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## REFERENCES

- Albornoz L, Alvarez D, Otao JC, Gadano A, Salviu J, Gerona S, Sorroche P, Villamil A, Mastai R (1999) Von Willebrand factor could be an index of endothelial dysfunction in patients with cirrhosis: relationship to degree of liver failure and nitric oxide levels. *J Hepatol* 30:451-455.
- Bernardo A, Ball C, Nolasco L, Moake JF, Dong J (2004) Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand factor multimers under flow. *Blood* 104:100-106.
- Carithers RL Jr, Herlong HF, Diehl AM, Shaw EW, Combes B, Fallon HJ, Maddrey WC (1989) Methylprednisolone therapy in patients with severe alcoholic hepatitis. A randomized multicenter trial. *Ann Intern Med* 110:685-90.
- French SW, Benson NC, Sun PS (1984) Centrilobular liver necrosis induced by hypoxia in chronic ethanol-fed rats. *Hepatology* 4:912-917.
- Fujimoto M, Uemura M, Kojima H, Ishii Y, Ann T, Sakurai S, Okuda K, Noguchi R, Adachi S, Kitano H, Hoppo K, Higashino T, Takaya A, Fukui H. (1999) Prognostic factors in severe alcoholic liver injury. *Alcohol Clin Exp Res* 23:33S-38S.
- Fujimoto M, Uemura M, Nakatani Y, Tsujita S, Hoppo K, Tamagawa T, Kitano H, Kikukawa M, Ann T, Ishii Y, Kojima H, Sakurai S, Tanaka R, Namisaki T, Noguchi R, Higashino T, Kikuchi E, Nishimura K, Takaya A, Fukui H. (2000) Plasma endotoxin and serum cytokine levels in patients with alcoholic hepatitis: Relation to severity of liver diseases. *Alcohol Clin Exp Res* 23:33S-38S.
- Fujimura Y, Matsumoto M, Yagi H, Yoshioka A, Matsui T, Titani K (2002) Von Willebrand factor-cleaving protease and Upshaw-Schulman syndrome. *Int J Hematol* 75:25-34.
- Fukui H, Brauner B, Bode JC, Bode C (1991) Plasma endotoxin concentrations in patients with alcoholic and non-alcoholic liver disease: reevaluation with an improved chromogenic assay. *J Hepatol* 12:162-169.
- Furlan M, Robles R, Lämmle B (1996) Partial purification and characterization of protease from human plasma cleaving von Willebrand factor to fragments produced by *in vivo* proteolysis. *Blood* 87:4223-4234.
- Furlan M, Robles R, Solenthaler M, Wassmer M, Sandoz P, Lämmle B (1997) Deficient activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. *Blood* 89:3097-3103.
- Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, Krause M, Scharer I, Aumann V, Mittler U, Solenthaler M, Lämmle B (1998) von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med* 339:1578-1584.
- Grignani G, Maiolo A (2000) Cytokines and hemostasis. *Haematologica* 85:967-972.
- Haber PS, Warner R, Seth D, Gorrell MD, McCaughan GW (2003) Pathogenesis and management of alcoholic hepatitis. *Gastroenterol Hepatol* 18:1332-1344.
- Ishii K, Furudera S, Kumashiro R, Koga Y, Hamada T, Sata M, Abe H, Tanikawa K (1993) Clinical and pathological features, and the mechanism of development in severe alcoholic hepatitis, especially in comparison with acute type fulminant hepatitis. *Alcohol* 1B:97-103.
- Itatsu T, Oide H, Watanabe S, Tateyama M, Ochi R, Sato N (1998) Alcohol stimulates the expression of L-type voltage-operated  $Ca^{2+}$  channels in hepatic stellate cells. *Biochem Biophys Res Commun* 251:533-537.
- Kavakli K (2002) Plasma levels of the von Willebrand factor-cleaving protease in physiological and pathological conditions in children. *Pediatr Hematol Oncol* 19:467-478.
- Langley PG, Hughes RD, Williams R (1985) Increased factor VIII complex in fulminant hepatic failure. *Thromb Haemostasis* 54:693-696.
- Lee TP, Bouhassira EE, Lyubsky S, Tsai HM (2002) ADAMTS13, the Von Willebrand factor cleaving metalloprotease, is expressed in the perisinusoidal cells of the liver (abstract). *Blood* 100(suppl 1):1938.
- Maddrey WC, Boitnott JK, Bedine MS, Weber FL, Jr., Mezey E, White RI Jr (1978) Corticosteroid therapy of alcoholic hepatitis. *Gastroenterology* 75:193-199.
- Mannucci PM, Canciani MT, Forza I, Lussana F, Lattuada A, Rossi E (2001) Changes in health and disease of the metalloproteinase that cleaves von Willebrand factor. *Blood* 98:2730-2735.
- Matsumoto M, Yagi H, Ishizashi H, Wada H, Fujimura Y (2004) The Japanese experience with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. *Semin Hematol* 41:68-74.
- Moake JL, Turner NA, Stathopoulos NA, Nolasco L, Hellums JD (1986) Involvement of large von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. *J Clin Invest* 78:1456-1461.
- Mookerjee RP, Sen S, Davies NA, Hodges SJ, Williams R, Jalan R (2003) Tumor necrosis factor alpha is an important mediator of portal and systemic haemodynamic derangements in alcoholic hepatitis. *Gut* 52:1182-1187.
- Mori Y, Wada H, Gabazza EC, Minami N, Nobori T, Shiku H, Yagi H, Ishizashi H, Matsumoto M, Fujimura Y (2002) Predicting response to plasma exchange in patients with thrombotic thrombocytopenic purpura with measurement of vWF-cleaving protease activity. *Transfusion* 42:572-80.
- Moschowitz E (1924) Hyaline thrombosis of the terminal arterioles and capillaries: a hitherto undescribed disease. *Proc NY Pathol Soc* 24:21-24.
- Nanji AA, Tahan SR, Khwaja S, Yacoub LK, Sadrzadeh H (1996) Elevated plasma levels of hyaluronic acid indicate endothelial cell dysfunction in the initial stages of alcoholic liver diseases in the rat. *J Hepatol* 24:368-374.
- Oshita M, Takei Y, Kawano S, Yoshihara H, Hijioka T, Fukui H, Goto M, Masuda E, Nishimura Y, Fusamoto H, Kamada T, (1993) Roles of endothelin-1 and nitric oxide in the mechanism for ethanol-induced vasoconstriction in rat liver. *J Clin Invest* 91:1337-1342.
- Park YD, Yoshioka A, Kawa K, Ishizashi H, Yagi H, Yamamoto Y, Matsumoto M, Fujimura Y (2002) Impaired activity of plasma von Willebrand factor-cleaving protease may predict the occurrence of hepatic veno-occlusive disease after stem cell transplantation. *Bone Marrow Transplant* 29:789-794.
- Plaimauer B, Zimmermann K, Volkel D, Antoine G, Kerschbaumer R, Jenab P, Furlan M, Gerritsen H, Lämmle B, Schwarz HP, Scheifflinger F (2002) Cloning, expression and characterization of the von Willebrand factor-cleaving protease (ADAMTS 13). *Blood* 100:3626-3632.
- Pugh RNH, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R (1973) Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg*; 60:646-649.
- Ruggeri ZM (1997) von Willebrand factor. *J Clin Invest* 100:S41-46.
- Soejima K, Mimura N, Hirashima M, Maeda H, Takayoshi H, Nakazaki T, Nozaki C (2001) A novel human metalloproteinase synthesized in the liver and secreted into the blood: Possibly, the von Willebrand factor-cleaving protease? *J Biochem* 130:475-480.
- Takada A A Japanese study group for alcoholic liver disease. A new diagnostic criteria of alcoholic liver disease. *Jpn Acta Hepatol Japonica* 1993;34:888-896.
- Trey C, Burns DG, Saunders SJ (1966) Treatment of hepatic coma by exchange blood transfusion. *N Engl J Med* 274:473-481.
- Tsai H-M Physiological cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood* 1996;87:4235-4244.

Tsai HM, Lian EC (1998) Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med* 339:1585-1594.

Urashima S, Tsutsumi M, Nakase K, Wang JS, Takada A (1993) Studies on capillarization of the hepatic sinusoids in alcoholic liver disease.

*Alcohol Alcohol* 28:77-84.

Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K (2001) Structure of von Willebrand factor-cleaving protease (ADAMTS 13), metalloproteinase involved in thrombotic thrombocytopenic purpura. *J Biol Chem* 276:41089-41163.

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

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## 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.

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## 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<sub>2</sub> synthesis [1], and the thienopyridine derivatives ticlopidine and clopidogrel block the ADP receptor P2Y<sub>12</sub> [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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**Table 1.** Patient characteristics

	Age years	Sex	Underlying disease	Complica- tion
<b>Ticlopidine</b>				
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
<b>Aspirin</b>				
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) I $\alpha$  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

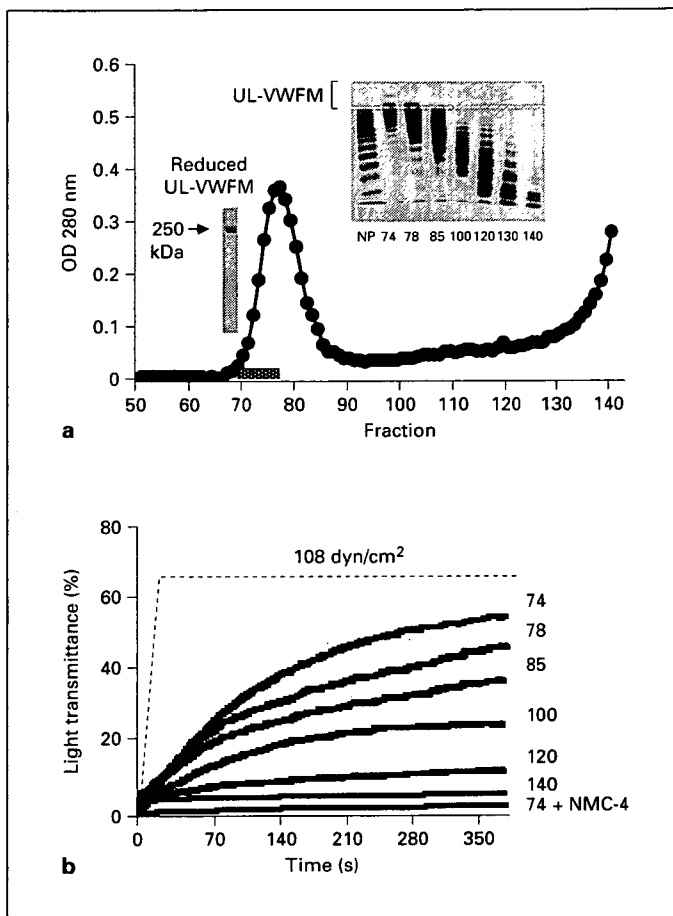
Twenty patients with cerebral ischemia who were being treated with antiplatelet drugs were enrolled in this study as listed in table 1 after informed consent had been obtained. Diagnoses were made based on neurological examinations, routine laboratory data, brain computed tomography, brain magnetic resonance 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<sup>®</sup>, Daiichi Pharmaceutical Corp.) or 81 mg aspirin (Bufferin<sup>®</sup>, 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 antecubital 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<sub>3</sub>-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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 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  $\epsilon$ -aminocaproic 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 electrophoresis. 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  $30 \times 10^8$ /ml and each purified VWF fraction at a final concentration of  $5 \mu\text{g/ml}$  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<sub>1</sub> (PGE<sub>1</sub>; Sigma-Aldrich), and were then washed twice in platelet-washing buffer (113 mmol/l NaCl, 4.3 mmol/l K<sub>2</sub>HPO<sub>4</sub>, 4.2 mmol/l Na<sub>2</sub>HPO<sub>4</sub>, 24.4 mmol/l NaH<sub>2</sub>PO<sub>4</sub> and 5.5 mmol/l glucose, pH 6.5) that contained 1 U/ml

apyrase and 1 mmol/l PGE<sub>1</sub>. The platelet pellets were next resuspended in HEPES-Tyrode's buffer (138 mmol/l NaCl, 2.8 mmol/l KCl, 2 mmol/l CaCl<sub>2</sub> and 10 mmol/l HEPES, pH 7.4) at a concentration of  $3 \times 10^8$ /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<sup>2</sup>, the purified UL-VWFM was added to the platelet suspensions at a final concentration of 5  $\mu\text{g/ml}$  in a total volume of 400  $\mu\text{l}$ . 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%.

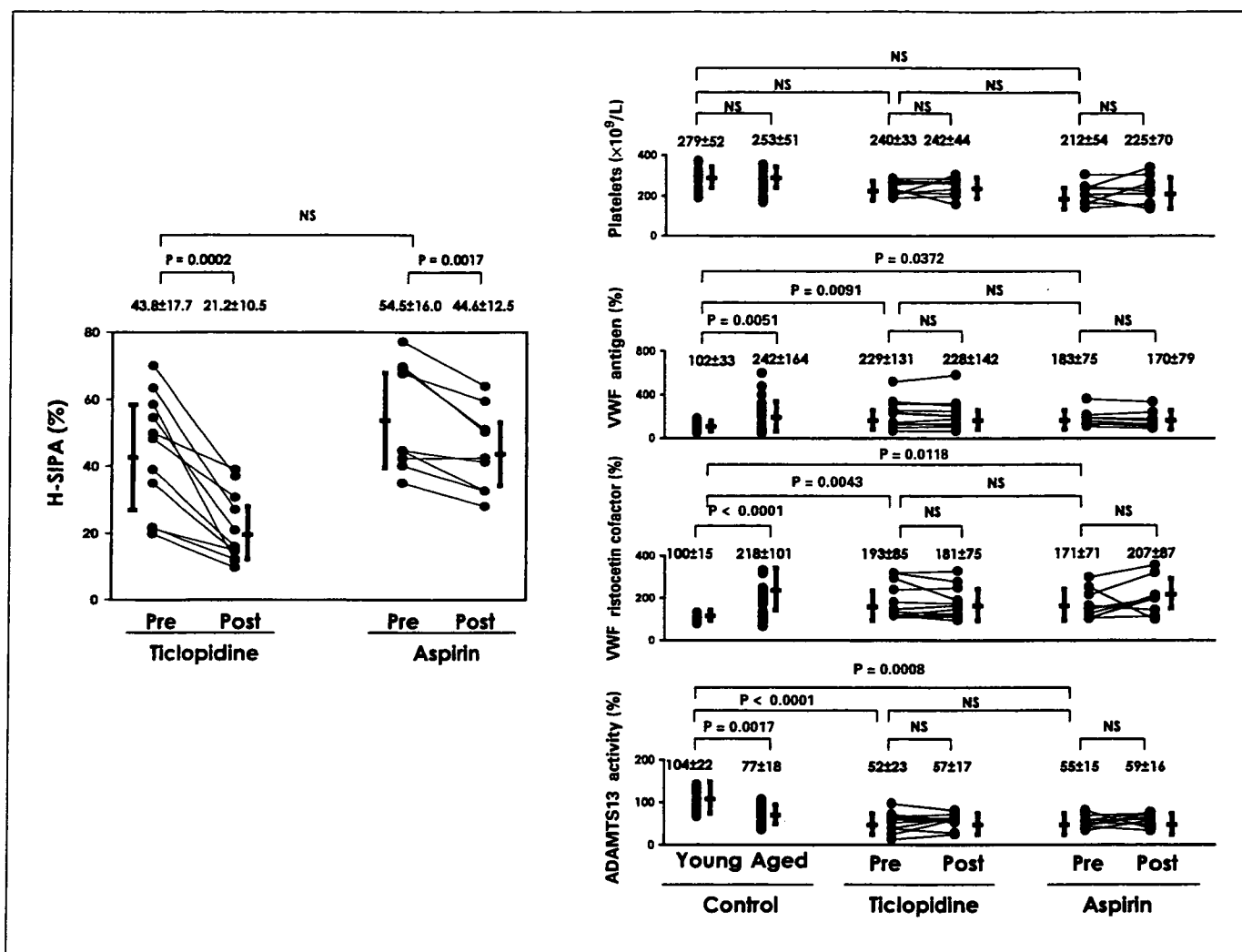
#### Statistical Analysis

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  $\pm$  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  $\mu\text{g/ml}$  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  $\mu\text{g/ml}$  (fig. 1) or with the anti-GP-Ib monoclonal antibody AP-1 at a final concentration of 10  $\mu\text{g/ml}$  (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<sup>2</sup> 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  $43.8 \pm 17.7\%$  before and  $21.2 \pm 10.5\%$  after treatment ( $p < 0.001$ ) as shown in figure 2. Furthermore, for the patients treated with aspirin, H-SIPA was also inhibited to

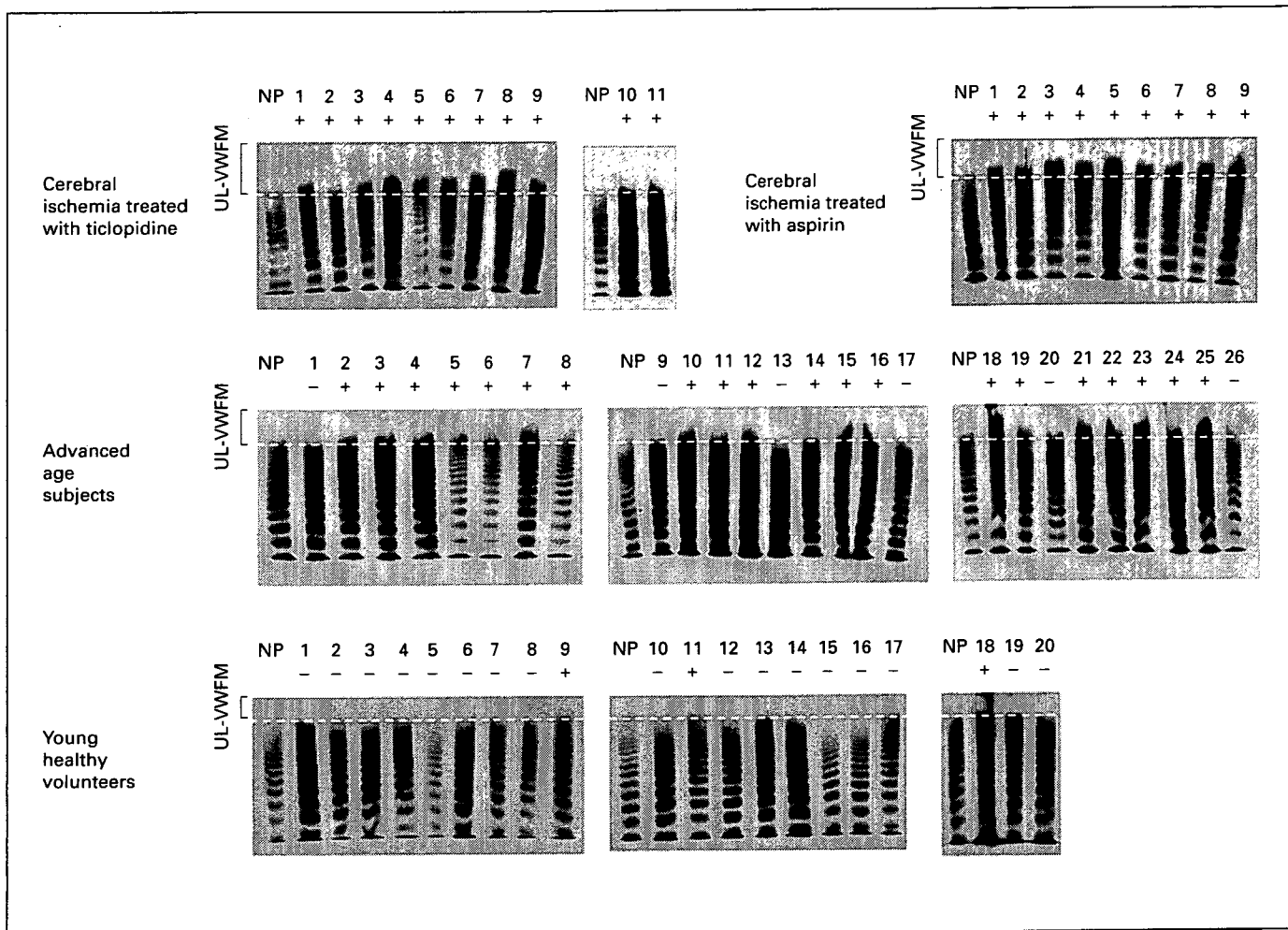


**Fig. 2.** Comparison of H-SIPA, platelets and VWF. For the patients treated with ticlopidine, H-SIPA was dramatically inhibited after treatment as was the case for patients treated with aspirin, although to a lesser extent. The plasma VWF antigen, ristocetin cofactor and ADAMTS13 activity in patients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

some extent but less than that after ticlopidine treatment, and the value was  $54.5 \pm 16.0\%$  before and  $44.6 \pm 12.5\%$  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 co-

factor 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 re-

cently 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 Iba 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.

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## References

- 1 Alberts MJ, Bergman DL, Molner E, Jovanovic BD, Ushiwata I, Teruya J: Antiplatelet effect of aspirin in patients with cerebrovascular disease. *Stroke* 2004;35:175-178.
- 2 Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, Zhang FL, Gustafson E, Monsma FJ, Wiekowski MT, Abbondanzo SJ, Cook DN, Bayne ML, Lira SA, Chintala MS: Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest* 2001;107:1591-1598.
- 3 Turner NA, Moake JL, McIntire LV: Blockade of adenosine diphosphate receptors P2Y<sub>12</sub> and P2Y<sub>1</sub> is required to inhibit platelet aggregation in whole blood under flow. *Blood* 2001;98:3340-3345.
- 4 Goto S, Noriko T, Eto K, Ikeda Y, Handa S: Functional significance of adenosine 5'-diphosphate receptor (P2Y<sub>12</sub>) in the platelet activation initiated by binding of von Willebrand factor to platelet GP1b $\alpha$  induced by conditions of high shear rate. *Circulation* 2002;105:2531-2536.
- 5 Uchiyama S, Yamazaki M, Maruyama S, Handa M, Ikeda Y, Fukuyama M, Itagaki I: Shear-induced platelet aggregation in cerebral ischemia. *Stroke* 1994;25:1547-1551.
- 6 Moake JL, Turner NA, Stathopoulos NA, Nolasco LH, Hellums JD: Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. *J Clin Invest* 1986;78:1456-1461.
- 7 Fujimura Y, Matsumoto M, Yagi H, Yoshioka A, Matsui T, Titani K: von Willebrand factor-cleaving protease and Upshaw-Schulman syndrome. *Int J Hematol* 2002;75:25-34.
- 8 Quinn MJ, Fitzgerald DJ: Ticlopidine and clopidogrel. *Circulation* 1999;100:1667-1672.
- 9 Fujimura Y, Usami Y, Titani K, Niinomi K, Nishino K, Takase T, Yoshioka A, Fukui H: Studies on anti-von Willebrand factor (vWF) monoclonal antibody NMC-4, which inhibits both ristocetin- and botrocetin-induced vWF binding to platelet glycoprotein Ib. *Blood* 1991;77:113-120.
- 10 Fujimura Y, Ikeda Y, Miura S, Yoshida E, Shima H, Nishida S, Suzuki M, Titani K, Taniuchi Y, Kawasaki T: Isolation and characterization of jararaca GPIb-BP, a snake venom antagonist specific to platelet glycoprotein Ib. *Thromb Haemost* 1995;74:743-750.
- 11 Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, Anbo H, Kawai Y, Watanabe K, Itagaki I, Sakai K, Ruggeri ZM: The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest* 1991;87:1234-1240.
- 12 Bartlett A, Dormandy KM, Hawkey CM, Stableforth P, Voller A: Factor VIII-related antigen: Measurement by enzyme immunoassay. *Br Med J* 1976;24:994-996.
- 13 Macfarlane DE, Stibbe J, Kirby EP, Zucker MB, Grant RA, McPherson J: A method for assaying von Willebrand factor (ristocetin cofactor). *Thromb Diath Haemorrh* 1975;34:306-308.
- 14 Ruggeri ZM, Zimmerman TS: Variant von Willebrand's disease: Characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. *J Clin Invest* 1980;65:1318-1325.
- 15 Budde U, Schneppenheim R, Plendl H, Dent J, Ruggeri ZM, Zimmerman TS: Luminographic detection of von Willebrand factor multimers in agarose gels and on nitrocellulose membranes. *Thromb Haemost* 1990;63:312-315.
- 16 Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrie PA, Brenner B, Krause M, Scharer I, Aumann V, Mittler V, Solenthaler M, Lammle B: Von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *N Engl J Med* 1998;339:1578-1584.
- 17 Kinoshita S, Yoshioka A, Park Y-D, Ishizashi H, Konno M, Funado M, Matsui T, Titani K, Yagi H, Matsumoto M, Fujimura Y: Upshaw-Schulman syndrome revisited: A concept of congenital thrombotic thrombocytopenic purpura. *Int J Hematol* 2001;74:101-108.
- 18 Mannucci PM, Canciani MT, Forza I, Lussana F, Lattuada A, Rossi E: Changes in health and disease of the metalloprotease that cleaves von Willebrand factor. *Blood* 2001;98:2730-2735.
- 19 Sun L, Feng S, Resendiz JC, Lu X, Durante W, Kroll MH: Role of the Pyk2-MAP kinase-cPLA2 signaling pathway in shear-dependent platelet aggregation. *Ann Biomed Eng* 2004;32:1193-1201.



## 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 cyto-

plasmic processes, features common to hepatic stellate cells (HSCs). Double-labeling experiments revealed that the ADAMTS13-positive cells also expressed  $\alpha$ -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)

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## 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.<sup>1-4</sup> VWF is synthesized in vascular endothelial cells, and released into the plasma as unusually large VWF multimers (UL-VWFMs).<sup>5,6</sup> Usually, UL-VWFMs are rapidly degraded into smaller VWF multimers by ADAMTS13. Deficiency of ADAMTS13 caused either by mutations of the *ADAMTS13* gene<sup>1,7</sup> or by inhibitory autoantibodies against ADAMTS13<sup>8,9</sup> 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).<sup>5-9</sup>

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.<sup>2</sup> In situ hybridization analysis revealed that the mRNA signals were expressed exclusively in the perisinusoidal cells,<sup>10</sup> 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.<sup>11</sup> Thus, specification and/or localization of the cells that produce this enzyme in the liver should have clinical impor-

tance, 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,  $18.3 \times 10^4/\text{mm}^3$ ; range,  $8.8\text{-}32.3 \times 10^4/\text{mm}^3$ ). 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.

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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.

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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<sup>12</sup> to produce monoclonal antibodies (A10 and C7) against ADAMTS13 in mice following standard procedures.<sup>13</sup> The immunoglobulin subclasses of A10 and C7 were IgG2b- $\kappa$  and IgG1- $\kappa$ , 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

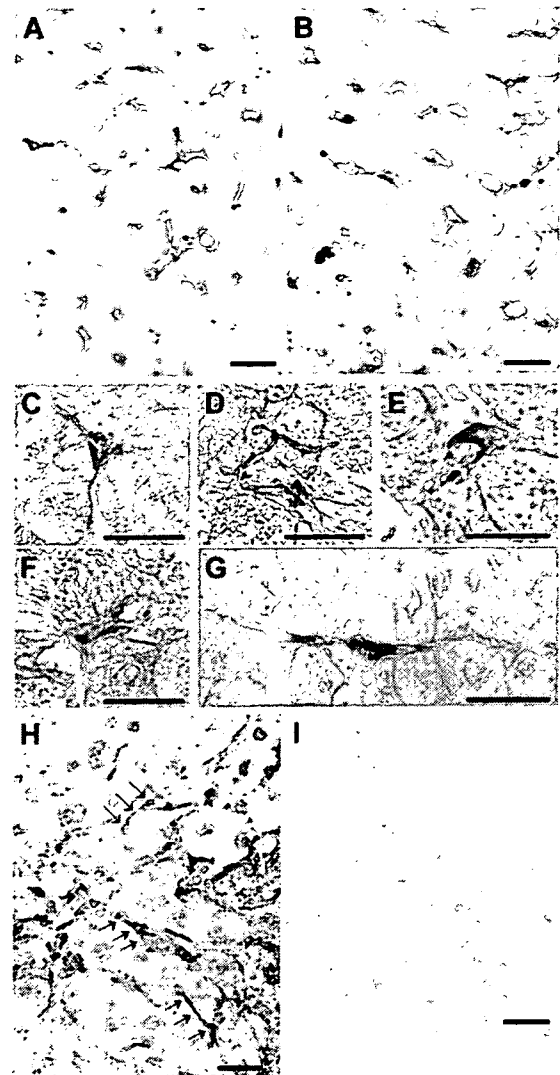
Fresh liver specimens obtained from the 8 patients were fixed in 4% paraformaldehyde solution before frozen sections were prepared. The sections were incubated with primary antibodies (A10 and C7) overnight at 4°C. Bound primary antibodies were subsequently immunodetected using a standard avidin-biotin-peroxidase complex technique. To simultaneously detect A10- and anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) immunoreactivity, sequential incubations using the following reagents were performed: A10 antibodies and anti- $\alpha$ -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 fluorescence 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). Digoxigenin (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.<sup>14</sup> 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).

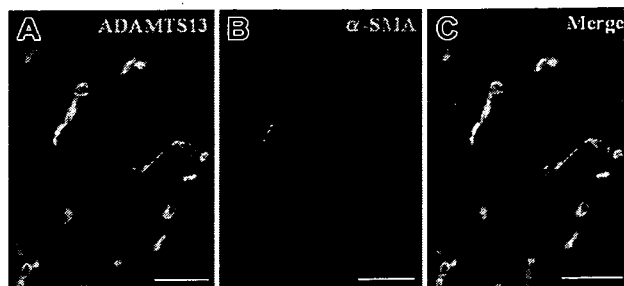
## Results and discussion

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).<sup>15,16</sup> 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



**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,  $\times 200$ ; panels C-G: original magnification,  $\times 400$ . 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,  $\times 200$ ). When sense probes were used, we observed no significant staining (I; original magnification,  $\times 200$ ). (Bar = 30  $\mu$ m in A-I.)

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- $\alpha$ -SMA antibodies. The microfilament protein  $\alpha$ -SMA has been recognized as a specific marker for stellate cells<sup>17</sup>; activated stellate cells in pathologic livers are strongly  $\alpha$ -SMA-positive, whereas in normal adult human liver, some stellate cells are  $\alpha$ -SMA-positive.<sup>18</sup> Immunofluorescence labeling with anti-ADAMTS13 antibodies showed intense green fluorescence in perisinusoidal cells that



**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  $\alpha$ -SMA immunoreactivity, reflecting the fact that these cells were activated HSCs (B). Colocalization of ADAMTS13 (A) and  $\alpha$ -SMA (B) in single cells yielded yellow color in the merged figure (C). (Original magnification,  $\times 400$ ; bar = 30  $\mu$ m in A-C.)

were irregular in shape with spotty, oval, unipolar, and bipolar cytoplasmic processes extending between hepatocytes (Figure 2A).  $\alpha$ -SMA antibodies revealed that these cells were also positive for the HSC marker (Figure 2B). Colocalization of ADAMTS13 and  $\alpha$ -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.<sup>17</sup> 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.<sup>11,19</sup> 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.<sup>20</sup> This is particularly evident in the sinusoids of patients at the early stages of alcoholic liver diseases,<sup>21</sup> 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.

## References

- Levy GG, Nichols WC, Lian EC, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*. 2001;413:488-494.
- Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS 13), metalloproteinase involved in thrombotic thrombocytopenic purpura. *J Biol Chem*. 2001;276:41089-41163.
- Soejima K, Mimura N, Hirashima M, et al. A novel human metalloproteinase synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease? *J Biochem*. 2001;130:475-480.
- Plaimauer B, Zimmermann K, Völkel D, et al. Cloning, expression and characterization of the von Willebrand factor-cleaving protease (ADAMTS 13). *Blood*. 2002;100:3626-3632.
- Ruggeri ZM. von Willebrand factor. *J Clin Invest*. 1997;99:559-564.
- Moake JL. Thrombotic microangiopathies. *N Engl J Med*. 2002;347:589-599.
- Kokame K, Matsumoto M, Soejima K, et al. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc Natl Acad Sci U S A*. 2002;99:11902-11907.
- Furlan M, Robles R, Galbusera M, et al. von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med*. 1998;339:1578-1584.
- Tsai H-M, Lian ECY. Antibody to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med*. 1998;339:1585-1594.
- Lee TP, Bouhassira EE, Lyubsky S, Tsai HM. ADAMTS13, the Von Willebrand factor cleaving metalloproteinase, is expressed in the perisinusoidal cells of the liver [abstract]. *Blood*. 2002;100:497a-498a. Abstract 1938.
- Mannucci PM, Canciani MT, Forza I, Lussana F, Lattuada A, Rossi E. Changes in health and disease of the metalloproteinase that cleaves von Willebrand factor. *Blood*. 2001;98:2730-2735.
- Soejima K, Matsumoto M, Kokame K, et al. ADAMTS13 cysteine-rich/spacer domains are functionally essential for von Willebrand factor cleavage. *Blood*. 2003;102:3232-3237.
- Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975;256:495-497.
- Tatsumi K, Haga S, Matsuyoshi H, et al. Characterization of cells with proliferative activity after a brain injury. *Neurochem Int*. 2005;46:381-389.
- Ito T, Nomoto M. Über die Kupfferschen Sternzellen und die "Fettspeicherungszellen" (fat storing cells) in der Blutkapillarenwand der menschlichen Leber. *Okajima Folia Anat Jpn*. 1952;24:243-258.
- Wake K. "Stemzellen" in the liver: perisinusoidal cells with special reference to storage of vitamin A. *Am J Anat*. 1971;132:429-462.
- Geerts A. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis*. 2001;21:311-335.
- Kawada N. The hepatic perisinusoidal stellate cell. *Histol Histopathol*. 1997;12:1069-1080.
- Albornoz L, Alvarez D, Otaso JC, et al. Von Willebrand factor could be an index of endothelial dysfunction in patients with cirrhosis: relationship to degree of liver failure and nitric oxide levels. *J Hepatol*. 1999;30:451-455.
- Knittel T, Neubauer K, Armbrust T, Ramadori G. Expression of von Willebrand factor in normal and diseased rat livers and in cultivated liver cells. *Hepatology*. 1995;21:470-476.
- Urashima S, Tsutsumi M, Nakase K, Wang JS, Takada A. Studies on capillarization of the hepatic sinusoids in alcoholic liver disease. *Alcohol Alcoholism*. 1993;28:77-84.

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

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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  $\mu\text{g/mL}$  and 6.92  $\mu\text{g/mL}$ , 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  $\mu\text{g/mL}$ , respectively. When the cutoff value was set at the average + 1 SD of those of the control (D-dimer, about 1.8  $\mu\text{g/mL}$ ; SF, about 1.4  $\mu\text{g/mL}$ ), 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  $\mu\text{g/mL}$ ; SF, 6.9  $\mu\text{g/mL}$ ), 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  $\mu\text{g/mL}$  of D-dimer or 6.9  $\mu\text{g/mL}$  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

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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  $\mu\text{g/mL}$  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 ( $55.9 \pm 18.1$  years, mean  $\pm$  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 ( $59.8 \pm 17.4$  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  $\gamma$  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  $\text{A}\alpha$  chain [( $\text{A}\alpha$ -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  $\pm$  SD. Differences between groups were examined for statistical

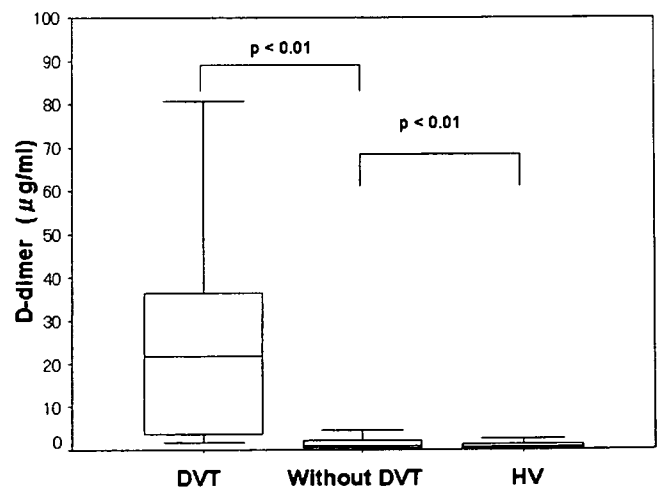


Fig. 1. Box plots of plasma concentrations of D-dimers in patients with DVT, those without DIC, and healthy volunteers (HV).

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  $\mu\text{g/mL}$ , respectively (Fig. 2A), with a 95% confidence interval (CI) of 2.45  $\mu\text{g/mL}$ . In patients without DVT, the maximum, minimum, and median values were 15.53, 0.24, and 0.96  $\mu\text{g/mL}$ , respectively, with a 95% CI of 6.92  $\mu\text{g/mL}$  (Fig. 2B). In patients with DVT, the respective

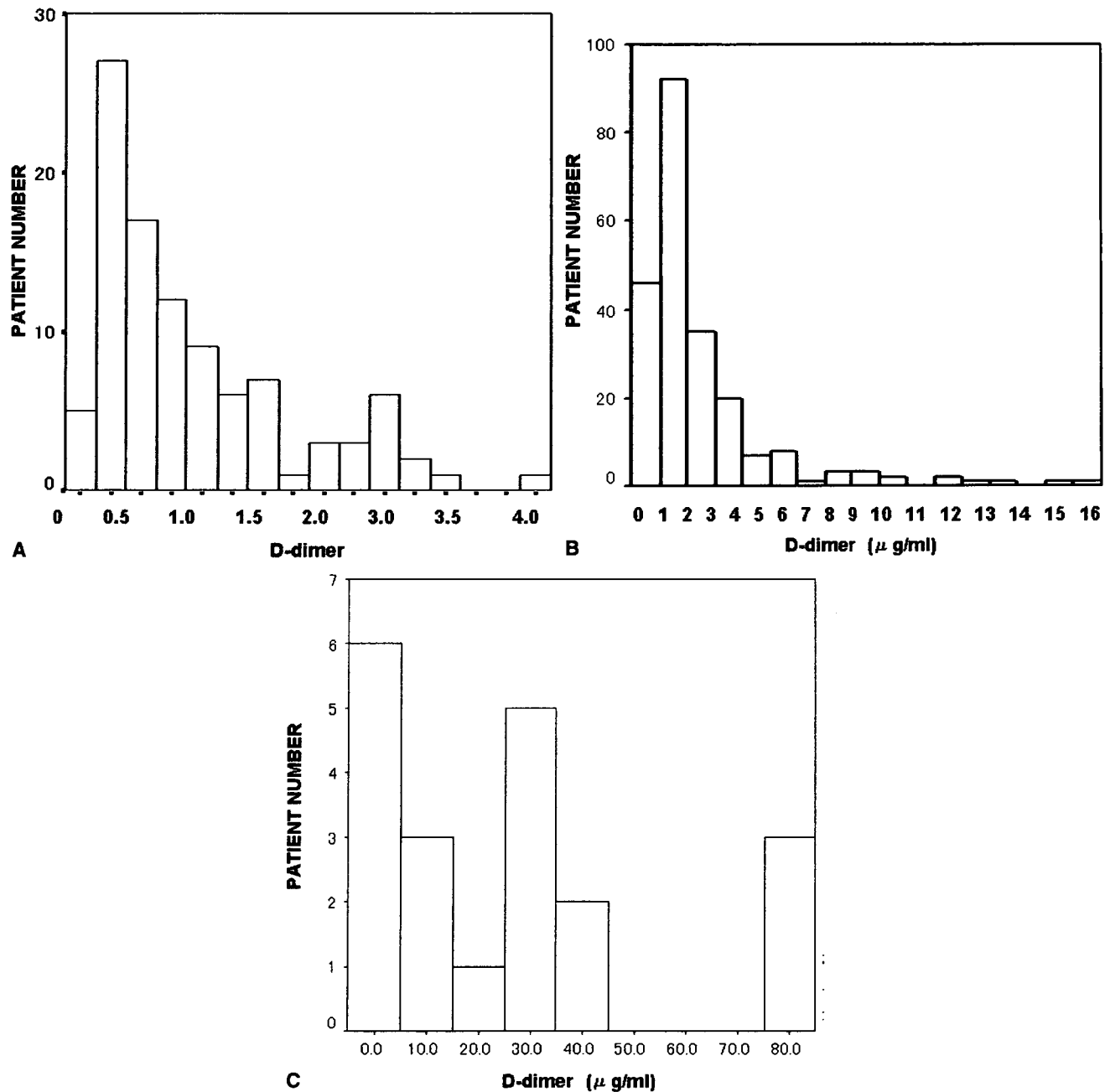


Fig. 2. Box plots of plasma concentrations of D-dimer in (A) healthy volunteers (HV), (B) patients without DVT, and (C) those with DVT.

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)
<b>D-dimer</b>				
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)
<b>SF</b>				
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).

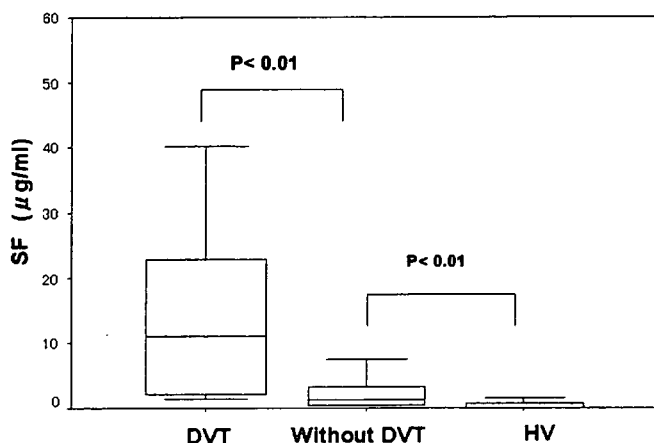


Fig. 3. Box plots of plasma concentrations of SF in patients with DVT, patients without DIC, and healthy volunteers (HV).

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

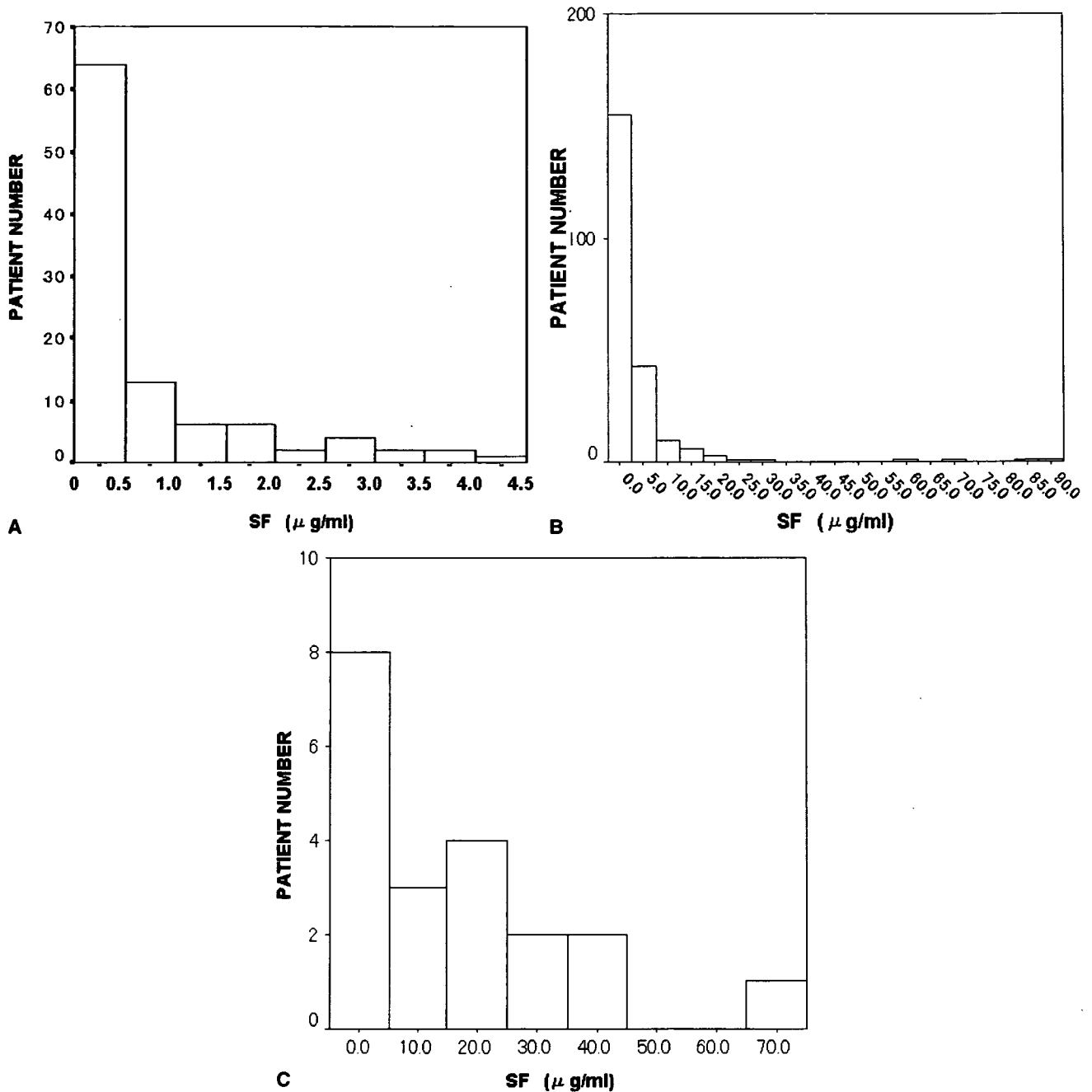


Fig. 4. Box plots of plasma concentrations of SF in (A) healthy volunteers, (B) patients without DVT, and (C) patients with DVT.

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



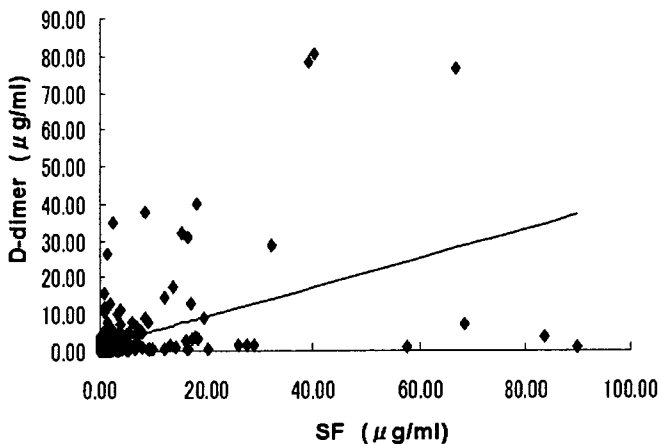


Fig. 5. Correlation between plasma concentrations of SF and D-dimer.

in patients with DVT, suggesting that the cutoff value is less than the above values. When the cutoff values were set at the average + 1 SD (D-dimer, 1.8 µg/mL; SF, 1.4 µg/mL) of those of healthy volunteers, 1 and 0 patient with DVT was overlooked, respectively. In these cases, the sensitivity was 95% for D-dimer and 100% for SF, with respective specificities of 61.9% and 53.8%. The number of patients requiring further vascular imaging studies diminished from 243 to 104 patients based on the use of D-dimer and to 123 patients based on SF. The estimated sensitivity in our study was similar to that in previous report using other D-dimer kits (0.5 µg/mL cutoff value) [6]. With regard to the difference between SF and D-dimer, SF reflects early phase of DVT/PE while D-dimer reflects secondary fibrinolysis after clot formation. In our study, plasma D-dimer concentrations correlated well with those of SF, suggesting that secondary fibrinolysis might occur immediately after clot formation. When the cutoff values were set at 2.5 µg/mL for D-dimer and 6.9 µg/mL for SF, only one DVT patient was overlooked, and the sensitivity and specificity were 95% and 69.5%, suggesting that both measurements of D-dimer and SF increase the sensitivity and specificity for the diagnosis of DVT/PE. The overlooked patient had chronic PE and he had small size thrombus.

However, as the hemostatic abnormalities are more significant in DVT patients with PE than those without PE [21], the difference between patients with PE and without PE was not significant in this study. This is probably because our study was too small and the PE was not severe.

Our data also suggest that both D-dimer and SF are useful for the diagnosis of DVT as NPV to reduce further examinations. However, the estimated cutoff values of these parameters for the diagnosis of DVT

are higher in Japan than in Europe and in the U.S. Accordingly, we believe it is important to state the cutoff value of D-dimer in each measurement kit.

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## REFERENCES

1. Lehman CM, Wilson LW, Rodgers GM. Analytic validation and clinical evaluation of the STA LIATEST immunoturbidimetric D-dimer assay for the diagnosis of disseminated intravascular coagulation. *Am J Clin Pathol* 2004;122:178–184.
2. Wada H, Sakuragawa N, Shiku H. Hemostatic molecular markers before onset of disseminated intravascular coagulation in leukemic patients. *Semin Thromb Hemost* 1998;24:293–297.
3. Minamikawa K, Wada H, Wakita Y, et al. Increased activated protein C-protein C inhibitor complex levels in patients with pulmonary embolism. *Thromb Haemost* 1994;71:192–194.
4. Le Gal G, Bounameaux H. Diagnosing pulmonary embolism: running after the decreasing prevalence of cases among suspected patients. *J Thromb Haemost* 2004;2:1244–1246.
5. Kline JA, Mitchell AM, Kabrheil C, Richman PB, Courtney DM. Clinical criteria to prevent unnecessary diagnostic testing in emergency department patients with suspected pulmonary embolism. *J Thromb Haemost* 2004;2:1247–1255.
6. Linkins LA, Bates SM, Ginsberg JS, Kearon C. Use of different D-dimer levels to exclude venous thromboembolism depending on clinical pretest probability. *J Thromb Haemost* 2004;2:1256–1260.
7. Tanigawa M, Wada H, Minamikawa K, et al. Decreased protein C inhibitor after percutaneous transluminal coronary angioplasty in patients with acute myocardial infarction. *Am J Hematol* 1995;49:1–5.
8. Taylor FB Jr, Toh CH, Hoots WK, Wada H, Levi M. Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. *Thromb Haemost* 2001;86:1327–1330.
9. Nieuwenhuizen W. A reference material for harmonization of D-dimer assays. *Thromb Haemost* 1997;77:1031–1033.
10. Dempfle CE, Zips S, Ergül H, Heene DL, FACT Study Group. The fibrin assay comparison trial (FACT). *Thromb Haemost* 2001;85:671–678.
11. Heit JA, Silverstein MD, Mohr DN, et al. The epidemiology of venous thromboembolism in the community. *Thromb Haemost* 2001;86:452–463.
12. Oger E. Incidence of venous thromboembolism: a community-based study in Western France. EPI-GETBP Study Group. Groupe d'Etude de la Thrombose de Bretagne Occidentale. *Thromb Haemost* 2000;83:657–660.
13. Giuntini C, Di Ricco G, Marini C, Melillo E, Palla A. Pulmonary embolism: epidemiology. *Chest* 1995;107(Suppl 1):3S–9S.
14. Manganelli D, Palla A, Donnamaria V, Giuntini C. Clinical features of pulmonary embolism. Doubts and certainties. *Chest* 1995; 107(Suppl 1):25S–32S.
15. Courtney DM, Kline JA. Identification of prearrest clinical factors associated with outpatient fatal pulmonary embolism. *Acad Emerg Med* 2001;8:1136–1142.
16. Wells PS, Anderson DR, Rodger M, et al. Evaluation of D-dimer in the diagnosis of suspected deep-vein thrombosis. *N Engl J Med* 2003;349:1227–1235.

17. Fedullo PF, Tapson VF. The evaluation of suspected pulmonary embolism. *N Engl J Med* 2003;349:1247–1255.
18. Wada H, Wakita Y, Nakase T, et al. Increased plasma soluble fibrin monomer levels in patients with disseminated intravascular coagulation. *Am J Hematol* 1996;51:255–260.
19. Matsuda M, Terukina S, Yamazumi K, Maekawa H, Soe G. A monoclonal antibody that recognizes the NH<sub>2</sub>-terminal conformation of fragment D. In: Matsuda M, Iwanaga S, Takada A, Henschen A, editors. *Fibrinogen 4—Current basic and clinical aspects: proceedings of the International Fibrinogen Workshop*, Kyoto, Japan, 27–28 August 1989. New York: Elsevier Science Publishing, Inc.; 1990. p 43–48.
20. Soe G, Kohno I, Inuzuka K, Itoh Y, Matsuda M. A monoclonal antibody that recognizes a neo-antigen exposed in the E domain of fibrin monomer complexed with fibrinogen or its derivatives: its application to the measurement of soluble fibrin in plasma. *Blood* 1996;88:2109–2117.
21. Yamada N, Wada H, Nakase T, et al. Hemostatic abnormalities in patients with pulmonary embolism compared with that in deep vein thrombosis. *Blood Coagul Fibrinolysis* 1995;6:627–633.
22. Wada H, Sakuragawa N, Shiku H. Hemostatic molecular markers before onset of disseminated intravascular coagulation in leukemic patients. *Semin Thromb Hemost* 1998;24:294–297.
23. Wada H, Mori Y, Shimura M, et al. Poor outcome in disseminated intravascular coagulation or thrombotic thrombocytopenic purpura patients with severe vascular endothelial cell injuries. *Am J Hematol* 1998;58:189–194.
24. Wada H, Mori Y, Kaneko T, et al. Elevated plasma levels of vascular endothelial cell markers in patients with hypercholesterolemia. *Am J Hematol* 1993;44:112–116.
25. Kline JA, Wells PS. Methodology for a rapid protocol to rule out pulmonary embolism in the emergency department. *Am Emerg Med* 2003;42:266–275.

## Usefulness of Measurement of Reticulated Platelets for Diagnosis of Idiopathic Thrombocytopenic Purpura

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**Summary:** Reticulated platelets (RP) and large platelets (LP) were measured by an automated hematology analyzer (modified R-2000) in 287 healthy volunteers and 131 patients with thrombocytopenia or thrombocytosis. RP was significantly higher in patients with idiopathic thrombocytopenic purpura (ITP), especially in active phase, while RP was markedly lower in patients with essential thrombocytosis (ET) or chronic myelocytic leukemia (CML). LP was significantly higher in patients with ITP, especially in active phase, while LP was markedly lower in patients with aplastic anemia (AA), ET, or CML. In

ITP, RP and LP were significantly higher in patients positive for anti-glycoprotein (Gp) IIb/IIIa antibody. RP and LP were poorly correlated with platelet-associated IgG (PAIgG). RP and LP were poorly correlated with plasma thrombopoietin levels, and negatively correlated with platelet count. These results show that RP reflects the pathology of thrombocytopenic disorders, and that measurement of RP is useful for the differential diagnosis and analysis of platelet kinetics.

**Key Words:** Idiopathic thrombocytopenic purpura—Reticulated platelets—Large platelets.

Thrombocytopenia is a common hematologic abnormality that is caused by decreased production, enhanced destruction, and sequestration of platelets. It is difficult to determine the pathogenesis of thrombocytopenia. The presence of normal or increased numbers of megakaryocytes in the bone marrow essentially excludes the diagnosis of hypoplastic thrombocytopenia due to aplastic anemia (AA) (1) or amegakaryocytic thrombocytopenia (2). No simple diagnostic test exists to diagnose thrombocytopenias caused by enhanced destruction of platelets such as idiopathic thrombocytopenic purpura (ITP) (3,4), disseminated intravascular coagulation (DIC) (5), and thrombotic thrombocytopenic purpura

(TTP) (6). Platelets were found to have a coarse, punctuated reticulum (reticulated platelets; RP) using a new methylene blue dye in 1969 (7). It was suggested that those cells might contain increased amounts of cytoplasmic RNA, reflecting thrombopoiesis in bone marrow. Subsequently, it was reported that platelet nucleic acid could be detected with flow cytometry utilizing thiazole orange as a dye (8). RNA-rich platelets were reported to be readily measurable by flow cytometry and to provide information on thrombopoietic activity in thrombocytopenic patients (9). Analysis of RP was found to provide a good estimate of the rate of platelet production in bone marrow (10). ITP is an autoimmune disease characterized by increased platelet clearance caused by anti-platelet autoantibodies (4). In this disorder, platelet production is normal or increased but platelet destruction in the spleen is enhanced. Patients with ITP usually have increased megakaryocytes in the bone marrow, and are believed to have high RP (11,12) and

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normal TPO levels (13,14). On the other hand, patients with AA have low counts of platelets, leukocytes, and erythrocytes in peripheral blood and hypoplastic bone marrow.

In the diagnosis of immune thrombocytopenia, flow cytometric analysis of RP was reported to be better than determination of platelet-associated IgG (PAIgG) (15). The flow cytometric analysis of RP has been suggested to be useful for evaluating thrombocytopenia. Recently, an instrument for fully automated measurement of RP (modified R-2000 fully automated reticulocyte counter) capable of measuring the RP in peripheral whole blood immediately was developed.

In this study, we measured the RP in healthy volunteers and patients with underlying diseases causing thrombocytopenia, especially ITP, to examine the usefulness of RP measurement for differential diagnosis of thrombocytopenic disorders.

## MATERIALS AND METHODS

RP was measured in 287 healthy volunteers (144 females and 143 males; median age, 32 years; range, 21–60 years). RP was also measured in 131 patients with thrombocytopenia (86 females and 45 males) due to various diseases: 76 with ITP, 37 with AA, 5 with systemic lupus erythematosus (SLE), 2 with myelodysplastic syndrome (MDS), and 11 patients with essential thrombocythemia (ET) or chronic myelogenous leukemia (CML). The study protocol was approved by the Human Ethics Review Committees of Mie University School of Medicine. ITP was diagnosed by thrombocytopenia, normal bone marrow with an increased number of megakaryocytes and lack of other disease causing thrombocytopenia; the disease condition was defined as follows: active phase, less than 50,000/ $\mu$ L platelet count; partial remission (PR), 50,000–120,000/ $\mu$ L platelet counts with or without treatment; complete remission (CR), more than 120,000/ $\mu$ L platelet count without treatment. Aplastic anemia was diagnosed by thrombocytopenia, anemia, and leukocytopenia without other disease causing pancytopenia; active phase, less than 50,000/ $\mu$ L platelet count; partial remission (PR), 50,000–100,000/ $\mu$ L platelet count with or without treatment; complete remission (CR), more than 100,000/ $\mu$ L platelet count without treatment.

RP was measured by a previously described method (16). Briefly, approximately 2 mL of

whole blood was collected from healthy volunteers or patients and was anticoagulated with ethylene diamine tetraacetic acid (EDTA)-2K. All blood samples were kept at room temperature until analysis. Counts of RP are reported to be stable during 2–6 hours after collection (17). Total platelet counts and ratio of RP were determined by the R-2000 (Sysmex Inc., Kobe, Japan) with special software (17,18). For measurements of RP, 10  $\mu$ L of blood sample was aspirated by the R-2000 and automatically mixed with 40  $\mu$ L of 2.6% auramine O in 95.9% ethylene glycol solution (Ret-Search, Sysmex Inc.) to stain the RNA present in reticulocytes and RP. The samples were then irradiated by an argon laser beam at a wavelength of 488 nm. The resulting fluorescence intensity and forward light scatter were measured with flow cytometry (Fig. 1) (17). The ratio of RP was expressed as the percentage of total platelet count. The time required to complete all these measurements was approximately 90 seconds.

Serum TPO concentration was measured by a previously described sandwich ELISA (13,19). PAIgG was measured using alkaline phosphatase-conjugated anti-human IgG (Sigma Co, St Louis, MO) with a competitive enzyme immunoassay (12). Antibody to platelet glycoproteins (GP) IIb/IIIa was detected with a platelet antibody screening kit (GTI PAKPLUS, Wisconsin). Patient or donor serum was incubated in microtiter wells that had been pre-coated with platelet glycoproteins. This allows antibody, if present, to bind. Unbound immunoglobulins were then removed by washing. An alkaline phosphatase labeled anti-human globulin reagent was added to the wells. After a brief incubation period, microtiter wells were washed to remove any unbound anti-human globulin, and the enzyme substrate PNPP (p-nitrophenyl phosphate) was added. Following a short incubation, the reaction was stopped by sodium hydroxide solution, and the optical density of the color produced was measured in an ELISA reader at a wavelength of 405 or 410 nm.

## Statistical Analysis

Results are expressed as mean  $\pm$  SD. Differences between groups were evaluated by one-way ANOVA and the Bonferroni test. For assessment of correlations, coefficients of correlation were determined by Pearson's correlation coefficient and linear regression analysis. In both cases, a level of  $p < 0.05$  was considered significant.