mortality among the groups treated with each therapy.[91,92] In two prospective phase 2 trials of eculizumab in which 37 patients with aHUS received eculizumab for 26 weeks and during long-term extension phases, 17 patients showed a low-platelet count and renal damage (in trial 1) and 20 patients showed renal damage without a decrease in the platelet count of more than 25% for at least 8 weeks during PE or plasma infusion (in trial 2).[92] Consequently, eculizumab treatment resulted in an increase in the platelet count in trial 1 and a TMA event-free status in 80% of the patients in trial 2. In addition, eculizumab inhibited complement-mediated TMA and was found to be associated with a significant time-dependent improvement in the renal function among the patients with aHUS (►**Tables 3** and **4**).

Antiplatelet drugs are appropriate for patients with standard cardiac or neurological ischemic symptoms without severe thrombocytopenia. Therefore, these agents may be administered in patients who exhibit a recovery in their platelet count. A clinical trial of ARC1779 to target the A1-domain of VWF for the treatment of TTP has been started.[94] As TMA involves a thrombotic state, prophylactic platelet transfusions should be withheld; however, such transfusions should not be suspended in TMA patients with severe thrombocytopenia who develop severe bleeding or require surgical intervention.

Concluding Remarks

Although typical TTP, USS, aHUS, STEC–HUS, and other types of TMA have similar symptoms, the pathological mechanisms of and optimal treatments for TMA are different, indicating that a differential diagnosis of TMA is important. As several issues remain in understanding the pathology of TMA, the establishment of the optimal management for each TMA requires further evidence.

Note

All authors have no competing interests.

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LETTER TO THE EDITOR

Antithrombin and mortality in severe pneumonia patients with sepsis-associated disseminated intravascular coagulation: an observational nationwide study: comment

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See also Tagami T, Matsui H, Horiguchi H, Fushimi K, Yasunaga H. Antithrombin and mortality in severe pneumonia patients with sepsis-associated disseminated intravascular coagulation: an observational nationwide study. *J Thromb Haemost* 2014; **12**: 1470–9 and Tagami T, Matsui H, Yasunaga H. Antithrombin and mortality in severe pneumonia patients with sepsis-associated disseminated intravascular coagulation: an observational nationwide study: reply. *J Thromb Haemost* 2015; DOI: 10.1111/jth.12821.

We read with interest the recent article by Tagami *et al.* [1] about a retrospective, large, nationwide database study of the effects of antithrombin (AT) on disseminated intravascular coagulation (DIC) in patients with severe pneumonia and sepsis-associated DIC. This study retrospectively demonstrated that the 28-day mortality was lower in the AT administration group than in the control group. The KyberSept trial [2] prospectively demonstrated that AT administration could not improve the mortality of patients with severe sepsis. In a subclass analysis of the KyberSept trial for DIC [3], the mortality of the septic patients with DIC was lower in the AT group than in the control group. Gando *et al.* reported the validation of the scoring systems for DIC [4] and then we suggested that antithrombotic therapy may worsen sepsis in the early stage of the disease while improving hemostatic abnormalities [5]. The death in DIC cases is divided into DIC-associated and non–DIC-associated deaths, and the former can be improved by treatment with antithrombotic therapy. The death in the patients with no recovery from DIC is termed DIC-associated death in this letter. In a reanalysis of our previous study of sepsis [6], a decreased AT concentration was frequently associated with a high rate of non-recovery from DIC and a poor outcome, especially DIC-associated death (Fig. 1), suggesting that severe AT deficiency may cause an increase in DIC-associated death. Although antithrombotic therapy, including AT, might not improve the non–DIC-associated deaths of DIC patients without AT deficiency, the possibility remains that AT administration to severely septic patients with DIC and AT deficiency may improve the survival outcomes. The International Society of Thrombosis and Haemostasis guidance for the diagnosis and treatment of DIC [7] indi-

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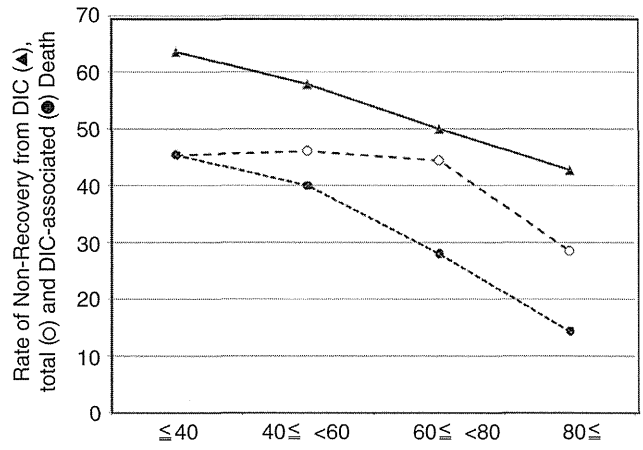

Fig. 1. The rates of non-recovery from DIC and the total and DIC-associated deaths. Closed triangles, non-recovery from DIC; open circles, total deaths; closed circles, DIC-associated deaths.

cates that the administration of AT, recombinant thrombomodulin, or activated protein C may be considered in DIC patients but that further prospective evidence from randomized controlled trials confirming a benefit is required. Finally, the AT concentration is important for diagnosing DIC and for decreasing DIC-associated deaths.

Addendum

T. Aota, T. Matsumoto, K. Suzuki, H. Imai, and N. Katayama analyzed references 1–5, respectively. All members discussed this letter to the editor. H. Wada wrote the letter to the editor based on the analysis.

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Disclosure of Conflict of Interests

The authors state that they have no conflicts of interest.

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Letter to the editor

Anti-Xa activity in VTE patients treated with fondaparinux

Dear Editor:

We previously reported that an anti-Xa level of > 0.33 mg/l on day 1 is associated with the risk of withdrawal of fondaparinux due to increased bleeding in patients undergoing major orthopedic surgery with prophylaxis, whereas the anti-Xa levels on day 4 and 8 are not correlated with this risk [1]. The anti-Xa level has been reported to be correlated with weight, height, body mass index and antithrombin activity [2], and the cause of increased bleeding in these patients is suggested to be hyperfibrinolysis [3]. Anti-Xa inhibitors, such as fondaparinux, have been used for prophylaxis as well as treatment in cases of venous thromboembolism (VTE) [4]. However, there are few reports regarding the anti-Xa activity in patients with VTE treated with fondaparinux. Therefore, we examined the Xa activity in 16 patients with deep vein thrombosis (DVT) or pulmonary embolism (PE) treated with fondaparinux. Eleven patients received 7.5 mg of fondaparinux and 5e patients with a low weight or old age received 3 or 5 mg of fondaparinux. No bleeding symptoms were observed among the patients treated with fondaparinux, and the median (95% confidence interval) anti-Xa activity was 1.34 mg/L (0.62–1.99 mg/L). The anti-Xa activity was not found to correlate with weight or the creatinine level, suggesting that the physicians selected the appropriate dose of treatment in accordance with the patient's condition. Both the symptoms and the computed tomography and echography findings improved, and the soluble fibrin and D-dimer levels significantly decreased after the administration of fondaparinux. Although the sample size was small, treatment with fondaparinux may be safe in VTE patients without a history of surgery and an anti-Xa activity level was <1.99 mg/l (Table 1).

Acknowledgments

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Table 1
Subjects.

	Sex	Age	Weight	Creatinine	Dose	Anti-Xa activity (mg/L)	Duration	Bleeding	Thrombosis	Efficacy
1	M	50	66.6	0.77	7.5 mg	1.1	7	Negative	PE/DVT	Useful
2	F	54	76.5	0.56	7.5 mg	1.56	8	Negative	DVT	Useful
3	F	76	54	0.41	7.5 mg	1.57	5	Negative	PE/DVT	Useful
4	F	72	67.1	0.67	7.5 mg	0.69	7	Negative	PE	Useful
5	F	64	58	0.72	7.5 mg	1.07	9	Negative	PE	Useful
6	M	48	68.4	0.7	7.5 mg	0.93	5	Negative	PE, DVT	Useful
7	F	60	51	0.69	7.5 mg	1.49	9	Negative	DVT	Useful
8	F	72	43.6	0.72	5 mg	1.99	8	Negative	PE	Useful
9	F	85	42	0.75	5 mg	1.1	8	Negative	DVT	Useful
10	M	53	87.8	0.85	7.5 mg	1.80	6	Negative	DVT	Useful
11	F	90	45.8	0.75	3 mg	1.47	6	Negative	PE	Useful
12	F	65	49.5	0.66	5 mg	1.89	7	Negative	PE/DVT	Useful
13	M	37	56.1	0.79	7.5 mg	1.23	11	Negative	DVT	Useful
14	M	67	62.5	1.1	7.5 mg	1.45	2	Negative	PE	Useful
15	M	30	88	0.78	7.5 mg	0.62	11	Negative	DVT	Useful
16	F	89	46.3	1.2	5 mg	1.14	5	Negative	DVT	Useful

DVT: deep vein thrombosis, PE: pulmonary embolism.

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Antithrombin or thrombomodulin administration in severe pneumonia patients with sepsis and disseminated intravascular coagulation: comment on two papers

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Key words; DIC, antithrombin, thrombomodulin, mortality, pneumonia

Correspondence/Findings: Is AT more effective than rhTM?

Dear editor

We are afraid that the recent papers by Tagami, et al. in the ***Journal of Thrombosis and Haemostasis***, [1, 2] may have caused a misunderstanding for physicians and other readers, namely the notion that antithrombin (AT) may be more effective than recombinant human thrombomodulin (rhTM) for the treatment of disseminated intravascular coagulation (DIC) in patients with pneumonia. The authors reported on the efficacy of AT [1] or rhTM [2] in this clinical setting using the same Japanese Diagnosis Procedure Combination

(DPC) database. The mortality was significantly lower in the AT group than in the control group, but there was no significant difference in the mortality between the rhTM group and the control group, thereby incorrectly suggesting that AT may be more effective than rhTM for the treatment of DIC due to pneumonia.

Although Tagami T, et al. used the same DPC database, they analyzed the efficacy of AT or rhTM using different patients groups. The mortality was about 44.3% in the AT study [1] and about 36.9% in the rhTM study [2] (**Table 1**), which may suggest that the pneumonia and/or DIC in the patients was more severe in the AT study than in the rhTM study. Prospective and controlled clinical trials of either AT [3] or rhTM [4] for severe sepsis did not clearly show efficacy of these drugs so far. It has also been suggested that AT might be effective in severe cases with DIC only [5-7]. The reason for the difference in study population between the two studies may be due to the fact that some patients were excluded, including patients classified as having "not severe pneumonia" in both studies and those suffering "death within 2 days" in the rhTM study. There were 4,416 patients more excluded patients with "not severe pneumonia" in the rhTM study compared to the AT study. It should be explained why such different exclusion criteria were used in these two studies. If patients who were "death within 2 days" were added to the rhTM study, mortality increased to 42.3% in, which was still lower than mortality in the AT study, potentially indicating that these 4,416 patients who were excluded because they had "not severe pneumonia" may have had a higher mortality than the eligible

patients in the rhTM study. Hence, the discrepancies between the two studies deserve an explanation.

The International Society of Thrombosis and Haemostasis guidance for the diagnosis and treatment of DIC [8] indicates that the administration of AT, recombinant thrombomodulin or activated protein C may be considered in DIC patients, but that further prospective evidence based on the findings of randomized controlled trials confirming such a benefit is required. We strongly recommend that clear and accurate head-to-head comparisons between AT and thTM treatment will be carried out as soon as possible.

Addendum

T. Aota, T. Matsumoto, K. Suzuki, H. Imai and N. Katayama analysed the reference 1 and 2, respectively and read reference 1-6. All members discussed this letter to editor. H. Wada wrote the letter to editor based on above analysis.

Competing interests

The authors have no competing interests to declare in association with this study.

Acknowledgments

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Table 1 Subjects evaluated in the AT study [1] and the rhTM study [2]

	AT study				rhTM study		
Pneumonia and DIC	35872			Pneumonia and DIC	35872		
Not severe pneumonia	—20136			Not severe pneumonia	—24552 = —(20136 + 4416)		
Met exclusion criteria	—4349			Met exclusion criteria	—3147		
Age < 18 years	—1471			Started rhTM > 2 days after A	—672		
Started AT > 7 days after A	—491			Death within 2days	—592		
Used AT for > 7 days	—350			Age < 18 years	—329		
				Used rhTM for > 7 days	—238		
	All	AT G	Control G		All	rhTM G	Control G
Eligible patients	9075	2663	6412	Eligible patients	6342	1280	5062
Mortality	44.3%	40.8%	45.7%	Mortality	36.9%	37.0%	36.9%
				Mortality (plus death	42.3%		

				within 2day)*			
Propensity-matched group	4388	2194	2194	Propensity-matched group	2280	1140	1140
Mortality	42.4%	40.6%	45.1%	Mortality	37.3%	37.6%	37.0%

A; administration, G: group

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Regular Article

Elevated plasma levels of soluble platelet glycoprotein VI (GPVI) in patients with thrombotic microangiopathy

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ABSTRACT

Background: Thrombotic microangiopathy (TMA) is caused by various conditions, such as decreased a ADAMTS13 level, activated or injured vascular endothelial cells or activated platelets. This study examined the soluble platelet glycoprotein VI (sGPVI) levels in patients with TMA to evaluate the activation of platelets in thrombotic states.
Materials and Methods: The plasma levels of sGPVI, ADAMTS13 activity, von Willebrand factor (VWF) and VWF propeptide (VWFpp) were measured in patients with TMA.
Results: The plasma levels of sGPVI were significantly higher in postoperative patients, patients with TMA and those with disseminated intravascular coagulation (DIC) than in those without thrombosis. The plasma levels of sGPVI were the highest in patients with TMA without markedly reduced ADAMTS13 and those were significantly reduced after plasma exchange.
Conclusion: The measurement of sGPVI level is therefore considered to be important for the diagnosis and evaluation of TMA.

Introduction

Thrombotic microangiopathies (TMAs) are defined by the association of acute mechanical hemolytic anemia, thrombocytopenia, and visceral ischemic manifestations related to the formation of platelet thrombi in the microcirculation [1–3]. TMAs are caused by various conditions, such as markedly decreased a ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I domain 13) level and unusually large multimers of Von Willebrand factor (ULM-VWF) [4], activated or injured vascular endothelial cells, activated platelets, or etc. ADAMTS13 [5] is a zinc metalloprotease that specifically cleaves ULM-VWF at the Tyr (1605)- Met(1606) boundary located in the A2 region of VWF [6,7], suggesting that ULM-VWF cause multiple platelet thrombi due to TMA. The diagnosis of TMA has improved remarkably by developing the method of ADAMTS13 measurement [8,9].

In the activated or damaged vascular endothelial cells, plasma levels of soluble thrombomodulin [10], P-selectin [11], VWF and VWF propeptide (VWFpp) [12] were reported in patients with TMA. After the secretion of VWFpp and VWF into plasma from endothelial cells in response to several physiological or pathological stimuli, VWFpp dissociates from VWF [13,14]. It is reported that VWFpp is more useful for the diagnosis of TMA than VWF or TM [11,15].

Platelet glycoprotein VI (GPVI), a type I transmembrane glycoprotein of immunoreceptor family, is constitutively associated and expressed with the Fc receptor γ-chain (FcRγ), an immunoreceptor tyrosine-based activation motif-bearing (ITAM-bearing) receptor [16,17]. On ligand-induced cross linking of GPVI/FcRγ, this ITAM enables Lyn-dependent activation of Syk kinase and promulgation of downstream signaling cascade. Upon platelet activation, the platelet surface GPVI was cleaved off by protease, such as ADAM10, resulting in down regulating platelet reactivity and releasing of the soluble form GPVI (sGPVI) [18–20]. The sGPVI has recently received much attention as a platelet activation marker, because, i) it is specifically expressed on platelet, ii) the level of sGPVI in plasma are increased in patients with thrombosis, iii) the production mechanism is dependent on platelet activation. Indeed, several groups developed an immunoassay for sGPVI, and reported that the sGPVI would be a useful biomarker for disease caused by platelet activation such as acute coronary syndrome (ACS) and stroke [21–25].

This study, examined the activation of platelets by measuring of sGPVI level in 70 patients with TMA in comparison to 40 healthy

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volunteers, 46 patients without thrombosis, 15 postoperative patients, 13 with disseminated intravascular coagulation (DIC).

Materials and Methods

The plasma levels of sGPVI, ADAMTS13 activity, VWF and VWFpp were measured in 70 with TMA (37 females and 33 males, median age: 25-75%tile; 55.5 years old: 34-72 years old) from April 1, 1990 to March 31, 2012 in Mie University Hospital, in comparison to 40 healthy volunteers (14 females and 26 males, 22.0 years old: 22.0-26.0 years old), 46 patients without thrombosis (23 females and 23 males, 52.0 years old: 30.0-68.0 years old), including 18 patients with hematological diseases, nine with infections, seven with liver diseases, six with solid cancers, four with autoimmune diseases and two with renal diseases, 15 postoperative patients following orthopedic surgery (11 females and 4 males, 71.0 years old: 66.5-74.8 years old), 13 with DIC (2 females and 11 males, 59.0 years old: 39.0-69.5 years old). TMA was diagnosed according to the diagnostic criteria of TMA: (1) thrombocytopenia (less than 12 X 10^4/μl), (2) hemolytic anemia (less than 11.0 g/dl of hemoglobin) due to the microangiopathy (presence of fragmented red cells, elevated total bilirubin, and LDH), (3) neurological dysfunction, (4) renal failure, and (5) fever [26]. The patients with (1) and (2) who had an ADAMTS 13 activity of less than 10%, who had an O-157 infection, and who had clinical symptoms, such as (3) or/and (4), were diagnosed with TMA. These TMA patients were classified into 4 groups; 6 patients with atypical HUS (HUSa), which has a frequent relapse and familial history for TMA, 5 patients with hematological TMA (TMA-H), which is due to bone mallow transplantation, 27 patients with ADAMTS13-related TMA (TMA-A), where the ADAMTS13 level was less than 10%; and 32 patients with TMA other (TMA-O), the cause of which was not known, which was associated with vascular endothelial cell damage. DIC was diagnosed using an International Society of Thrombosis Haemostasis overt-DIC diagnostic criteria [27]. The patients who had a complete remission and survived, were called survivors. The patients who did not have a complete remission and died within one year were called non-survivors.

The study protocol was approved by the Human Ethics Review ommittees of Mie University School of Medicine, and signed informed consent was obtained from each patient.

Measurement of ADAMTS13, VWF and VWFpp

ADAMTS13 was measured using a FRETS-VWF73, which was chemically synthesized by the Peptide Institute, Inc. (Osaka, Japan) according to the method described by Kokame et al. [8,9]. TM was measured with a Thrombomodulin "MKI" EIA kit (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). VWF and VWFpp levels were measured with a VWF&Propeptide assay kit (GTi DIAGNOSTiCs, Waukesha, USA)[12,15].

Measurement of sGPVI in plasma

Recombinant sGPVI was used as calibrator in the assay. The extracellular region of human GPVI fused with histidine-tag at the C-terminus was transiently expressed by COS-1 cell, and the cultured supernatant was purified by Ni-column chromatography. The level of sGPVI in plasma was quantified by sandwich ELISA, which consists of two mouse anti-GPVI monoclonal antibodies, F1232-7-1 and F1232-10-2 which could recognize the extracellular domain I (D1) N-terminal loop and the extracellular domain D2 loop of GPVI, respectively. [28,29]. Briefly, a 96-well microtiter plate (Nunc-immuno Module Maxsorp, Thermo, Waltham, USA) was coated with F1232-7-1 diluted in phosphate buffer saline, pH7.4 (PBS), and then was blocked with 5% stabilguard (SurModics, Eden Prairie, USA) and 3.2% sucrose in PBS. The calibrators consisting of recombinant sGPVI and human plasma diluted in the sample dilution buffer (0.1% bovine serum albumin, 0.05% Tween20 and 0.3 mol/L NaCl in PBS) were added in duplicate to wells and the plate was incubated at room temperature for 2 h. After the plate was washed five times with wash buffer (0.05% Tween20 in saline), the captured sGPVI was detected by applying the peroxidase-labeled F1232-10-2 Fab in the dilution buffer (6% rat serum, 1% mouse serum, 0.3% BSA and 0.05% Tween 20 in PBS) for 1 h at room temperature. Then, the wells were washed five times with the wash buffer, and the level of sandwiched sGPVI was determined by adding tetramethyl benzidine for 20 min at room temperature, with the enzyme reaction being terminated by lowering the pH. The absorbance of each well at 450 nm was measured using a microplate reader (reference 620 nm or longer). Using this assay, quantitative measurements are available within 3 h. The standard curve was linear from 0.156-5.0 ng/mL and the intra-assay and inter-assay variation were less than 10%. The limit of detection and the limit of quantification in the assay were 0.067 and 0.156 ng/mL, respectively.

Identification of sGPVI in plasma

sGPVI in plasma was confirmed by immunoprecipitation and western blotting. Briefly, each plasma was incubated with F1232-7-1 coupled bead, which is prepared with NHS-activated sepharose (GE healthcare, Buckinghamshire, UK), for 1 h at room temperature. The bead was washed twice with phosphate buffer (pH7.4), and treated with Tris-SDS buffer containing 2-mercaptoethanol. After centrifugation, the supernatant was subjected to SDS-PAGE using 10 % Bis-Tris Gel (Life Technologies, Carisbad, USA), and electrotransferred to nitrocellulose membrane. The membrane was immunoblotted with a rabbit anti-GPVI antibody [29]. The blots were probed with the HRP-conjugated goat anti-rabbit immunoglobulins antibody (Dako, Glostrup, Denmark), developed with ECL-Plus (GE healthcare), and imaged with CCD cameras.

Statistical analysis

The data are expressed as the median (25% tile-75% tile). Differences between groups were examined for significance using the Mann-Whitney U test. A P-value of less than 0.05 was considered to indicate a significant difference. The correlations between sGPVI and hemostatic markers were examined using the Spearman's rank correlation coefficient.

All statistical analyses were performed using Stat flex, version 6, software package (Artec Co Ltd, Osaka, Japan,).

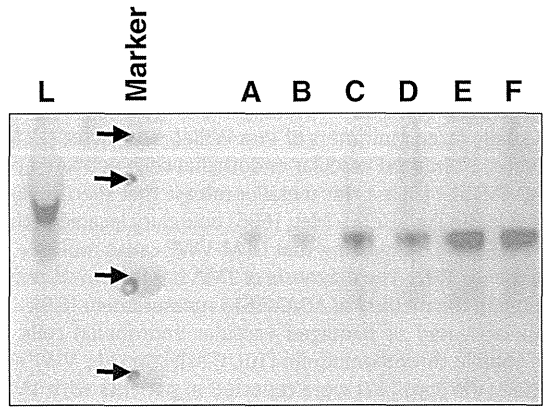

Fig. 1. The immunoprecipitation and Western blotting analyses of sGPVI in the plasma and washed platelet lysate. The immunoprecipited samples derived from plasma were analyzed by a Western blotting analysis using a rabbit anti-GPVI antibody. The samples in lanes A and B were supplied from healthy controls, while those examined in lanes C, D, E and F were supplied from TTP patients. The levels of sGPVI in the plasma were 7.47, 7.13, 20.0, 22.9, 40.4 and 44.4 ng/mL, respectively. The sample in lane L was washed platelet lysate, and the molecular weight marker was from Bio-Rad. The arrows from top to bottom indicate the 100, 75, 50 and 37 kDa, respectively.

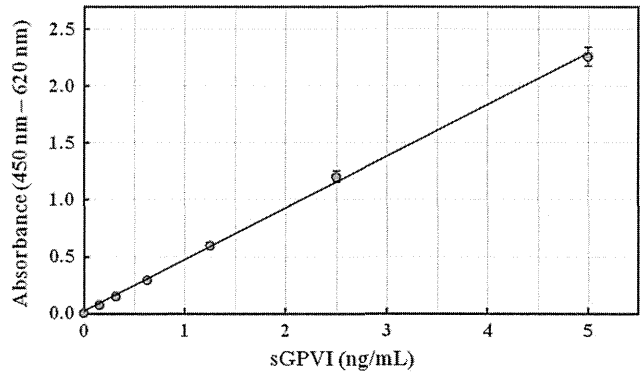

Fig. 2. A typical calibration curve for the assay. Various concentrations of recombinant sGPVI were used to generate as calibration curve. The values are expressed as the means ± SD (n = 4).

Results

The immunoprecipitation and western blotting in healthy volunteers (Lane A and B) and TTP patients (Lane C ~ F) show a single band and molecular weight 55 KD. Each level of sGPVI in plasma was 7.47, 7.13, 20.0, 22.9, 40.4 and 44.4 ng/mL, respectively (Fig. 1). The standard curve was linear from 0.156-5.0 ng/mL and the intra-assay and inter-assay variation were less than 10%. The limit of detection and the limit of quantification in the assay were 0.067 ng/ml and 0.156 ng/mL, respectively (Fig. 2).

The plasma levels of sGPVI (median; 2.5-97.5%tile) were 11.4 ng/mL (5.9-19.5) ng/mL in healthy volunteers. Plasma levels of sGPVI (median; 25.0-75.0%tile) were significantly higher in patients without TH (16.2 ng/mL 12.6-22.5 ng/mL), post operation (31.6 ng/mL; 28.3-35.1 ng/mL), DIC (44.5 ng/mL; 36.6- 60.8 ng/mL), TMA (40.8 ng/mL; 32.9-56.7 ng/mL) than in healthy volunteers (11.4 ng/mL; 9.1-14.8 ng/mL)($p < 0.001$, respectively, Fig. 3). Those were significantly higher in patients with post operation than in patients without TH ($p < 0.001$), and significantly higher in those with TMA and in those with DIC than in those without TH ($p < 0.001$, respectively). Plasma levels of sGPVI were significantly higher in patients with TMA-A (36.8 ng/mL; 30.5-49.0 ng/mL) and TMA-O (51.9 ng/mL; 37.4-66.3 ng/mL) than in patients without TH ($p < 0.001$, respectively, Fig. 4). Those were significantly higher in patients with TMA-O than in patients with aHUS (33.2 ng/mL; 19.5-50.7 ng/mL, $p < 0.05$) and TMA-H (14.2 ng/mL; 6.1-21.4 ng/mL, $p < 0.001$). On one day after treatments such as plasma exchange, plasma levels of GPVI were significantly reduced in comparison to the onset of TMA ($p < 0.001$, Fig. 5).

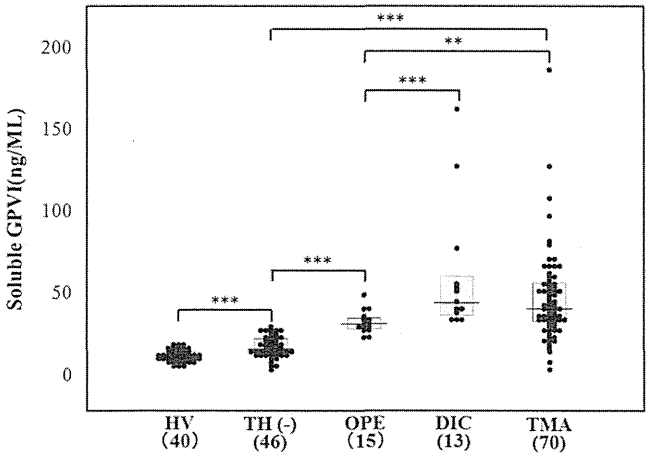

Fig. 3. The plasma levels of soluble GPVI in healthy volunteers, patients without thrombosis, postoperative patients, and patients with DIC or TMA HV; health volunteers, TH(-); patients without thrombosis, OPE; postoperative patients, DIC; patients with disseminated intravascular coagulation, TMA; patients with thrombotic microangiopathy. ***; $p < 0.001$, **; $p < 0.01$.

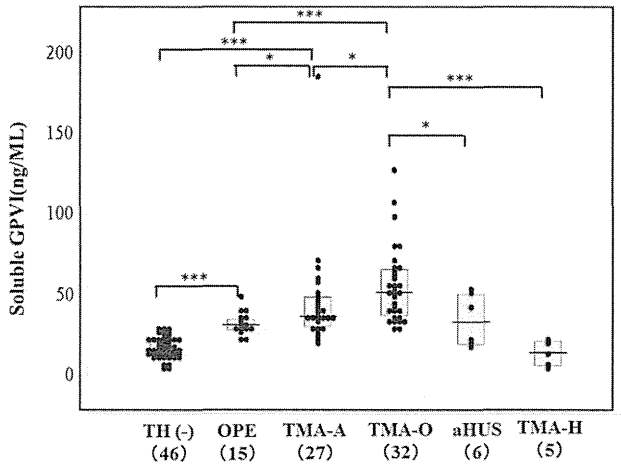

Fig. 4. The plasma levels of soluble GPVI in patients without thrombosis, postoperative patients and patients with TMA-A, TMA-O, aHUS or TMA-H. TH(-); patients without thrombosis, OPE; postoperative patients, TMA-A: TMA-ADAMTS13; patients with thrombotic microangiopathy (TMA) due to markedly reduced ADAMTS13, TMA-O: TMA-Other; patients with TMA due to other causes, aHUS; patients with atypical HUS, TMA-H; patients with TMA due to hematological malignancy. ***; $p < 0.001$, *; $p < 0.05$.

There was no significant difference in plasma levels of ADAMTS13, sGPVI, VWF and VWFpp/VWF ratio between TMA patients with survivor or non-survivor. Plasma VWFpp levels were significantly higher in the non-survivor (339 %; 263-441 %) than in the survivor (200 %; 165-241 %, $p < 0.001$, Table 1). Plasma levels of sGPVI were not well correlated with ADAMTS13, VWF, VWFpp, VWFpp/VWF ratio and TM in the patients with TMA (Table 2), but the correlation between ADAMTS13 and TM, between VWF and VWFpp, VWFpp and VWFpp/VWF ratio and VWFpp and TM was significant in the patients with TMA. The plasma sGPVI levels were not well correlated with the platelet counts ($Y = 50.0 - 1.40X$, $r = -0.5405$). The patients were divided into two groups; a normal platelet count group and a low platelet count group (Fig. 6).

Discussion

Hypercoagulable and thrombotic states including platelet activation might exist in patients with TMA [11,26]. An activation of coagulation system can be examined by measuring soluble fibrin and thrombin-antithrombin complex [30], and an activations or injuries of vascular endothelial cells can be examined by measuring VWFpp and TM [12],

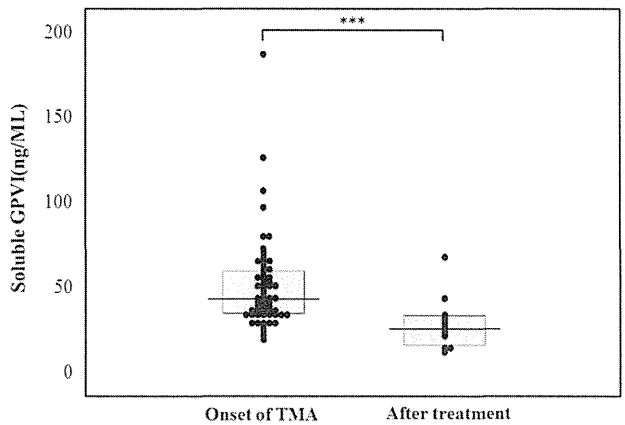

Fig. 5. The plasma levels of soluble GPVI in patients at the onset of TMA and one day after treatment of plasma exchange. ***; $p < 0.001$.

Table 1
The plasma levels of ADAMTS13 activity, sGPVI, VWF, VWFpp and the VWFpp/VWF ratio in surviving and non-surviving TMA patients.

	Survivor	Non-survivor	
ADAMTS13 (%)	65.0 (19.4 – 94.7)	35.2 (17.5 – 61.3)	NS
sGPVI (ng/mL)	56.6 (43.2 – 136.3)	40.4 (32.7 – 58.8)	NS
VWF (%)	197 (144 – 229)	225 (193 - 299)	NS
VWFpp (%)	200 (165 – 241)	339 (263 – 441)	P < 0.001
VWFpp/VWF ratio	1.07 (0.98 – 1.42)	1.30 (1.02 – 1.96)	NS

NS; not significant.
Survivor: patients who had a complete remission and survived.
Non-survivor: patients who did not have a complete remission and died within one year.

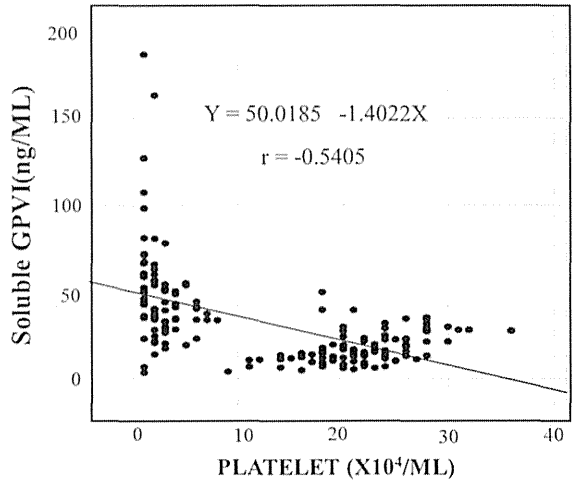

Fig. 6. The relationship between the plasma soluble GPVI level and the platelet count.

While an activation of platelet is difficult to be examined by routine tests. Although β-thromboglobin (β-TG) and platelet factor 4 (PF4) might be reflect to the platelet activation, those tests need to careful procedure and a low temperature centrifuge. This sGPVI assay is stable, easy and fast in comparison toβ-TG and PF4. A sGPVI exist in plasma and it is considered to be released from activated platelets. The normal range of plasma sGPVI was from 5.9 ng/mL to 19.5 ng/mL. Plasma sGPVI levels were significantly increased in patients with post operation, those with DIC and those with TMA, thus suggesting that plasma sGPVI levels increased in a thrombotic state which activates platelets. Several groups have already reported the development of an immunoassay for determination of sGPVI in plasma [31,32], but, we assumed that there are some discrepancy between each assay. The one group reported that the level of sGPVI was elevated in platelet activated disease [22,23,33] such as DIC, stroke and SAP which cause shear-dependent platelet activation. The other group represented that the levels of sGPVI was more negatively associated with the development of ACS, such as non-ST-elevation myocardial infarction and ST-elevation myocardial infarction, and they speculated the reason why the released sGPVI would bind to the atherosclerotic plaque and protect from atherothrombosis [32,34]. The cause for the difference is unclear, it might be due to a sample preparation, characteristic features of antibody or complexity of assay. These differences might reflect the different diseases and/or stages of disease studied in the different patient groups.

Plasma levels of sGPVI were the highest in those with TMA-O among TMA group. The one of causes might be activated platelet exception with activation of VWF/ADAMTS13 system. Markedly elevated VWFpp levels were also reported in the patients with TMA-O [13], indicating that severe vascular endothelial injuries existed in this state. Although TMA-H is also associated with severe vascular endothelial cell injuries, the patients with TMA-H had severe thrombocytopenia. Therefore, the consumption of platelets in TMA-H patients is not significant compared to that in patients with TMA-O, and subsequently, the plasma levels of sGPVI did not significantly increase in the patients with TMA-H. After treatments such as plasma exchange, plasma levels of GPVI were significantly reduced in comparison to the onset of TMA, suggesting that plasma levels of sGPVI might be useful to evaluate the efficacy of treatment for TMA. There was no significant difference in plasma levels of sGPVI between TMA patients with survivor or non-survivor, thus suggesting that the degree in the activation of platelets may not affect the outcome. However, the VWFpp levels were significantly high in the poor outcome patients with TMA [13]. As VWFpp is reflected to vascular endothelial injuries, sGPVI might not be a marker for vascular endothelial injuries. Plasma levels of sGPVI were not well correlated with ADAMTS13, VWF, VWFpp and TM in the patients with TMA, suggesting sGPVI might be new marker for reflecting the activation of platelet but not reflecting the activation or injury of vascular endothelial cells. As the plasma levels of ADAMTS13 are not closely correlated with the platelet counts, not only a reduced ADAMTS13 level, but also other factors, may activate platelets.

The measurement of sGPVI might be useful for assessing the activation of platelets in patients with acute myocardial infarction, cerebral infarction and DIC, and for the evaluating the efficacy of anti-platelet agents.

Conflict of Interest Statement

The study was funded by Mochida Pharmaceutical CO., LTD and the sGPVI ELISA system was provided from the company. KN contributed to

Table 2
The correlations among the ADAMTS13, sGPVI, VWF and VWFpp levels, the VWFpp/VWF ratio and TM.

	ADAMTS13	sGPVI	VWF	VWFpp	VWFpp/ VWF ratio	TM
ADAMTS13		0.129 (NS)	0.194 (NS)	0.126 (NS)	- 0.134 (NS)	0.462 (p < 0.001)
sGPVI	0.129 (NS)		- 0.177 (NS)	- 0.144 (NS)	- 0.096 (NS)	0.158 (NS)
VWF	0.194 (NS)	- 0.177 (NS)		0.688 (p < 0.001)	- 0.199 (NS)	0.524 (p < 0.001)
VWFpp	0.126 (NS)	- 0.144 (NS)	0.688 (p < 0.001)		0.492 (p < 0.001)	0.602 (p < 0.001)
VWFpp/ VWF ratio	- 0.134 (NS)	- 0.096 (NS)	- 0.199 (NS)	0.492 (p < 0.001)		0.114 (NS)
TM	0.462 (p < 0.001)	0.158 (NS)	0.524 (p < 0.001)	0.602 (p < 0.001)	0.114 (NS)	

NS; not significant.
The values indicate the Spearman's rank correlation coefficient.

the measurement and western blotting analysis of sGPVI, but was not involved in the interpretation of the results. KN and YH are employees of Mochida Pharmaceutical CO., LTD.

All authors have no othere COI.

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