the epitope resides on the VWF A1 domain and inhibits its binding to platelet GP Ib [9].

Additional Assays

Assays for VWF antigen [12] and ristocetin cofactor [13] were performed in addition to SDS-0.9% agarose gel electrophoresis followed by Western blotting with luminographic detection of VWFM [14, 15]. Plasma ADAMTS13 activity was assayed by the modified method of Furlan et al. [16] based on VWFM analysis [17]. The activity of pooled normal plasma was defined as 100%.

Statistical Analysis

Paired and unpaired comparisons between the two groups were performed using the Student's t test for which $p < 0.05$ was judged to indicate statistical significance. All experimental data are presented as means ± SD.

Results and Discussion

From SDS-1.2% agarose gel electrophoretic analysis, the initial half void volume fractions (F71–78) were found to possess UL-VWFM and also showed a single 250-kD band by SDS-5% polyacrylamide gel electrophoretic analysis under reducing conditions (fig. 1a). Furthermore, H-SIPA using a mixture of normal WPs and each purified VWF fraction at 5 μg/ml final concentration was dependent on its multimeric size. Maximum aggregation was observed when using F74 VWF, and this was completely blocked by the anti-VWF monoclonal antibody NMC-4 at a final concentration of 10 μg/ml (fig. 1) or with the anti-GP-Ib monoclonal antibody AP-1 at a final concentration of 10 μg/ml (not shown). Thus, the pooled fractions (F71–78) were used as the source of UL-VWFM for subsequent studies of H-SIPA, and the percent light transmittance at 360 s after the generation of high shear stress at 108 dyn/cm^2 was recorded as the maximum aggregation value.

For the patients treated with ticlopidine, H-SIPA was dramatically but not totally inhibited, and the value was 43.8 ± 17.7% before and 21.2 ± 10.5% after treatment ($p < 0.001$) as shown in figure 2. Furthermore, for the patients treated with aspirin, H-SIPA was also inhibited to

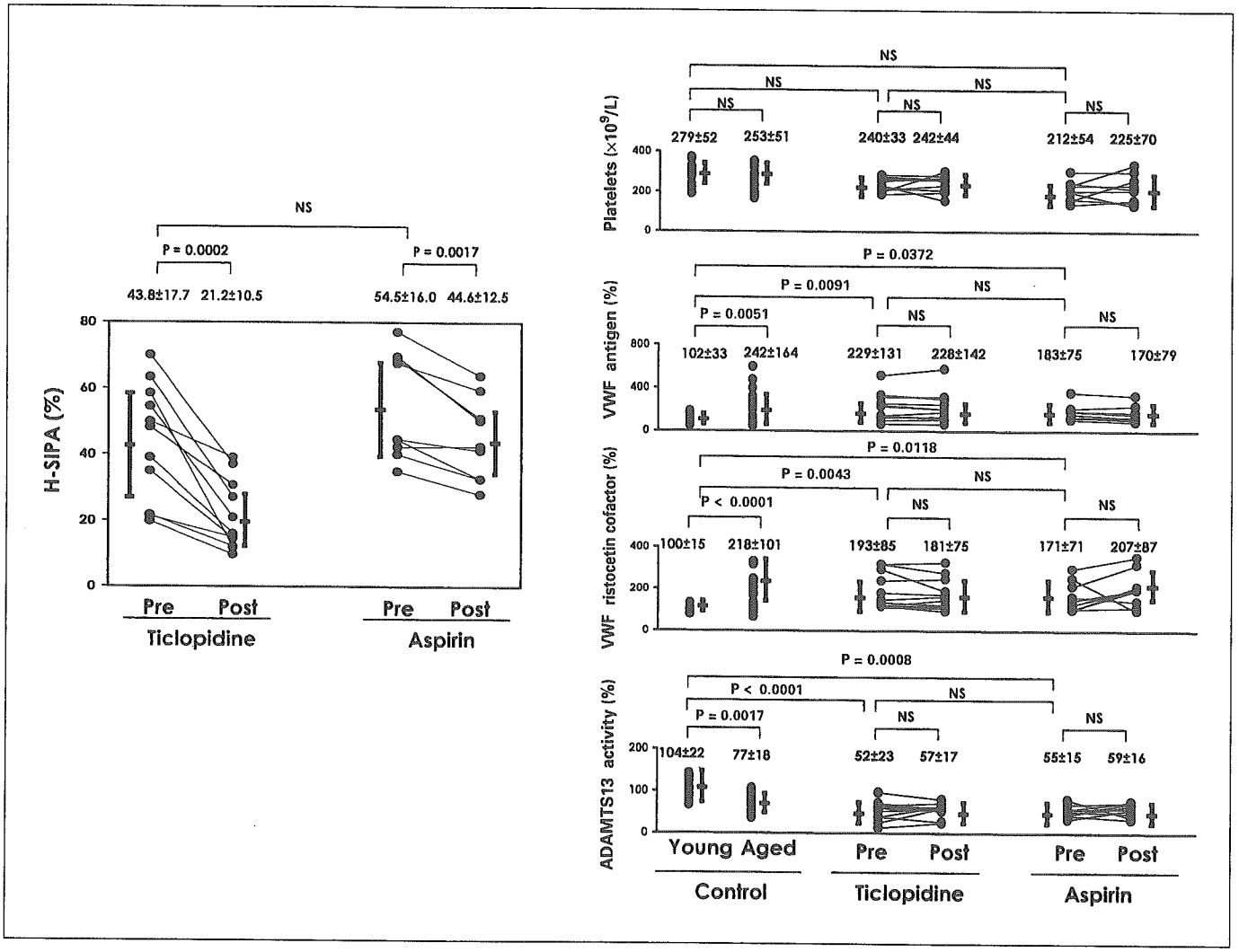

Fig. 2. Comparison of H-SIPA, platelets and VWF. For the patients treated with ticlopidine, H-SIPA was dramatically inhibited after treatment as was the case for patients treated with aspirin, although to a lesser extent. The plasma VWF antigen, ristocetin cofactor and ADAMTS13 activity in patients from both groups were significantly unchanged before and after treatment.

some extent but less than that after ticlopidine treatment, and the value was 54.5 ± 16.0% before and 44.6 ± 12.5% after treatment (p = 0.0017), which is in contrast to the results of Uchiyama et al. [3], who found a remarkable inhibition of H-SIPA in patients treated with ticlopidine but no significant inhibition in those treated with aspirin using a PRP system.

Since H-SIPA in the PRP system is influenced by the nature of the platelets and multiple plasma factors, we analyzed the plasma VWF of both patient groups. As shown in figure 2, both groups exhibited a remarkably increased level of plasma VWF antigen and ristocetin cofactor compared with young healthy volunteers. The plasma VWF antigen and ristocetin cofactor of patients with cerebral ischemia were significantly unchanged before and after treatment, but a remarkable increase in both was observed in subjects of advanced age. Furthermore, a significant decrease in plasma ADAMTS13 activity compared with young healthy volunteers was noted for both patient groups, which was unchanged before and after treatment. A remarkable decrease was also observed for subjects of advanced age. The reason why the activity of plasma ADAMTS13 decreased in both patients with cerebral ischemia and advanced-age subjects is consid-

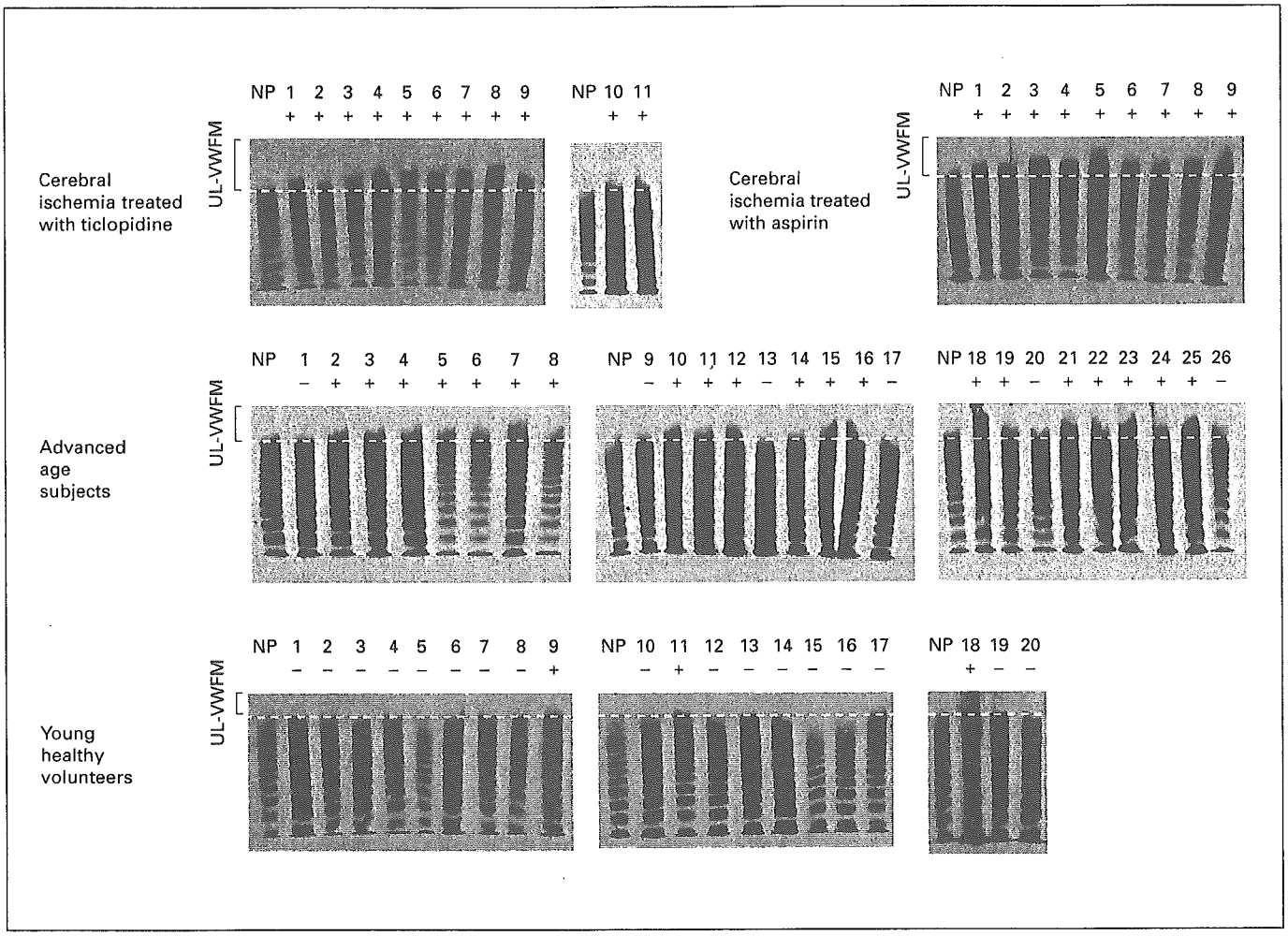

Fig. 3. The detection of UL-VWFM by SDS-0.9% agarose gel analysis. All patients with cerebral ischemia in both groups had UL-VWFM, and UL-VWFM was also detected in 20 of 26 of subjects of advanced age, whereas it was only seen in 3 out of 20 young healthy volunteers. NP = Normal human plasma.

ered to be decreased production of ADAMTS13 in the liver or consumption of ADAMTS13 to degrade very high amounts of VWF antigen. Mannucci et al. [18] speculated that the consumption of ADAMTS13 degraded a large amount of VWF antigen in order to explain the inverse correlation between ADAMTS13 activity and VWF antigen in healthy and various pathological conditions. Decreased activity of ADAMTS13 together with a large amount of VWF antigen may induce the appearance of UL-VWFM in plasma, which may result in a risk factor for cerebral ischemia to occur.

Our assay using the patient's WPs was successful at determining platelet function because it excluded the effect of plasma factors. Likewise, Sun et al. [19] have recently reported that something in the plasma was responsible for the phenomenon of shear aggregation 'aspirin resistance'.

Next, we examined the UL-VWFM of patients with cerebral ischemia by SDS-0.9% agarose gel electrophoresis as shown in figure 3. All patients with cerebral ischemia in both groups had UL-VWFM, and this confirmed the observed increase in plasma VWF antigen and ristocetin cofactor in patients with cerebral ischemia. UL-VWFM was also detected in 20 of 26 subjects of advanced age, whereas it was only seen in 3 of 20 young healthy volunteers.

Cerebral ischemia is caused by platelet thrombosis induced by high shear stress. Recent studies revealed that

VWF and its interaction with the platelet receptors GP Ibα and GP IIb/IIIa play a role in platelet thrombosis induced by this type of stress [4]. In this study, we found that the enhancement of H-SIPA in patients with cerebral ischemia is corrected for by taking ticlopidine and aspirin, and that ticlopidine has a stronger effect than does aspirin. These drugs seem to solely affect platelet function. In addition, subjects of advanced age possessed UL-VWFM more frequently than did young subjects. These results indicate that people of advanced age may be more susceptible to developing cerebral ischemia.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (to M.M., H.I. and Y.F.), and from the Ministry of Health and Welfare of Japan for Blood Coagulation Abnormalities H14-02 (to Y.F.).

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Brief report

Localization of ADAMTS13 to the stellate cells of human liver

Masahito Uemura, Kouko Tatsumi, Masanori Matsumoto, Masao Fujimoto, Tomomi Matsuyama, Masatoshi Ishikawa, Taka-aki Iwamoto, Toshio Mori, Akio Wanaka, Hiroshi Fukui, and Yoshihiro Fujimura

Although the chromosomal localization (9q34) of the gene encoding the human form of ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13) and its exclusive expression in the liver have been established, the cells that produce this enzyme are yet to be determined. We investigated the expression of ADAMTS13 mRNA and protein in fresh frozen specimens obtained during liver biopsies of 8 patients with liver diseases. In situ hybridizations to localize ADAMTS13 mRNA showed positive signals exclusively in perisinusoidal cells with irregularly elongated dendritic processes extending between hepatocytes. Furthermore, ADAMTS13 was detected immunohistochemically in perisinusoidal cells, whereas no staining was observed in hepatocytes. The positive cells varied in shape from unipolar to dendritic with irregularly elongated cytoplasmic processes, features common to hepatic stellate cells (HSCs). Double-labeling experiments revealed that the ADAMTS13-positive cells also expressed α-smooth muscle actin, confirming that these cells were activated HSCs. These results suggest that HSCs may be major cells producing ADAMTS13 in human liver. (Blood. 2005;106:922-924)

Introduction

ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13) is a metalloproteinase that specifically cleaves the multimeric von Willebrand factor (VWF) between Tyr1605 and Met1606 within the VWF A2 domain.[1-4] VWF is synthesized in vascular endothelial cells, and released into the plasma as unusually large VWF multimers (UL-VWFMs).[5,6] Usually, UL-VWFMs are rapidly degraded into smaller VWF multimers by ADAMTS13. Deficiency of ADAMTS13 caused either by mutations of the *ADAMTS13* gene[1,7] or by inhibitory autoantibodies against ADAMTS13[8,9] increases the plasma levels of UL-VWFMs, which leads to platelet clumping and/or thrombi under high shear stress, resulting in thrombotic thrombocytopenic purpura (TTP).[5-9]

Northern blot analysis indicated that the 4.6-kilobase ADAMTS13 mRNA was exclusively expressed in the liver, and a 2.4-kilobase ADAMTS13 mRNA was also expressed in placenta and skeletal muscle.[2] In situ hybridization analysis revealed that the mRNA signals were expressed exclusively in the perisinusoidal cells,[10] but without addressing the type of cells expressing ADAMTS13. Moreover, a substantial decrease of plasma ADAMTS13 activity in patients with chronic liver disease has been associated with its disease progression, but not always with the serum levels of enzymes produced by hepatocyte.[11] Thus, specification and/or localization of the cells that produce this enzyme in the liver should have clinical importance, and may help elucidate the pathogenesis of sinusoidal microcirculatory disturbances and/or thrombotic complications in patients with liver diseases.

In this study, we have clearly shown that ADAMTS13 is produced specifically in hepatic stellate cells, formerly called Ito cells, by both in situ hybridization techniques and immunohistochemical analysis using 2 novel mouse monoclonal antibodies specific for ADAMTS13.

Study design

Patients

This study examined 8 patients with liver disease (6 women and 2 men; mean age, 54.6 years; range, 43-72 years) including 4 patients with hepatitis C virus (HCV)–related chronic hepatitis, one patient with hepatitis B virus–related chronic hepatitis, one patient with primary biliary cirrhosis, one patient with autoimmune hepatitis, and one patient with a drug-induced liver injury who had undergone laparoscopies or percutaneous needle biopsies. Laboratory findings of these patients showed well-preserved functional liver capacity and platelet counts (mean, $18.3 \times 10^4/mm^3$; range, $8.8\text{-}32.3 \times 10^4/mm^3$). Informed consent was provided by the patients and their families before the biopsies. The protocol used in this study was approved by the Nara Medical University Hospital Ethics Committee, Nara, Japan.

From the Third Department of Internal Medicine, the Departments of Anatomy and Blood Transfusion Medicine, and the Radioisotope Research Center, Nara Medical University, Kashihara, Nara, Japan.

Submitted January 12, 2005; accepted March 22, 2005. Prepublished online as *Blood* First Edition Paper, April 26, 2005; DOI 10.1182/blood-2005-01-0152.

Supported in part by research grants from the Japanese Ministry of Education, Culture, and Science (Y.F. and M.M.) and from the Ministry of Health and Welfare of Japan for Blood Coagulation Abnormalities H14-02 (Y.F.).

M.U., K.T., and A.W. performed immunostaining and in situ hybridization and prepared this manuscript. T. Matsuyama, M.I., and M.F. collected liver specimens. M.M., T.I., and T. Mori prepared murine anti-ADAMTS13 monoclonal antibody and characterized it. H.F. directed this study. Y.F. designed and directed this study throughout.

The online version of the article contains a data supplement.

Reprints: Yoshihiro Fujimura, Department of Blood Transfusion Medicine, Nara Medical University, 840 Shijo-cho, Kashihara City, Nara, 634-8522, Japan; e-mail: yfujimur@naramed-u.ac.jp.

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Production and characterization of anti-ADAMTS13 murine monoclonal antibodies

Full-length wild-type recombinant (r) ADAMTS13 that was purified by anti-FLAG (fludarabine, cytarabine, and granulocyte colony–stimulating factor) M2 agarose affinity chromatography (Sigma, Saint Louis, MO) was used as an immunogen[12] to produce monoclonal antibodies (A10 and C7) against ADAMTS13 in mice following standard procedures.[13] The immunoglobulin subclasses of A10 and C7 were IgG2b-κ and IgG1-κ, respectively. These antibodies were purified with a Protein A column (Amersham Biosciences, Uppsala, Sweden). These 2 monoclonal antibodies were able to detect endogenous plasma ADAMTS13 as a 190-kDa/180-kDa doublet band by Western blot analysis under nonreducing conditions. The epitopes recognized by the A10 and C7 antibodies were determined to reside in the disintegrin-like domain, and the seventh and eighth thrombospondin type-1 domains, respectively. Detailed characterizations of these antibodies are shown as supplementary data (Figure S1, available on the *Blood* website; see the Supplemental Figure link at the top of the online article).

Immunohistochemistry

Fresh liver specimens obtained from the 8 patients were fixed in 4% paraformaldehyde solution before frozen sections were prepared. The sections were incubated with primary antibodies (A10 and C7) overnight at 4°C. Bound primary antibodies were subsequently immunodetected using a standard avidin-biotin-peroxidase complex technique. To simultaneously detect A10- and anti–α-smooth muscle actin (α-SMA) immunoreactivity, sequential incubations using the following reagents were performed: A10 antibodies and anti–α-SMA antibodies (DAKO, Kyoto, Japan) followed by Alexa 488–conjugated anti–mouse immunoglobulin G (Invitrogen, Carlsbad, CA) and Alexa 546–conjugated anti–rabbit IgG (Molecular Probes). These labeled sections were observed with a Nikon Labphoto-2 fluorescent microscope and imaged with an MRC-600 confocal laser-scanning microscope system (Bio Rad Laboratories, Tokyo, Japan). Figures were assembled using Confocal Assistant software (Bio Rad Laboratories).

In situ hybridization

The cDNA encoding human ADAMTS13 was kindly provided by Dr Kenji Soejima (Chemo-Thero-Therapeutic Institute, Kumamoto, Japan). Digoxygenin (DIG)–labeled cRNA probes (sense and antisense) were transcribed using either T3 (sense) or T7 (antisense) RNA polymerase and a plasmid with an insert corresponding to nucleotides 3710-4237 of the full-length human ADAMTS13 transcript (Genbank accession no. AB069 698). All prehybridization procedures have been previously described.[14] To visualize the DIG-labeled probes, the sections were incubated with alkaline-phosphatase–conjugated anti-DIG antibodies (Roche Diagnostics KK, Tokyo, Japan) followed by 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate solution (Roche).

Results and discussion

When immunostaining was performed with A10 antibodies on liver specimens from a patient with HCV-related chronic hepatitis, dense brown staining was observed in perisinusoidal cells inside the lobule (Figure 1A). Furthermore, when the same liver section was stained with C7 antibodies, staining patterns were similar to those of A10 antibodies (Figure 1B). The varied morphologic features of these positive cells were consistent with those previously described for perisinusoidal stellate cells (Figure 1C-G).[15,16] Control specimens treated with unspecific mouse IgG instead of primary antibodies displayed no significant staining (data not shown). In situ hybridizations using antisense probes for ADAMTS13 revealed strongly positive labeling only in perisinusoidal cells with irregularly elongated cytoplasmic processes extending between hepatocytes (Figure 1H), whereas control specimens treated with sense probes showed no significant staining (Figure 1I). These ADAMTS13 mRNA–positive cells resembled HSCs morphologically, suggesting that HSCs produce ADAMTS13. In order to evaluate whether the ADAMTS13-positive cells truly would be HSCs, we performed double immunofluorescence immunohistochemistry with A10 anti-ADAMTS13 antibodies and anti–α-SMA antibodies. The microfilament protein α-SMA has been recognized as a specific marker for stellate cells[17]; activated stellate cells in pathologic livers are strongly α-SMA–positive, whereas in normal adult human liver, some stellate cells are α-SMA–positive.[18] Immunofluorescence labeling with anti-ADAMTS13 antibodies showed intense green fluorescence in perisinusoidal cells that

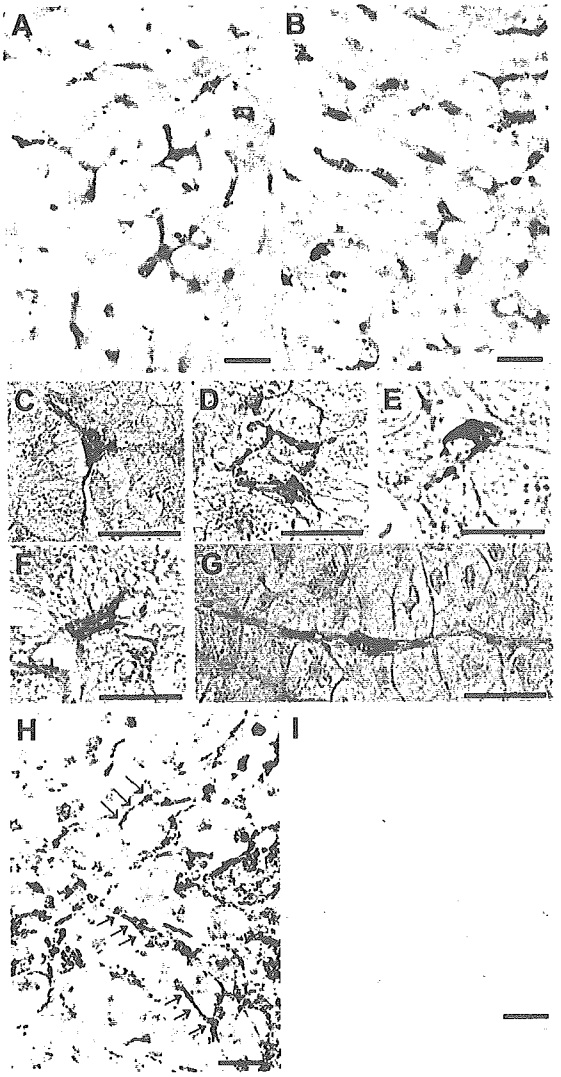

Figure 1. ADAMTS13 protein and mRNA expression in frozen sections of a liver specimen from a patient with hepatitis C–related chronic hepatitis. Immunostaining using ADAMTS13-specific monoclonal antibodies (A10) showed dense brown staining in perisinusoidal cells, but not in hepatocytes, inside the lobule (A). When the same liver section was stained with C7 antibodies, staining patterns were similar to those of A10 antibodies (B). The positive cells had a variety of morphologic forms. Examples shown here include a tripolar cell with long processes extending between hepatocytes (C), combined cells surrounding a hepatocyte (D), a dome-shaped cell (E), a bipolar cell with short processes (F), and a unipolar cell with long processes (G). Panels A-B: original magnification, × 200; panels C-G: original magnification, × 400. ADAMTS13 mRNA expression was examined using in situ hybridization. Strongly positive labeling was seen only in perisinusoidal cells, which were unipolar to dendritic in shape with irregularly elongated cytoplasmic processes extending between hepatocytes (H, arrows; original magnification, × 200). When sense probes were used, we observed no significant staining (I; original magnification, × 200). (Bar = 30 μm in A-I.)

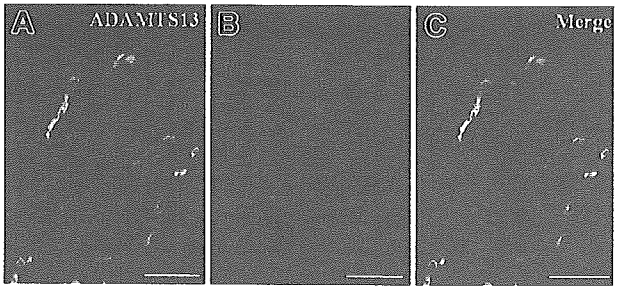

Figure 2. Confocal laser-scanning immunofluorescence microscopy using frozen sections of a liver specimen from a patient with hepatitis C–related chronic hepatitis. Immunofluorescence labeling with anti-ADAMTS13 antibodies showed intense green fluorescence in the perisinusoidal cells, which were irregular in shape with spotty, oval, unipolar, and bipolar cytoplasmic processes extending between the hepatocytes (A). Red fluorescence indicated α-SMA immunoreactivity, reflecting the fact that these cells were activated HSCs (B). Colocalization of ADAMTS13 (A) and α-SMA (B) in single cells yielded yellow color in the merged figure (C). (Original magnification, × 400; bar = 30 μm in A-C.)

were irregular in shape with spotty, oval, unipolar, and bipolar cytoplasmic processes extending between hepatocytes (Figure 2A). α-SMA antibodies revealed that these cells were also positive for the HSC marker (Figure 2B). Colocalization of ADAMTS13 and α-SMA in single cells clearly indicate that stellate cells produce ADAMTS13 in the liver (Figure 2C). Considering that a similar staining pattern was obtained with both A10 and C7 antibodies against ADAMTS13 using the same liver sections (Figure 1A-B), it would seem that the HSCs may produce full-length ADAMTS13 protein.

HSCs have many functions, including vitamin A storage, liver fibrogenesis, and regulation of sinusoidal blood flow. These cells are also rich sources of bioactive mediators for maintaining homeostasis in the microenvironment of the hepatic sinusoid.[17] HSCs are located in the space of Disse adjacent to endothelial cells. It is, therefore, of particular interest that HSCs produce ADAMTS13. In patients with liver cirrhosis, a remarkably high level of plasma VWF has been noted.[11,19] Immunostaining with anti-VWF antibodies has shown the presence of this protein in the sinusoidal lining cells and at the scar-parenchyma interface in cases of liver cirrhosis.[20] This is particularly evident in the sinusoids of patients at the early stages of alcoholic liver diseases,[21] indicating the capillarization of the sinusoidal endothelial cells. Considering that ADAMTS13 is synthesized in the HSCs and its substrate, UL-VWFM, is produced in transformed vascular endothelial cells, the deficiency of plasma ADAMTS13 activity in liver diseases may play an important role in sinusoidal microcirculatory disturbances and subsequent development of liver injury. It will be necessary to clarify the intralobular heterogeneity of ADAMTS13 expression in HSCs associated with the activity of plasma ADAMTS13 in different stage of liver diseases.

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American Journal of Hematology 79:274–280 (2005)

Diagnosis of Deep Vein Thrombosis by Plasma-Soluble Fibrin or D-Dimer

Satoshi Ota,[1] Hideo Wada,[2*] Tsutomu Nobori,[2] Toshihiko Kobayashi,[3] Midori Nishio,[4] Yukiko Nishioka,[4] Maki Noda,[4] Akane Sakaguchi,[4] Yasunori Abe,[4] Junji Nishioka,[4] Ken Ishikura,[1] Norikazu Yamada,[1] and Takeshi Nakano[1]

[1] First Department of Internal Medicine, Mie University School of Medicine, Mie-ken, Japan
[2] Department of Laboratory Medicine, Mie University School of Medicine, Mie-ken, Japan
[3] Second Department of Internal Medicine, Mie University School of Medicine, Mie-ken, Japan
[4] Central Laboratory, Mie University School of Medicine, Mie-ken, Japan

The present study was designed to determine the cutoff values of D-dimer and soluble fibrin (SF) for the diagnosis of deep venous thrombosis (DVT) and pulmonary embolism (PE) in Japanese patients. Plasma levels of these molecules were measured in 243 patients suspected of having DVT and 100 healthy volunteers (controls). Out of 243 patients, 20 patients were diagnosed with DVT. In the control group, plasma levels of D-dimer and SF did not show normal distribution, and the 95% confidence intervals (CI) of D-dimer and SF were 2.45 μg/mL and 6.92 μg/mL, respectively. Plasma levels of D-dimer and SF of patients with DVT were significantly higher than of those without DVT. In patients with DVT, the minimum values of D-dimer and SF were 1.71 and 1.44 μg/mL, respectively. When the cutoff value was set at the average + 1 SD of those of the control (D-dimer, about 1.8 μg/mL; SF, about 1.4 μg/mL), 1 and 0 patient with DVT was overlooked, respectively. The sensitivity and specificity of D-dimer and SF for DVT were 95% and 100%, and 61.9% and 53.8%, respectively. When the cutoff value was set at 95% CI of the control (D-dimer, 2.5 μg/mL; SF, 6.9 μg/mL), 2 and 9 patients with DVT were overlooked, respectively. The sensitivity and specificity of D-dimer and SF were 90% and 50%, and 77.6% and 88.3%, respectively. When the cutoff values set at 2.5 μg/mL of D-dimer or 6.9 μg/mL of SF, 1 DVT patient was overlooked, with sensitivity and specificity of 95% and 69.5%. Our data suggest that both D-dimer and SF are useful markers for the diagnosis of DVT and that measurement of both D-dimer and SF increases the sensitivity and specificity for the diagnosis of DVT/PE. Am. J. Hematol. 79:274–280, 2005. © 2005 Wiley-Liss, Inc.

Key words: DVT; SFI; D-dimer; negative predictive value (NPV)

INTRODUCTION

D-dimer is widely used clinically for the detection of in-vivo fibrin formation, and plasma levels of D-dimer are reported to be increased in patients with disseminated intravascular coagulation (DIC) [1,2], deep vein thrombosis (DVT)/pulmonary embolism (PE) [3–6], and acute myocardial infarction [7]. D-dimer has been adopted by the International Society of Thrombosis and Haemostasis as a diagnostic criterion of overt DIC [8]. Because the issue of standardization of D-dimer assays remains to be resolved, several studies [9,10] were designed to generate basic data for standardization of D-dimer.

Pulmonary embolism is a common, frequently undiagnosed, and potentially fatal cause of several common symptoms; such as dyspnea and chest pain [11–15]. Because PE is a fatal disease often caused by

Contract grant sponsor: Japanese Ministry of Health, Labor and Welfare; Contract grant sponsor: Japanese Ministry of Education, Science, Sports and Culture

*Correspondence to: A/Prof. Hideo Wada, M.D., Department of Laboratory Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu-city, Mie-ken 514-8507, Japan.
E-mail: wadahide@clin.medic.mie-u.ac.jp

Received for publication 12 November 2004; Accepted 17 February 2005

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ajh.20396

DVT, early evaluation of DVT [16] and PE [17] is important clinically. D-dimer is reported to be a negative predictor for DVT and in Europe and North America, and D-dimer levels less than 0.5 μg/mL are indicative of absence of DVT/PE [16]. On the other hand, plasma levels of soluble fibrin (SF), produced by thrombin-catalyzed cleavage of fibrinogen, were reported to be significantly high in patients with DIC [18].

The present study was designed to evaluate the cutoff values of D-dimer and SF in the diagnosis of DVT and PE as negative predictive values (NPV). For this purpose, we determined plasma levels of these molecules in 20 patients with DVT or PE, 223 patients without, and 100 healthy volunteers.

MATERIALS AND METHODS

Subjects

From 1 January 2002 to 31 December 2003, 243 patients (55.9 ± 18.1 years, mean ± SD; 150 females and 93 males) were diagnosed with suspected DVT or PE at Mie University School of Medicine. The underlying diseases in these patients were orthopedic conditions in 81, malignancies in 59, autoimmune diseases in 17, ulcerative colitis in 13, benign tumors in 12, kidney diseases in 6, liver diseases in 5, infections in 4, liver transplantation in 4, trauma or burn in 4, diabetes mellitus in 3, pregnancy-related complications in 3, and other diseases in 12. The study protocol was approved by the Human Ethics Review Committees of the participating institutions, and a signed consent form was obtained from each subject. Among this group, DVT or PE was subsequently confirmed in 20 patients (59.8 ± 17.4 years; 12 females and 8 males) based on ultrasonographic or venographic findings, while the remaining 223 patients were confirmed to be free of DVT/PE by ultrasonography. B-Mode ultrasonography with compression and color Doppler imaging were performed for the common femoral veins, the superficial femoral veins, the popliteal veins, and the calf veins of each lower extremity. Augmentation by calf squeezing or Valsalva's maneuver were included as needed. The criteria for the diagnosis of DVT were loss of compressibility of the vein, presence of intraluminal echogenicity, and absence of venous flow with calf squeezing. All studies were performed using an Aplio (Toshiba Medical Systems Corp., Tokyo, Japan) sonographic scanner with a linear transducer of frequency 6 MHz. All studies were performed by physicians or sonographers experienced in evaluating leg DVT.

The DVT/PE group included 9 patients with orthopedic conditions, 6 patients with cancers, 2 with kidney diseases, 1 with autoimmune disease, and 2 without underlying diseases. Each of the 20 patients with DVT or PE was treated with 20,000–40,000 units of heparin and urokinase or tissue-type plasminogen activator (t-PA). Plasma levels of SF and D-dimer were measured in these patients at the onset of DVT or PE and also in those without DVT at ultrasonography. The same parameters were also measured in 100 healthy volunteers (HV; mean age, 41.5 years; range, 20–58 years; 47 males and 53 females) as control; HVs had no history of venous thromboembolism or any complications known to affect the hemostatic system.

Measurement of Plasma Concentrations of D-Dimer and Soluble Fibrin

Blood samples were obtained immediately after the diagnosis of DVT or PE and before the administration of tissue-type plasminogen activator, urokinase, or heparin. For D-dimer determination, JIF23 monoclonal antibody, which recognizes the plasmin-digested N-terminus of the γ chain on the D region, was used for latex agglutination [19]. SF was also determined by the latex agglutination method using monoclonal antibody IF-43. Monoclonal antibody IF-43 recognizes a segment of the fibrin Aα chain [(Aα-17-78) residue segment] exposed in the E region of fibrin monomer (FM) when the FM molecule binds the D region of another FM or fibrinogen. The antibody is coated for the SF assay [20].

Statistical Analysis

Data are expressed as mean ± SD. Differences between groups were examined for statistical

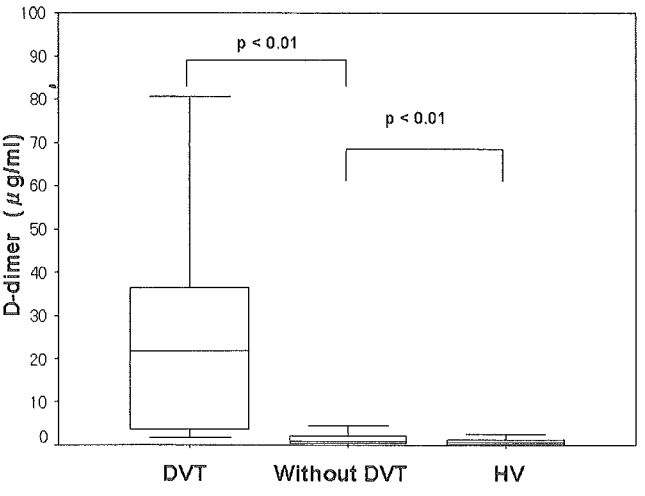

Fig. 1. Box plots of plasma concentrations of D-dimers in patients with DVT, those without DIC, and healthy volunteers (HV).

significance using the Mann–Whitney *U*-test, while correlation between two variables was tested by Pearson's correlation analysis. A *P* value less than 0.05 denoted the presence of a statistically significant difference. All statistical analyses were performed using the SPSS II software package (SPSS Japan, Tokyo, Japan).

RESULTS

Plasma concentrations of D-dimer were significantly higher in patients with DVT than those without DVT ($P < 0.01$), and those concentrations were also significantly higher in patients free of DVT than in healthy volunteers ($P < 0.01$, Fig. 1). In healthy volunteers, plasma concentrations of D-dimer did not show a normal distribution, and the maximum, minimum, and median values were 3.66, 0, and 0.66 μg/mL, respectively (Fig. 2A), with a 95% confidence interval (CI) of 2.45 μg/mL. In patients without DVT, the maximum, minimum, and median values were 15.53, 0.24, and 0.96 μg/mL, respectively, with a 95% CI of 6.92 μg/mL (Fig. 2B). In patients with DVT, the respective

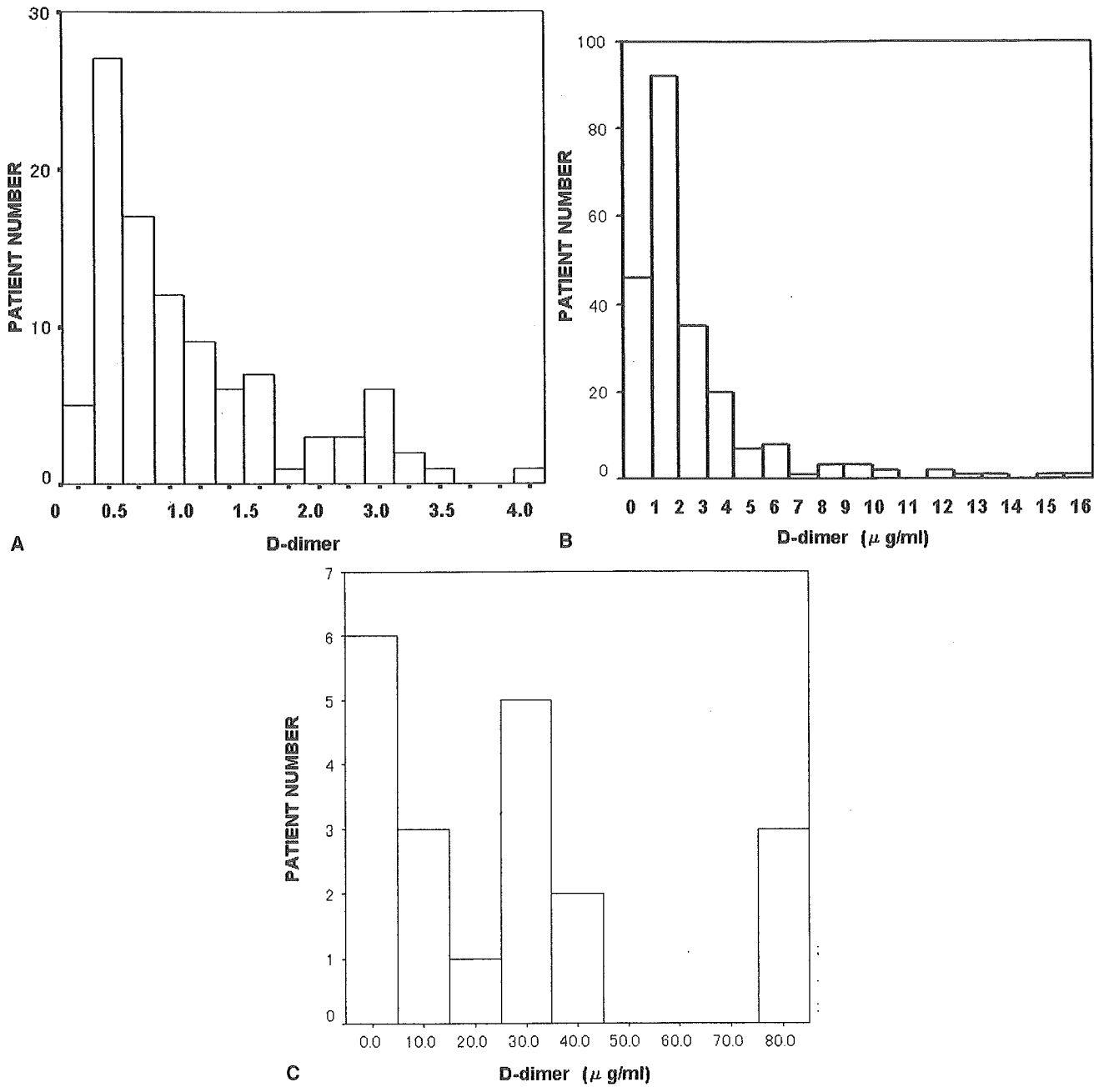

Fig. 2. Box plots of plasma concentrations of D-dimer in (A) healthy volunteers (HV), (B) patients without DVT, and (C) those with DVT.

TABLE I. Diagnosis of DVT by D-Dimer or SF

	Cutoff value (μg/mL)	DVT (n = 20)	Without DVT (n = 223)	Total (n = 243) (miss for DVT)
D-dimer				
Average + 1 SD of healthy volunteers	>1.8	19 (95.0%)	85 (38.1%)	104 (1)
95% CI of healthy volunteers	>2.5	18 (90%)	50 (22.4%)	68 (2)
95% CI of patients without DVT	>7.0	14 (70%)	12 (5.4%)	26 (6)
SF				
Average + 1 SD of healthy volunteers	>1.40	20 (100%)	103 (46.2%)	123 (0)
95% CI of healthy volunteers	>6.9	11 (55%)	26 (11.7%)	37 (6)
95% CI of patients without DVT	>16.3	8 (40%)	12 (5.4%)	20 (12)
SF > 6.9 μg/mL or D-dimer > 2.5 μg/mL		19 (95.0%)	68 (30.5%)	87 (1)

values were 80.60, 1.71, and 17.47 μg/mL (Fig. 2C). One patient with DVT was not diagnosed by D-dimer when the cutoff value was set at the average + 1 SD value (about 1.8 μg/mL) of the healthy volunteers. Accordingly, the sensitivity and specificity were 95% and 61.9%, respectively, and 104 patients required further examination (Table I). When the cutoff value of D-dimer was set at the 95% CI of healthy volunteers (2.5 μg/mL), 2 patients with DVT were incorrectly diagnosed and 60 patients required further examination.

Plasma concentrations of SF were significantly higher in patients with DVT than those without DVT ($P < 0.01$), and the latter was significantly higher than in healthy volunteers ($P < 0.01$, Fig. 3). In healthy volunteers, plasma concentrations of SF did not show a normal distribution, and the maximum, minimum, median, and 95% CI values were 3.97, 0, 0, and 6.92 μg/mL, respectively (Fig. 4A). The respective concentrations in patients without DVT were 90.00, 0.30, 1.25, and 16.31 μg/mL (Fig. 4B), and concentrations in those with DVT were 66.72, 1.44, 13.69, and 40.30 μg/mL (Fig. 4C). None of the patients with DVT was overlooked when the cutoff value was set as the average + 1 SD value (about 1.4 μg/mL) of the healthy volunteers. Accordingly, the sensitivity and specificity were 100% and 53.8%, respectively, and 123 patients required further examination (Table I). When the cutoff value of plasma SF was set at the 95% CI of healthy volunteers (about 6.9 μg/mL), 9 patients with DVT were overlooked and 37 patients required further examination. When the cutoff values were set at 2.5 μg/mL of D-dimer and 6.9 μg/mL of SF, 1 DVT patient who had chronic PE was overlooked, and the sensitivity and specificity were 95% and 69.5%, respectively.

Out of 20 patients with DVT, 7 were associated with PE. The maximum, minimum, and median values of plasma D-dimer were 32.00, 1.71, and 3.74 μg/mL, respectively, and those of plasma SF value were 16.5, 1.44, and 2.50 μg/mL, respectively. Plasma SF concentrations were significantly lower in DVT patients with PE than those without PE ($P < 0.05$).

Finally, plasma SF concentrations correlated well with plasma D-dimer concentrations ($r = 0.459$, $P < 0.01$, Fig. 5).

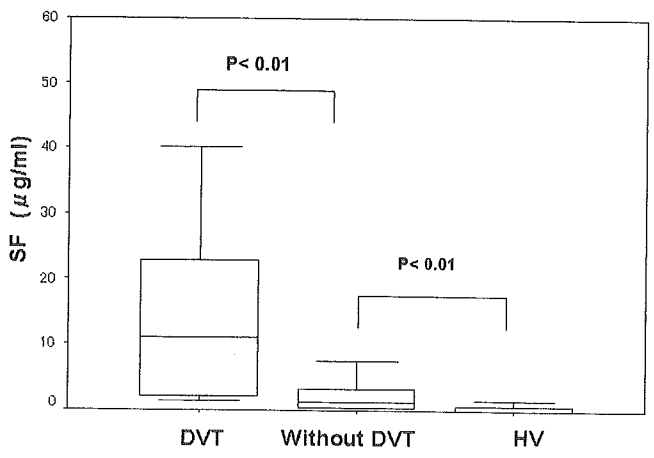

Fig. 3. Box plots of plasma concentrations of SF in patients with DVT, patients without DIC, and healthy volunteers (HV).

DISCUSSION

SF and D-dimer are considered as markers of hypercoagulable state, as both parameters are reported to be elevated in DVT [3,21], DIC [22,23], and hyperlipidemia [24]. In the present study, plasma concentrations of D-dimer and SF were significantly high in patients with DVT, but those levels were also significantly high in patients without DVT.

D-dimer can facilitate wider screening of PE and provides a higher rate of diagnosis of PE. However, the high false-positive rate of D-dimer can create the potential for detrimental increases in pulmonary vascular imaging, increased lengths of stay in overcrowded emergency departments, and increased false-positive diagnoses [25]. Therefore, cutoff values should be determined for D-dimer and SF for the

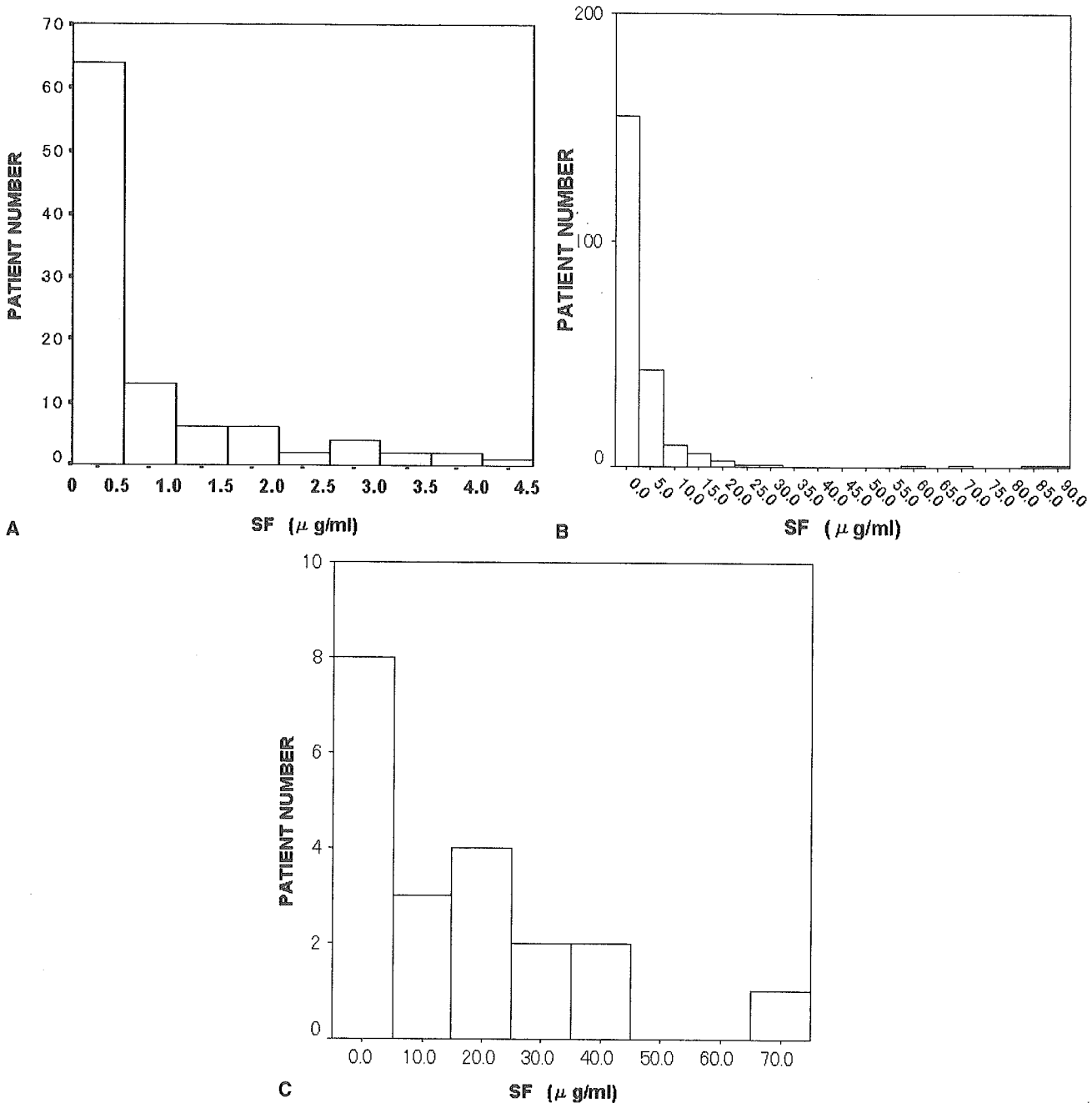

Fig. 4. Box plots of plasma concentrations of SF in (A) healthy volunteers, (B) patients without DVT, and (C) patients with DVT.

diagnosis of DVT/PE. In Europe and North America, D-dimer is measured by enzyme linked immunosorbent assay (ELISA), and values less than 0.5 μg/mL rule out the presence of DVT/PE [16]. However, in Japan, the D-dimer concentration is usually measured by LIA, and in many patients it is higher than 0.5 μg/mL; thus a cutoff value < 0.5 μg/mL is not useful as an NPV for DVT/PE in Japan. The difference in plasma D-dimer concentration between Europe/U.S. and Japan may depend on the measurement system, characteristics of the antibody, and the standard product used. As plasma concentrations of D-dimer and SF were not distributed normally in our population, the 95% CI values of these markers are considered the maximum value of normal range. When the cutoff values were set to the 95% CI value of healthy volunteers (D-dimer, 2.5 μg/mL; SF, 6.9 μg/mL), 2 and 9 patients with DVT were overlooked, respectively. Our results indicated that the minimum concentration of D-dimer was 1.71 μg/mL and that of SF was 1.44 μg/mL