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Potential Role of Enhanced Cytokinemia and Plasma Inhibitor on the Decreased Activity of Plasma ADAMTS13 in Patients With Alcoholic Hepatitis: Relationship to Endotoxemia

Masatoshi Ishikawa, Masahito Uemura, Tomomi Matsuyama, Masanori Matsumoto, Hiromichi Ishizashi, Seiji Kato, Chie Morioka, Masao Fujimoto, Hideyuki Kojima, Hitoshi Yoshiji, Tatsuhiro Tsujimoto, Chikara Takimura, Yoshihiro Fujimura, and Hiroshi Fukui

Background: Deficiency of ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13) results in an increase in unusually large von Willebrand factor multimer (UL-VWFM) of the plasma and finally causes microcirculatory disturbance. Our previous study demonstrated that the imbalance of increased UL-VWFM over decreased ADAMTS13 activity may contribute to the development of multiorgan failure in patients with alcoholic hepatitis (AH). The aim of this study was to explore the potential mechanism to reduce the activity of plasma ADAMTS13.

Methods: Plasma cytokine levels including interleukin (IL)-6, IL-8, and tumor necrosis factor-α (TNF-α), plasma endotoxin concentration, and the plasma inhibitor against ADAMTS13 were determined together with ADAMTS13 activity, VWF antigen (VWF:Ag), and UL-VWFM in 24 patients with AH and 5 patients with severe alcoholic hepatitis (SAH).

Results: The concentrations of IL-6, IL-8, and TNF-α on admission were significantly higher in patients with SAH than in those with AH and controls. The ADAMTS13 activity concomitantly decreased, and the VWF:Ag progressively elevated with increasing concentrations of these cytokines from normal range to over 100 pg/ml. Plasma endotoxin concentration was markedly higher in patients with SAH (mean 52.3 pg/ml) and AH (21.7 pg/ml) than in controls (7.9 pg/ml). The endotoxin concentration inversely correlated with ADAMTS13 activity and was higher in patients with UL-VWFM than those without. The inhibitor was detected in 4 patients with SAH (0.9 to 2.1 BU/ml) and 6 patients with AH (0.5 to 1.6 BU/ml). Patients with the inhibitor showed lower functional liver capacity, higher endotoxin concentration, and marked inflammatory signs than those without. At the recovery stage, the ADAMTS13 activity increased to normal range, the VWF:Ag decreased, and the UL-VWFM disappeared with the decrease in the concentrations of cytokines and endotoxin, and the disappearance of the inhibitor.

Conclusion: Decreased ADAMTS13 activity and increased VWF:Ag could be induced not only by pro-inflammatory cytokinemia, but also by its inhibitor, both of which may be closely related to enhanced endotoxemia in patients with AH and SAH.

Key Words: ADAMTS13, Cytokines, Inhibitor, Endotoxin, Alcoholic Hepatitis.

ALCOHOLIC HEPATITIS (AH) is a potentially life-threatening complication of alcoholic abuse, and its severe form, severe AH (SAH) frequently develops multi-organ failure with manifestations of acute hepatic failure, which is associated with high morbidity and mortality (Ishii et al., 1993; Maddrey et al., 1978; Mookerjee et al., 2003). The pathogenesis of AH is uncertain, but relevant factors include metabolism of alcohol to toxic products, oxidant stress, acetaldehyde adducts, the action of endotoxin on Kupffer cells, and impaired hepatic regeneration (Haber et al., 2003).

Recently, ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13) has been focused on the occurrence of thrombotic thrombocytopenic purpura (TTP) (Fujimura et al., 2002; Furlan et al., 1997; Tsai and Lian, 1998), which is characterized by thrombocytopenia, renal dysfunction, fluctuating neurological symptoms, microangiopathic hemolytic anemia, and fever (Moschcowitz, 1924). ADAMTS13 is a metalloproteinase that specifically cleaves the multimeric von Willebrand factor (VWF) between

From the Third Department of Internal Medicine (MI, MU, TM, CM, MF, HK, HY, TT, HF), Department of Blood Transfusion Medicine (MM, SK, YF), Department of Health and Sports Science (HI), Nara Medical University, Kashihara, Nara, Japan; and Asuka Hospital (CT), Takatoricho, Takaichigun, Nara, Japan.

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Reprint requests: Masahito Uemura, MD, Third Department of Internal Medicine, Nara Medical University, Kashihara, Nara, 634-8522, Japan; Fax: +81-744-24-7122; E-mail:muemura@naramed-u.ac.jp

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Tyr1605 and Met1606 within the VWF A2 domain (Levy et al., 2001; Plaimauer et al., 2002; Soejima et al., 2001; Zheng et al., 2001). VWF is synthesized in the vascular endothelial cells, and released into the plasma as "unusually large" VWF multimers (UL-VWFM) (Moake, 2002; Ruggeri, 1997). Deficiency of ADAMTS13 caused either by mutations of the *ADAMTS13* gene (Kokame et al., 2002) or by inhibitory autoantibodies against ADAMTS13 (Furlan et al., 1998; Tsai and Lian, 1998) increases the plasma levels of UL-VWFM, which leads to platelet clumping and/or thrombi under high shear stress, resulting in microcirculatory disturbance (Furlan et al., 1998; Moake, 2002; Ruggeri, 1997; Tsai and Lian, 1998). We recently demonstrated that the ADAMTS13 is produced exclusively in the hepatic stellate cells adjacent to the endothelial cells (Uemura et al., 2005a), where VWF is produced.

A little information has been available on the ADAMTS13 activity associated with liver diseases. The activity was low in the patients with liver cirrhosis (Mannucci et al., 2001; Uemura et al., 2008) and acute hepatitis (Kavakli, 2002). We showed the significant reduction in the ADAMTS13 activity in patients with hepatic veno-occlusive disease after stem cell transplantation (Park et al., 2002), and a prompt decrease in the protease activity associated with early adverse events including ischemia-reperfusion injury and/or acute graft rejection in living-donor related liver transplantation (Ko et al., 2006). In our previous reports, the ADAMTS13 activity was extremely low in the nonsurvivors with SAH and multiorgan failure, and the imbalance of increased production of UL-VWFM over decreased activity of ADAMTS13 may, in part, contribute to the progression of liver disturbance and the development of multiorgan failure through microcirculatory disturbance in SAH in addition to AH (Matsuyama et al., 2007; Uemura et al., 2005b). However, it remains unclear why the ADAMTS13 activity decrease in patients with AH.

Alternatively, endotoxemia due to hepatic reticuloendothelial dysfunction and increased intestinal permeability may be thought to trigger the enhancement of proinflammatory cytokines, which may cause systemic inflammatory response syndrome together with microcirculatory disturbance and finally lead to multiorgan failure in SAH (Fukui et al., 1991; Ishii et al., 1993; Mookerjee et al., 2003). It was, recently, demonstrated that inflammatory cytokines are associated with the decrease in the ADAMTS13 activity and the increase in UL-VWFM released from endothelial cells in vitro (Bernardo et al., 2004) and that inflammation-associated ADAMTS13 deficiency promotes formation of UL-VWFM in patients with sepsis (Bockmeyer et al., 2008), indicating the close linkage among cytokinemia, endotoxemia, and the ADAMTS13 activity in AH.

In the present study, we determined the plasma cytokine levels, plasma endotoxin concentration, and the inhibitor against the ADAMTS13, and tried to explore the potential mechanism to reduce the activity of plasma ADAMTS13 in patients with AH and SAH.

MATERIALS AND METHODS

Patients

The study was carried out in 28 patients with AH (26 men and 2 women; mean age: 55.1 years) and 5 patients with SAH (4 men and 1 woman; mean age: 41.2 years), who were principally same patients previously described (Matsuyama et al., 2007; Uemura et al., 2005b) (Table 1). All patients were originally admitted in our

Table 1. Clinical Data of Patients With Alcoholic Hepatitis

Variable	Alcoholic hepatitis	Severe alcoholic hepatitis	Normal range
Age (year)	55.1 (23–67)	41.2[b] (30–61)	
Sex (male/female)	26/2	4/1	
Serum total bilirubin (mg/dl)	4.4 (0.3–22.1)	13.5[c] (8.0–24.3)	0.3–1.1
Aspartate aminotransferase (IU/l)	180 (40–673)	320 (119–709)	12–32
Alanine aminotransferase (IU/l)	116 (25–407)	87 (63–165)	5–36
Lactate dehydrogenase (IU/l)	278 (132–450)	538[c] (283–836)	116–230
γ-Glutamyl transpeptidase (IU/l)	670 (37–2388)	472 (145–1000)	11–69
White blood cell count (/mm^3)	7,474 (3000–17100)	12,620[a] (3500–26600)	3,900–9,800
Polymorphonuclear neutrophil (/mm^3)	5,260 (1462–14877)	11,345[c] (3220–25004)	2,000–7,500
Hemoglobin (g/dl)	13.3 (9.1–17.0)	9.0[c] (7.3–11.1)	13.5–17.6
Platelet count (×10^4/mm^3)	16.8 (6.9–27.9)	8.8[a] (2.8–16.4)	13.1–36.2
C-reactive protein (mg/dl)	1.2 (0.1–13.8)	4.0 (0.5–12.2)	0–0.6
Serum albumin (g/dl)	4.0 (2.3–4.9)	3.0[c] (1.8–3.1)	3.8–5.0
Prothrombin time (%)	83 (58–100)	36[c] (27–39)	70–100
Blood urea nitrogen (mg/dl)	17 (4–60)	33[a] (11–89)	8–20
Serum creatinine (mg/dl)	1.0 (0.6–1.8)	2.8[c] (0.4–4.7)	0.3–0.9
Liver cirrhosis (+)	11	5	
Hepatic encephalopathy (Grade II-III)	0	3	
Renal failure/pneumonia/heart failure/DIC	0/0/0/0	4/4/3/1	
Treatment (FFP/prednisolone/HD)	–	5/2/1	
Outcome (alive/dead)	28/0	2/3	

DIC, disseminated intravascular coagulation; FFP, fresh frozen plasma; HD, hemodialysis.
[a]$p < 0.05$, [b]$p < 0.01$, and [c]$p < 0.005$ versus alcoholic hepatitis.

hospital between June 2001 and January 2006. Any patients with a known history of coagulopathies, sepsis, or platelet disorders were excluded from this study. The diagnosis of AH and SAH was based on the physical findings, laboratory tests, and confirmed by the liver histology in 2 patients with SAH and 11 patients with AH; the remaining 3 cases with SAH and 17 cases with AH were clinically diagnosed, according to the Diagnostic Criteria for Alcoholic Liver Injury, established by Takada, and a Japanese study group for alcoholic liver disease (1993). In brief, the etiological diagnosis of alcoholics with liver disease was classified into 3 groups: alcohol alone, combination with alcohol and virus, and others. In the alcohol alone group, virus markers were negative, and serum transaminase decreased less than 80 units during 4 weeks after abstinence. Serum γ-glutamyl transpeptidase (γ-GTP) also decreased either 1.5 times of normal value or less than 40% of the initial levels, during 4 weeks after abstinence. In addition, in the absence of liver histology, AH was clinically diagnosed in patients who showed augmented liver dysfunction following the increase in alcohol consumption, the increase in aspartate aminotransferase higher than alanine aminotransferase, and the increase in serum total bilirubin more than 2.0 mg/dl, in addition to more than 3 clinical features among abdominal pain, fever up, leukocytosis, the increase in alkaline phosphatase more than 1.5 times of normal value, and the increase in γ-GTP more than 2.0 times of normal value. The severity of SAH was estimated according to Maddrey score (Carithers et al., 1989). Hepatic encephalopathy was graded according to the classification of Trey and colleagues (1966). The diagnosis of disseminated intravascular coagulation (DIC) was made by the scoring system (Taylor et al., 2001). Standard therapy for patients with AH was abstinence from alcohol and supportive care including nutritional supplementation of at least 25 kcal/d, 1 g protein/kg/d, vitamins, and minerals via oral or enteral routes, but if difficulties arised, a parenteral route was used. All subjects gave informed consent to participate in the study. The study protocol was approved by the Nara Medical University Hospital Ethics Committee.

Assays of ADAMTS13 Activity, VWF Antigen, UL-VWFM, and Inhibitor Against ADAMTS13

Blood was taken from the patients on and/or during admission in plastic tubes with 1/10th volume of 3.8% sodium citrate as an anticoagulant. In 8 patients with AH and 2 survivors with SAH, a second plasma sample was taken between 7 and 90 days at the recovery stage when serum total bilirubin has been normalized and/or transaminase decreased within 2 times of normal range; in a nonsurvivor with SAH, plasma was sequentially taken every 2 week for 2 months until the terminal stage. Platelet-poor plasma was prepared by centrifugation of the plasma at $3000 \times g$ at 4°C for 15 minutes, and was stored in aliquots at −80°C until analysis. Plasma ADAMTS13 activity was assayed according to the method of Furlan et al. (1998) with slight modification (Mori et al., 2002). The detection limit of the activity was approximately 3%, and its normal value was 102 ± 23% (mean ± SD) (n = 60; 30 women and 30 men, 20 to 39 years old) (Mori et al., 2002). We, therefore, considered the activity low when it was less than 50% of the healthy subjects (mean—2SD). The plasma UL-VWFM was analyzed by SDS-0.9% agarose gel electrophoresis using 1 μl of samples (Park et al., 2002). The plasma VWF:Ag was measured by ELISA (Dako, Kyoto, Japan), and its normal level was 100 ± 53% (n = 60, 20 to 39 years of age). The inhibitor activity against ADAMTS13 was measured using heat-inactivated plasmas at 56°C for 30 minutes (Furlan et al., 1998; Tsai and Lian, 1998). One Bethesda's unit (BU) of the inhibitor was defined as the amount that reduces the ADAMTS13 activity to 50% of the control (Kasper et al., 1975), and its titer was estimated to be significant in more than 0.5 BU/ml.

Measurements of Cytokines

Plasma concentrations of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-8 were determined by Immunoassay Kits (BioSource International, Camarillo, CA).

Determination of Endotoxin

All blood specimens from 20 healthy controls (10 men and 10 women, 20 to 39 years old) and from patients with AH and SAH were obtained under aseptic conditions by peripheral venipuncture using pyrogen-free syringe and needles. The blood samples were mixed in pyrogen-free tubes with 1/10th volume of 3.8% sodium citrate as an anticoagulant, placed on ice, and transported immediately to the laboratory. Plasma was immediately separated in a refrigerated centrifuge at $3000 \times g$ at 4°C for 15 minutes, and stored at −20°C for subsequent analysis. Endotoxin activity was measured by a chromogenic substrate assay (Toxicolor LS–M Set, Seikagaku Kogyo Co., Tokyo, Japan) with kinetics analysis (Obayashi et al., 1985). In brief, 50 μl of plasma samples was mixed with 450 μl of 0.02% Triton X-100. The mixture was heated at 70°C for 10 minutes to inactivate the inhibitor reacted with endotoxin, and serial standard solution was made to final exogenous endotoxin concentration of 180, 90, 45, 22.5, 11.3, and 5.6 pg/ml. The absorbance was measured at 37°C every 15 second until 30 minutes by a microprocessor controlled reader (Wellreader, SK603; Seikagaku Co., Tokyo, Japan). Liner part of the kinetics curve was read and endogenous plasma endotoxin concentrations were calculated from the obtained standard curve. Determinations were done in duplicate, and the mean value was utilized.

Statistics

The differences between the paired and unpaired groups were analyzed using the Mann–Whitney U-test. Correlations were calculated with the Spearman rank test. Categorical data were analyzed using the chi-squared test (Fisher's exact test). The analysis was carried out using the statistical software Statview (version 5.0; SAS Institute, Cary, NC). The data are expressed as mean ± SD. A 2-tailed p-value less than 0.05 was considered significant.

RESULTS

Clinical Characteristics and Laboratory Values

The clinical data of patients with AH and SAH are shown in Table 1. The patients with SAH were younger than those with AH, and the gender was predominant in male both in patients with AH and SAH. Serum total bilirubin, lactate dehydogenase, white blood cell, and peripheral polymorphonuclear neutrophil (PMN) count were higher in patients with SAH than those with AH, whereas hemoglobin, platelet count, serum albumin, and prothrombin time were lower in patients with SAH than those in AH. Maddrey score of patients with SAH was 52 to 71 (mean: 60) on admission. Eleven of 24 patients with AH and all patients with SAH were complicated by liver cirrhosis (LC). All patients with AH survived, and 3 of 5 patients with SAH died of hepatic failure within 2 to 61 days. Three nonsurvivors with SAH showed hepatic encephalopathy of grade II to III, ascites, renal failure, pneumonia, and heart failure on admission, indicating the occurrence of multiorgan failure. One of them had DIC, but the others did not. Of the remaining 2 survivors with SAH, one was complicated by renal

failure and pneumonia, but not by hepatic encephalopathy, and the other had moderate ascites, but not multiorgan failure. All patients with SAH were treated with fresh frozen plasma (FFP) together with standard therapy. Of the 2 survivors, one completely recovered in 30 days and the other in 90 days. One of the 3 nonsurvivors was treated with hemodialysis because of acute renal failure, but finally died in 61 days. The other 2 was treated with prednisolone, but died within a week. In 3 nonsurvivors, plasma exchange was not performed because of systemic circulatory disturbance (Table 1).

Plasma ADAMTS13 Activity, VWF:Ag, and UL-VWFM

As previously reported (Matsuyama et al., 2007; Uemura et al., 2005b), the plasma ADAMTS13 activity on admission was significantly lower in patients with AH (61 ± 34%, $p < 0.001$) and SAH (24 ± 22%, $p < 0.001$) than in healthy subjects (102 ± 23%). The activity further decreased in patients with SAH as compared with those with AH ($p < 0.02$). The values of plasma VWF:Ag were higher in patients with AH (381 ± 207%, $p < 0.001$) and SAH (806 ± 326%, $p < 0.001$) than in healthy subjects (100 ± 53%), and it was higher in patients with SAH than those with AH ($p < 0.005$). The ratio of VWF:Ag to ADAMTS13 activity was higher in patients with AH (10.6 ± 11.6, $p < 0.001$) and SAH (102.2 ± 112.6, $p < 0.001$) than in healthy subjects (1.0 ± 0.4), and it was higher in patients with SAH than those with AH ($p < 0.005$). Plasma UL-VWFM was detected in 4 (80.0%) of 5 patients with SAH, and in 5 (17.9%) of 28 patients with AH, who had moderate deficiency of ADAMTS13 activity together with markedly high VWF values.

Plasma Cytokine Levels and Their Relationships to ADAMTS13 Activity, VWF:Ag, and UL-VWFM

Plasma IL-6 concentration on admission was significantly higher in patients with AH (25 ± 32 pg/ml, $p < 0.05$) and SAH (504 ± 681 pg/ml, $p < 0.01$) than in healthy subjects (< 7.8 pg/ml), and it was higher in patients with SAH ($p < 0.001$) compared with those with AH (Fig. 1*A*). Plasma concentration of IL-8 was significantly higher in patients with SAH (216 ± 304 pg/ml) than in healthy subjects (< 15.6 pg/ml, $p < 0.01$) and patients with AH (37 ± 77 pg/ml, $p < 0.05$), whereas it did not differ between patients with AH and healthy subjects (Fig. 1*B*). Plasma TNF-α concentration was higher in patients with SAH (29 ± 18 pg/ml) than those with AH (17 ± 6 pg/ml, $p < 0.005$) and healthy subjects (< 15.6 pg/ml, $p < 0.01$), although it did not differ between patients with AH and healthy subjects (Fig. 1*C*).

The ADAMTS13 activity on admission concomitantly decreased from the highest in patients with normal range of IL-6 (68 ± 31%) and IL-8 (70 ± 32%), to those with normal range to 100 pg/ml of IL-6 (37 ± 14%, $p < 0.02$) and IL-8 (37 ± 14%, $p < 0.02$), and to the lowest in those with more than 100 pg/ml of IL-6 (13 ± 10%, $p < 0.02$) and IL-8 (9 ± 7%, $p < 0.05$) (Fig. 2*A* and 2*B*). In addition, the

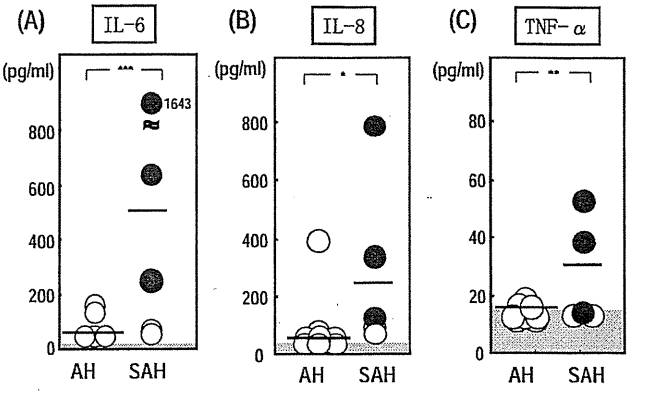

Fig. 1. Plasma levels of cytokines in the patients with alcoholic hepatitis (AH) and severe alcoholic hepatitis (SAH) on admission. The shaded area shows the normal range. The open circles indicate survivors and the closed circles indicate nonsurvivors. The concentrations of IL-6 (**A**), IL-8 (**B**), and TNFα (**C**) were significantly higher in the patients with SAH than those in AH. IL-6, interleukin 6; IL-8, interleukin 8; TNF-α, tumor necrosis factor-α; AH, alcoholic hepatitis; SAH, severe alcoholic hepatitis. $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$: significantly different from the 2 groups.

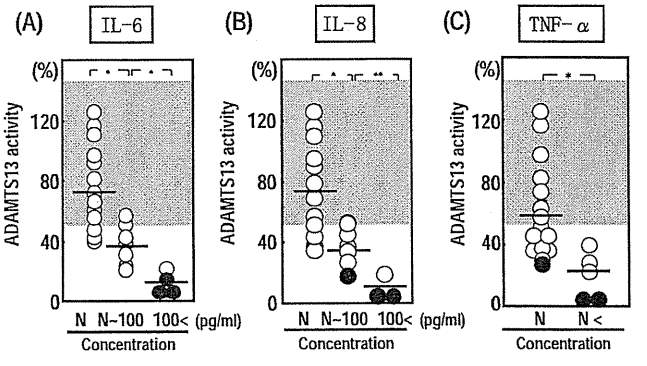

Fig. 2. Relationship between plasma cytokine levels and ADAMTS13 activity in the patients with alcoholic hepatitis and severe alcoholic hepatitis on admission. The shaded area shows the normal range. The open circles indicate survivors and the closed circles indicate nonsurvivors. The ADAMTS13 activity concomitantly decreased with increasing levels of plasma concentration of IL-6 (**A**) and IL-8 (**B**). In addition, the activity decreased in patients with higher TNF-α concentrations over normal range compared to those without (**C**). IL-6, interleukin 6; IL-8, interleukin 8; TNF-α, tumor necrosis factor-α; N, normal range. $^{*}p < 0.02$ and $^{**}p < 0.005$: significantly different from the 2 groups.

activity decreased in patients with higher TNF-α concentrations over normal range (22 ± 18%, $p < 0.02$) compared to those without (57 ± 31%) (Fig. 2*C*).

The VWF:Ag on admission progressively increased from the lowest in patients with normal range of IL-6 (298 ± 107%) and IL-8 (309 ± 107%), to those with normal range to 100 pg/ml of IL-6 (509 ± 232%, $p < 0.005$) and IL-8 (425 ± 190%, $p < 0.05$), and to the highest in those with more than 100 pg/ml of IL-6 (624 ± 394%, $p < 0.001$) and IL-8 (880 ± 354%, $p < 0.02$) (Fig 3*A* and 3*B*). In addition, the VWF:Ag increased in patients with higher TNF-α concentrations over normal range (609 ± 328%, $p < 0.02$) compared to those without (352 ± 178%) (Fig. 3*C*). The incidence of UL-VWFM was

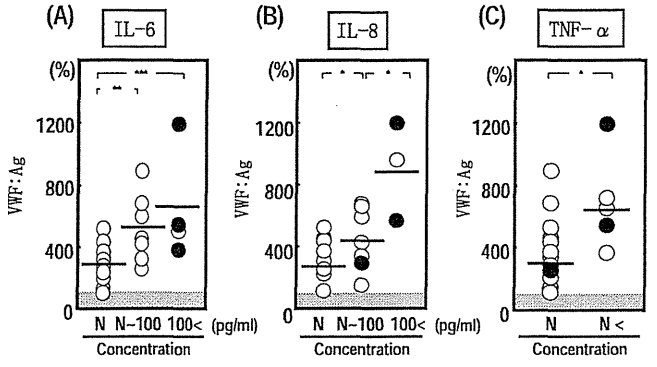

Fig. 3. Relationship between plasma levels of cytokines and VWF antigen (VWF:Ag) in the patients with alcoholic hepatitis and severe alcoholic hepatitis on admission. The shaded area shows the normal range. The open circles indicate survivors and the closed circles indicate nonsurvivors. The VWF:Ag concomitantly increased with increasing levels of plasma concentration of IL-6 (**A**) and IL-8 (**B**). In addition, the antigen increased in patients with higher TNF-α concentrations over normal range compared to those without (**C**). IL-6, interleukin 6; IL-8, interleukin 8; TNF-α, tumor necrosis factor-α; N, normal range. $*p < 0.05$, $**p < 0.005$, and $***p < 0.001$: significantly different from the 2 groups.

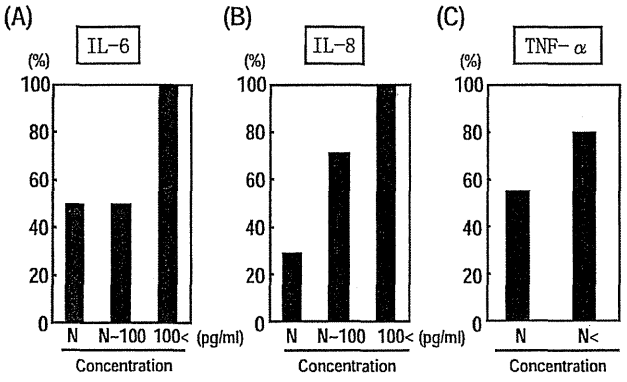

Fig. 4. Relationship between plasma levels of cytokines and the incidence of unusually large von Willebrand factor multimer (UL-VFWM) in the patients with alcoholic hepatitis and severe alcoholic hepatitis on admission. The incidence reached 100% in patients with higher concentration more than 100 pg/ml of IL-6 (**A**), and increased with increasing levels of plasma concentration of IL-8 (**B**). In addition, it increased in patients with higher TNF-α concentration over normal range than those without (**C**). IL-6, interleukin 6; IL-8, interleukin 8; TNF-α, tumor necrosis factor-α; N, normal range.

50% both in patients with normal range and normal range to 100 pg/ml of IL-6, and reached 100% in those with more than 100 pg/ml of IL-6 (Fig. 4*A*). The incidence concomitantly increased from the lowest in patients with normal range of IL-8 (30%), to those with normal range to 100 pg/ml of IL-8 (70%), and to the highest in those with more than 100 pg/ml of IL-8 (100%) (Fig. 4*B*). In addition, it tended to be higher in patients with higher TNF-α concentrations over normal range (80%) than those without (55%) (Fig. 4*C*).

Plasma Endotoxin Concentration and Their Relationships to ADAMTS13 Activity, VWF:Ag, and UL-VWFM

In normal healthy subjects, plasma endotoxin concentration was below 10 pg/ml, and averaged 7.9 ± 1.7 pg/ml.

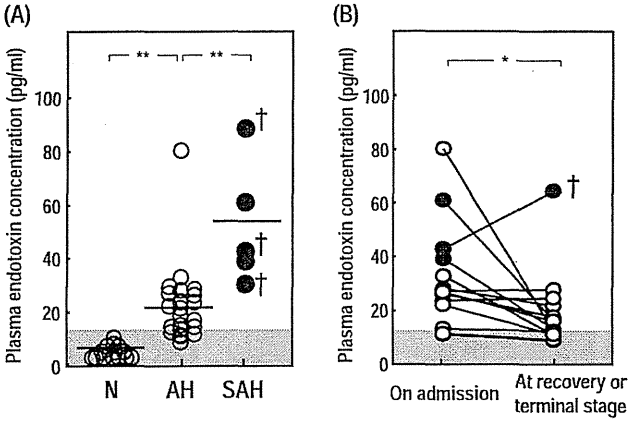

Fig. 5. Plasma endotoxin concentration in patients with alcoholic hepatitis (AH) and severe alcoholic hepatitis (SAH). The shaded area shows the normal range. The open circles indicate AH and the closed circles indicate SAH. The crosses indicate nonsurvivors. Plasma endotoxin concentration on admission was significantly higher in patients with AH and SAH than in normal subjects, and it was higher in patients with SAH compared to those with AH (**A**). The concentration on admission significantly decreased at the recovery phase in 8 patients with AH and 2 survivors with SAH, whereas a nonsurvivor with SAH showed further increase at the terminal stage (**B**). N, normal subjects; AH, alcoholic hepatitis; SAH, severe alcoholic hepatitis. $*p < 0.02$ and $**p < 0.001$: significantly different from the 2 groups.

The concentration on admission was significantly higher in patients with AH (21.7 ± 14.0 pg/ml, $p < 0.001$) and SAH (52.3 ± 23.1 pg/ml, $p < 0.001$) than in healthy subjects, and it was higher in patients with SAH ($p < 0.001$) as compared with those with AH (Fig. 5*A*). The concentration on admission significantly decreased at the recovery phase in 8 patients with AH and 2 survivors with SAH (31.0 ± 19.8 to 15.0 ± 6.0 pg/ml, $p < 0.02$), whereas a nonsurvivor with SAH showed further increase at the terminal stage (42.8 to 64.5 pg/ml) (Fig. 5*B*). The endotoxin concentration on admission inversely correlated with plasma ADAMTS13 activity ($r = -0.474$, $p < 0.01$) (Fig. 6), and was higher in patients with UL-VWFM than those without UL-VWFM (46.6 ± 24.0 vs. 18.5 ± 7.9 pg/ml, $p < 0.001$). In addition, plasma endotoxin concentration correlated positively with white blood cell count ($r = 0.486$, $p < 0.005$), PMN count ($r = 0.814$, $p < 0.001$), serum total bilirubin ($r = 0.493$, $p < 0.005$), blood urea nitrogen ($r = 0.677$, $p < 0.001$), and serum creatinine ($r = 0.749$, $p < 0.001$), and correlated inversely with hemoglobin ($r = -0.512$, $p < 0.005$) and prothrombin time ($r = -0.665$, $p < 0.001$).

Plasma Inhibitor Against ADAMTS13 and Its Relationship to ADAMTS13 Activity, VWF:Ag, Plasma Endotoxin Concentration, and Clinical Features

The plasma inhibitor against ADAMTS13 on admission was detected in 4 patients with SAH (80%, $p < 0.05$) and 6 patients with AH (21.4%). The inhibitory activity averaged 1.5 BU/ml (range 0.9 to 2.1 BU/ml) in SAH and 1.0 BU (0.5 to 1.6 BU/ml) in AH, respectively. Patients with the inhibitor showed lower ADAMTS13 activity (Fig. 7*A*),

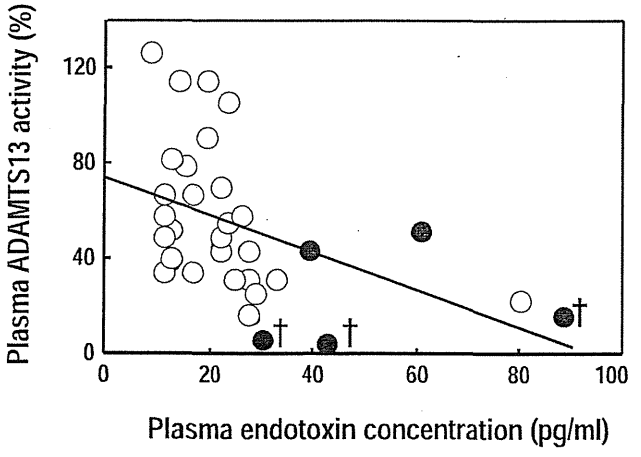

Fig. 6. Correlation between plasma endotoxin concentration and plasma ADAMTS13 activity in patients with alcoholic hepatitis (AH) and severe alcoholic hepatitis (SAH) on admission. The open circles indicate AH and the closed circles indicate SAH. The crosses indicate nonsurvivors. The endotoxin concentration inversely correlated with plasma ADAMTS13 activity ($r = -0.474$, $p < 0.01$).

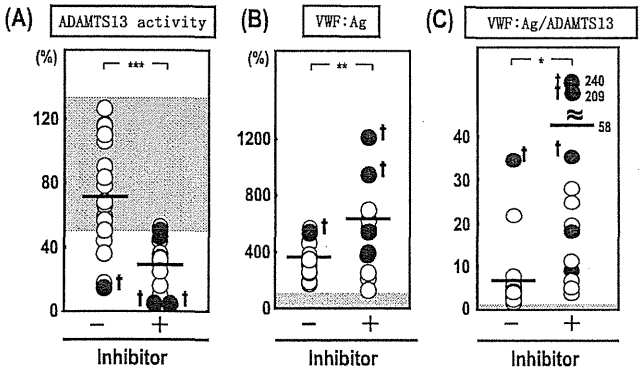

Fig. 7. Relationship of plasma inhibitor against ADAMTS13 to ADAMTS13 activity, VWF antigen (VWF:Ag), and the ratio of VWF:Ag to ADAMTS13 activity in patients with alcoholic hepatitis (AH) and severe alcoholic hepatitis (SAH) on admission. The shaded area shows the normal range. The open circles indicate AH and the closed circles indicate SAH. Crosses indicate nonsurvivors. Patients with the inhibitor showed lower ADAMTS13 activity (**A**), higher VWF:Ag (**B**), and higher ratio of VWF:Ag to ADAMTS13 activity (**C**) than those without. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$: significantly different from the 2 groups.

higher VWF:Ag (Fig. 7*B*), and higher ratio of VWF:Ag to ADAMTS13 activity (Fig. 7*C*) than those without (ADAMTS13 activity: 26 ± 15% vs. 68 ± 32%, $p < 0.001$; VWF:Ag: 609 ± 316% vs. 374 ± 199%, $p < 0.01$; the ratio of VWF:Ag to ADAMTS13 activity: 58.4 ± 88.2 vs. 7.3 ± 7.9, $p < 0.02$; respectively). In addition, patients with AH and SAH who had inhibitor showed lower serum albumin level and higher levels of serum total bilirubin, PMN count, C-reactive protein, and plasma endotoxin concentration than those with AH who had no inhibitor (Table 2).

Changes in Plasma ADAMTS13 Activity and Its Related Parameters During Hospitalization

At the recovery stage in survivors with AH and SAH, the ADAMTS13 activity significantly increased to normal range, the VWF:Ag decreased, and the UL-VWFM disappeared with the decrease in the concentrations of IL-6, IL-8, and endotoxin, and with the disappearance of the inhibitor against ADAMTS13 (Table 3). On the other hand, in a nonsurvivor with SAH, the activity of ADAMTS13 during FFP infusion showed transient increase but finally decreased, the VWF:Ag remained high, and the UL-VWFM was still present with the increase in the concentrations of IL-6, IL-8, TNF-α, and endotoxin, and the presence of the ADAMTS13 inhibitor at the terminal stage (Table 3).

DISCUSSION

In the present study, the ADAMTS13 activity gradually decreased, and the VWF:Ag progressively elevated with concomitant increase in concentrations of IL-6, IL-8, and TNF-α from normal range to over 100 g/ml, on admission (Figs. 2 and 3). The incidence of UL-VWFM detected in plasma became higher as concentrations of IL-6, IL-8, and TNF-α increased (Fig. 4).

At the recovery stage in survivors with AH and SAH, the ADAMTS13 activity significantly increased to normal range, the VWF:Ag decreased, and the UL-VWFM disappeared with the decrease in the concentration of IL-6 and IL-8, whereas in a nonsurvivor with SAH, the ADAMTS13 activity remained extremely in low levels, the VWF:Ag was still high, and the UL-VWFM was persistently present with the increase in concentrations of these cytokines (Table 3). These results indicate that the decrease in the ADAMTS13 activity and the increase in VWF:Ag in addition to UL-VWFM may be closely associated with increased proinflammatory cytokines including IL-6, IL-8, and TNF-α. It was, recently, demonstrated that IL-6 inhibited the action of ADAMTS13 under flow condition, and both IL-8 and TNF-α stimulated the release of UL-VWFM in a dose-dependent manner from human umbilical vein endothelial cells in vitro (Bernardo et al., 2004). Considering that high concentrations of proinflammatory cytokines such as IL-8 and TNFα are closely related to a poor outcome of AH (Fujimoto et al., 2000; Ishii et al., 1993; Mookerjee et al., 2003), enhanced cytokinemia may, in part, cause the decrease in the ADAMTS13 activity together with the increase in the VWF:Ag and UL-VWFM, finally resulting in the occurrence of multiorgan failure through microcirculatory disturbance in patients with SAH.

On the other hand, endotoxemia has been known to play an important role in the initiation and aggravation of AH through the enhancement of proinflammatory cytokines including IL-6, IL-8, and TNF-α (Fujimoto et al., 2000; Fukui et al., 1991; Ishii et al.,1993; Mookerjee et al., 2003). In our study, the concentrations of IL-6, IL-8, and TNF-α on

Table 2. Relationship of Presence or Absence of Plasma Inhibitor Against ADAMTS13 to Laboratory Findings and Plasma Endotoxin Concentration in Patients With Alcoholic Hepatitis

	Alcoholic hepatitis		Severe alcoholic hepatitis
Variable	Inhibitor (–) (n = 22)	Inhibitor (+) (n = 6)	Inhibitor (+) (n = 4)
Serum total bilirubin (mg/dl)	2.5 ± 4.0	11. 1 ± 10.0[b]	10.0 ± 2.7[b]
Polymorphonuclear neutrophil (/mm^3)	4063 ± 1750	8762 ± 3118[c]	7931 ± 4316[b]
C-reactive protein (mg/dl)	1.1 ± 2.1	4.6 ± 4.9[a]	4.3 ± 5.4[a]
Serum albumin (g/dl)	4.2 ± 1.1	3.3 ± 1.2[a]	3.1 ± 1.2[b]
Plasma endotoxin concentration (pg/ml)	17.3 ± 6.1	39.4 ± 23.0[b]	43. 3 ± 12.9[c]

[a]$p < 0.05$, [b]$p < 0.01$, and [c]$p < 0.001$ versus alcoholic hepatitis without inhibitor against ADAMTS13.

Table 3. *Changes in Plasma ADAMTS13 Activity and Its Related Parameters in Survivors and a Nonsurvivor in Patients With Alcoholic Hepatitis*

	Survivors (n = 10)		Nonsurvivors (n = I)		
Variables	On admission	Recovery state	On admission	During FFP infusion	Terminal stage
ADAMTS13 activity (%)	42 ± 14	72 ± 26[c]	4.5	12.0	4.5
VWF:Ag	533 ± 367	335 ± 241[a]	940	501	750
VWF:Ag/ADAMTS13	17.7 ± 19.5	5.6 ± 5.1[a]	209	42	167
UL-VWFM (positive/negative)	5/5	0/10[b]	1/0	1/0	1/0
Interleukin-6 (pg/ml)	21 ± 14	12 ± 7[a]	563	649	1756
Interleukin-8 (pg/ml)	28 ± 18	15 ± 13[b]	211	213	322
Tumor necrosis factor-α (pg/ml)	16.1 ± 1.8	<15.6	42	53	138
Plasma endotoxin concentration (pg/ml)	31.0 ± 19.8	15.0 ± 6.0[b]	42.8	55.2	64.5
Inhibitor against ADAMTS13 (positive/negative)	7/3	0/10[c]	1/0	1/0	1/0

VWF:Ag, von Willebrand factor; UL-VWFM, unusually large von Willebrand factor; FFP, fresh frozen plasma.
[a]$p < 0.05$, [b]$p < 0.02$, and [c]$p < 0.005$ versus on admission.

admission were significantly higher in patients with SAH than in those with AH and controls (Fig. 1). Plasma endotoxin concentration was higher in patients with SAH and AH than in healthy subjects, and was markedly higher in patients with SAH than in AH (Fig. 5*A*). The endotoxin concentration determined by the chromogenic substrate assay after pretreatment with detergent, Triton X-100, and heating at 70°C for 10 min was consistent with that described by the previous report (Fukui et al., 1991, Lumsden et al., 1988; Obayashi, 1984; Obayashi et al., 1985). The endotoxin concentration on admission inversely correlated with ADAMTS13 activity (Fig. 6), and was higher in patients with UL-VWFM than those without. At the recovery stage, the endotoxin concentration significantly decreased with increased ADAMTS13 activity and decreased VWF:Ag, and the disappearance of UL-VWFM together with the reduction of IL-6 and IL-8 concentrations (Table 3). These results indicate that enhanced endotoxemia may be closely related to the decrease in the ADAMTS13 activity and the appearance of UL-VWFM through the enhanced cytokinemia. This is the first report to demonstrate a potential linkage of endotoxemia to enhanced inflammatory cytokines and the imbalance of increased VWF:Ag over decreased activity of ADAMTS13 leading to systemic microcirculatory disturbance especially in patients with SAH. Recent study demonstrated that inflammation-associated ADAMTS13 deficiency promotes formation of UL-VWFM (Bockmeyer et al., 2008), and that severe secondary ADAMTS13 deficiency can be associated with sepsis-induced DIC and may contribute to the development of renal failure (Ono et al., 2006), which may support our data and hypothesis.

Alternatively, another mechanism to reduce the activity of ADAMTS13 is the presence of plasma inhibitor against ADAMTS13. In our study, the inhibitor on admission was detected in 80% in patients with SAH and 21.4% in patients with AH, and its inhibitory activity averaged 1.5 BU/ml in SAH and 1.0 BU/ml in AH. Patients with the inhibitor showed lower ADAMTS13 activity and higher VWF:Ag than those without (Fig. 7). At the recovery stage, the inhibitor detected in 5 patients disappeared with increased ADAMTS13 activity and decreased VWF:Ag, together with the decrease in concentrations of cytokines and endotoxin (Table 3). Interestingly, patients with AH in addition to SAH who had inhibitor showed higher levels of serum total bilirubin, PMN count, C-reactive protein, and plasma endotoxin concentration, and lower serum albumin level than those with AH who had no inhibitor (Table 2). These results indicate that the decrease in the ADAMTS13 activity may be caused by the presence of its inhibitor, which is closely related to lower functional liver capacity, marked inflammation, and enhanced endotoxemia in patients with AH and SAH. It was recently reported that the intravenous infusion of endotoxin to healthy volunteers brought the decrease in plasma ADAMTS13

activity together with the increase in VWF:Ag and the appearance of UL-VWFM during acute systemic inflammation (Reiter et al., 2005). From our results and the previous finding (Reiter et al., 2005), endotoxemia itself might be a candidate to reduce the plasma activity of ADAMTS13 together with inflammatory cytokines in patients with AH. It will be, then, necessary to clarify what kinds of the inhibitor would be involved in the association with inflammatory cytokines and endotoxin. We, recently, encountered 2 patient who developed TTP; one occurred in the course of hepatitis C virus (HCV)-related advanced liver cirrhosis (Yagita et al., 2005) and another did in a month after pegylated-interferon alpha-2a therapy in a HCV-related chronic hepatitis (Kitano et al., 2006). In both of them, plasma ADAMTS13 activity was extremely low, and the inhibitor against ADAMTS13 was detected in the patient's heated plasma (2.0 and 1.6 BU/ml, respectively) and purified IgG (0.19 and 0.4 BU/mg IgG, respectively). Furthermore, we could detect IgG-inhibitor by western blot in 4 patients with advanced liver cirrhosis, who showed extremely lower ADAMTS13 activity (<3% of controls), but had no apparent clinical features of TTP (Uemura et al., 2008). Of 108 patients with idiopathic TTP whose plasma samples were sent to our department of Blood Transfusion Medicine across Japan, the inhibitor was detected in 54 (79.4%) of 68 patients analyzed, and its inhibitor activity was 0.5 to 2.0 BU/ml in 33 cases (61.1%), and more than 2.0 BU/ml in remaining 21 cases (38.9%) (Matsumoto et al., 2004). Taken these considerations together, the inhibitor activity detected in our patients with SAH and AH would be enough to reduce the activity of plasma ADAMTS13.

As for the relationship of the treatment to ADAMTS13 activity and outcome, all AH patients treated with supportive care including nutritional supplementation survived with the increase in the ADAMTS13 activity. All 5 patients with SAH were treated with FFP infusion together with supportive care, and 2 of them survived, but remaining 3 did not. One of the nonsurvivors showed transient increase in ADAMTS13 activity during FFP infusion, but finally decreased, and the other 2 died of hepatic failure in spite of the administration of prednisolone within a week. The administration of FFP might be, in part, useful as the supplementation of ADAMTS13, but the effect might depend on the severity of liver disturbance and the degree of multiorgan failure prior to the administration.

In conclusion, decreased ADAMTS13 activity and increased VWF:Ag could be induced not only by enhanced cytokinemia, but also by its inhibitor, both of which are closely related to enhanced endotoxemia in patients with AH and SAH. The cytokinemia and the presence of inhibitor may cause the imbalance of the enzyme to substrate, resulting in multiorgan failure especially in patients with SAH. These results will raise the possibility of novel supportive therapies for patients with AH, such as ADAMTS13 supplementation or anti-inflammatory cytokine agents.

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CONFLICTS OF INTEREST STATEMENT

The authors have declared no conflicts of interest. [Correction added after online publication 16 December 2008: Conflicts of Interest Statement added.]

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Pivotal role of ADAMTS13 function in liver diseases

Masahito Uemura · Yoshihiro Fujimura · Saiho Ko · Masanori Matsumoto · Yoshiyuki Nakajima · Hiroshi Fukui

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Abstract The liver is a major source of clotting and fibrinolytic proteins, and plays a central role in thromboregulation. Patients with advanced liver diseases tend to bleed because of reduced plasma levels of several clotting factors and thrombocytopenia, but they do also exhibit thrombotic complications. ADAMTS13 is a metalloproteinase, produced exclusively in hepatic stellate cells, and specifically cleaves highly multimeric von Willebrand factor (VWF). VWF plays a pivotal role in hemostasis and thrombosis, and its function is dependent on its multimeric state. Deficiency of ADAMTS13 results in accumulation of unusually large VWF multimers (UL-VWFM) in plasma, in turn induces platelet clumping or thrombi under high shear stress, followed by microcirculatory disturbances. Considering that UL-VWFM, the substrate of ADAMTS13, is produced in transformed vascular endothelial cells at sites of liver injury, decreased ADAMTS13 activity may be involved in not only sinusoidal microcirculatory disturbances, but also subsequent progression of liver injuries, eventually leading to multiorgan failure. This concept can be applied to the development or aggravation of liver diseases, including liver cirrhosis, alcoholic hepatitis, veno-occlusive disease, and adverse events after liver transplantation. These results promise to bring further understanding of the pathophysiology of liver diseases, and offer new insight for development of therapeutic strategies.

Keywords ADAMTS13 · Von Willebrand factor · Liver cirrhosis · Alcoholic hepatitis · Veno-occlusive disease · Liver transplantation · Microcirculatory disturbance · Multiorgan failure

1 Introduction

The liver plays a central role in hemostasis by synthesizing clotting factors, coagulation inhibitors, and fibrinolytic proteins [1]. The hemostatic system is normally in a delicate balance between pro-hemostatic and anti-hemostatic processes [1]. Severe liver diseases are accompanied by multiple changes in the hemostatic system, and the alterations in the system may lead to either a bleeding or thrombosis [1, 2]. Bleeding is clinically evident but hypercoagulability is also an important role in many aspects including poor hepatic blood flow, vasculopathy, and portal and hepatic vein thrombosis, which are closely related to microcirculatory disturbance [2]. Deficiency of anticoagulant proteins and high levels of several procoagulant factors may favor hypercoagulability [2], but the mechanisms underlying this disorder have not been fully elucidated.

ADAMTS13 (*a* *d*isintegrin-like *a*nd *m*etalloproteinase with *t*hrombo*s*pondin type-1 motifs *13*) is a metalloproteinase that specifically cleaves multimeric von Willebrand factor (VWF) between Tyr1605 and Met1606 within its A2 domain [3, 4]. ADAMTS13 deficiency, caused either by mutations in the *ADAMTS13* gene [3–6] or by inhibitory

M. Uemura and Y. Fujimura contributed equally to this study.

M. Uemura (✉) · H. Fukui
Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan
e-mail: muemura@naramed-u.ac.jp

Y. Fujimura · M. Matsumoto
Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Nara 634-8522, Japan

S. Ko · Y. Nakajima
Department of Surgery, Nara Medical University, Kashihara, Nara 634-8522, Japan

autoantibodies against ADAMTS13 [7, 8], results in the accumulation of "unusually large" VWF multimers (UL-VWFM) in plasma; this, in turn, leads to platelet clumping and/or thrombi under high shear stress and subsequent microcirculatory disturbances.

In 2000, we reported that predominantly decreased ADAMTS13 activity (ADAMTS13:AC) in sick children with advanced cirrhotic biliary atresia could be fully restored after living donor liver transplantation, indicating that the liver is the major organ producing ADAMTS13 [9]. In 2001, three other groups indicated that ADAMTS13 mRNA was exclusively expressed in the liver by northern blot analysis [5, 10, 11]. Subsequently, we were able to demonstrate that ADAMTS13 was produced exclusively in hepatic stellate cells (HSCs) using both in situ hybridization and immunohistochemistry [12]. Platelets [13], vascular endothelial cells [14], and kidney podocytes [15] were also shown to be ADAMTS13-producing cells, but the relevance to the pathogenesis of thrombo-regulation in each organ remained unclear.

Since HSCs are the major ADAMTS13-producing cells in human liver [12], we will review the potential functional role of ADAMTS13 in association with the pathogenesis of liver diseases.

2 Hepatic microcirculation and hypercoagulability in liver diseases

Hepatic microcirculation compromises a unique system of capillaries, called sinusoids, which are lined by three different cell types: sinusoidal endothelial cells (SEC), HSC, and Kupffer cells [16]. The SEC modulates microcirculation between hepatocytes and the sinusoidal space through the sinusoidal endothelial fenestration. The SEC has tremendous endocytic capacity, including for VWF and the extracellular matrix, and secretes many vasoactive substances [16]. The HSC is located in the space of Disse adjacent to the SEC, and regulates sinusoidal blood flow by contraction or relaxation induced by vasoactive substances [17]. Kupffer cells are intrasinusoidally located in tissue macrophages, and secrete potent inflammatory mediators during the early phase of liver inflammation [16]. Intimate cell to cell interaction has been found between these sinusoidal cells and hepatocytes [16, 17].

Vascular endothelial cells play a pivotal role in hemostasis and thrombosis [3, 4]. VWF is a marker of endothelial cell activation (damage), and plays an essential role in hemostasis [3, 4]. In the normal state, VWF immunostaining is usually positive in large vessels, but negative in the SEC [18]. On the occurrence of liver injury accompanied by a necroinflammatory process, the SEC becomes positive for VWF, presumably in association with the capillarization of hepatic sinusoids [19]. Subsequently, platelets adhere to subendothelial tissue mediated by UL-VWFM [3, 4]. ADAMTS13 then cleaves UL-VWFM into smaller VWF multimers [3, 4]. This interaction of ADAMTS13 and UL-VWFM is, indeed, the initial step in hemostasis [3, 4]. Recent work has further shown that recombinant ADAMTS13 binds to recombinant CD36 and platelet membrane CD36 in vitro, demonstrating a role for this protein in localizing ADAMTS13 to endothelial cells expressing CD36, where ADAMTS13 regulates the cleavage of VWF [20].

In patients with fulminant hepatic failure and liver cirrhosis, circulating plasma VWF antigen (VWF:AG) levels are extremely high [21–23]. Many fibrin thrombi were found in the hepatic sinusoids in acute liver failure, suggesting a role for intravascular coagulation in the pathogenesis of hepatic necrosis [24]. In cirrhotic liver tissue [25] and even tissue from patients in early stages of alcoholic liver diseases [26], VWF immunostaining shows positive cells predominantly at the scar–parenchyma interface, within the septum, and in the sinusoidal lining. Portal or hepatic vein thrombosis is often observed in advanced cirrhosis [27, 28] and microthrombi formation was found in one or multiple organs in half of autopsied cirrhotics [29]. This hypercoagulable state in liver diseases may be involved in hepatic parenchymal extinction, the acceleration of liver fibrosis, and disease progression.

Considering that ADAMTS13 is synthesized in HSC [12] and its substrate, UL-VWFM, is produced in transformed SEC during liver injury [18], decreased plasma ADAMTS13:AC may involve not only sinusoidal microcirculatory disturbances, but also subsequent progression of liver diseases, eventually leading to multiorgan failure. Based on these findings, it is of particular interest to evaluate plasma ADAMTS13:AC in liver disease patients.

3 ADAMTS13 assays

The classic VWF multimer assay used to be the gold standard method for evaluating plasma ADAMTS13:AC; however, its major disadvantage was that it took several days to provide results [7]. In this regard, the discovery of a minimum 73 amino acid residue sequence within the VWF-A2 domain (VWF73) by Kokame et al. [30], which was prerequisite for the rapid cleavage by ADAMTS13, provided a breakthrough in developing novel methods to assay ADAMTS13:AC. Indeed, a convenient fluorescence method based on FRET-VWF73 is now widely used as the gold standard second generation method [31]. However, the sensitivity of FRET-VWF73 remains approximately 3% of the normal control, and the presence of hemoglobin, bilirubin, and/or chylomicron in samples significantly

influences the results [32]. To solve these problems, a unique method for determining ADAMTS13:AC, termed ADAMTS13-act-ELISA, was developed in our laboratory as a third generation method [33]. This assay was established after production of a novel murine monoclonal antibody to ADAMTS13, termed N-10, which specifically recognizes the Y1605 residue of the VWF-A2 domain, generated by ADAMTS13 cleavage [33]. The lower limit of this assay is 0.5% of the normal control. Developing an automated more rapid assay for ADAMTS13:AC and its usage in hospitals is urged to prevent unnecessary or harmful infusions of platelet concentrates to patients with masked thrombocytopenia, such as "subclinical TTP".

4 The physiological significance of ADAMTS13 in liver diseases

4.1 Liver cirrhosis

Sinusoidal microcirculatory disturbance in liver cirrhosis occurs when the normal hepatic structure is disrupted by fibrin deposition [19] or by impaired balance between the action of vasoconstrictors and vasodilators in hepatic vascular circulation [16]. Studies have shown that cirrhotic liver exhibits a hyperresponse to vasoconstrictors, including catecholamine, endothelin, and leukotrienes D_4 [16]. Now it is well-accepted that thrombocytopenia gradually progresses as functional liver capacity decreases (Fig. 1a). Previously, thrombocytopenia in liver cirrhosis has been speculated to be associated with hypersplenism [34] and decreased synthesis of thrombopoietin in the affected liver [35]. Our recent studies, however, have provided evidence considering that UL-VWFM accumulated in plasmas with far advanced cirrhotic patients enhances high shear-stress-induced platelet aggregation, resulting in thrombocytopenia [36].

Mannucci et al. [37] originally reported a significant reduction of plasma ADAMTS13:AC in advanced cirrhotics. Recently, we showed that ADAMTS13:AC decreased with increasing severity of cirrhosis [36] (Fig. 1b). The values determined by act-ELISA correlated well with those of the classical VWFM assay, and also closely correlated with ADAMTS13 antigen determined by the antigen-ELISA. These results confirmed that both ADAMTS13 activity and antigen decreased with increasing cirrhosis severity [36] (Fig. 1b, c). Our results are consistent with findings described by Feys et al. [38]. In sharp contrast, Lisman et al. [39] showed that both ADAMTS13 activity and antigen levels were highly variable; however, they did not distinguish between patients with varying degrees of cirrhosis. It is unclear why Lisman et al. reached the conclusions different from ours. One possible explanation relates to two distinct clinical settings: a majority of our

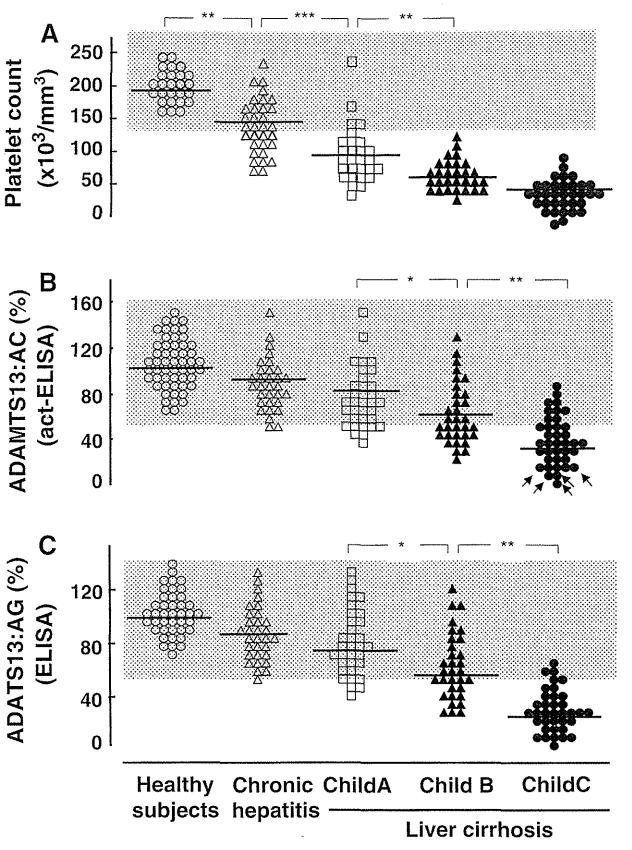

Fig. 1 Platelet counts and plasma levels of ADAMTS13:AC and ADAMTS13:AG in patients with chronic liver diseases. Platelet count decreased with the severity of chronic liver diseases, but no difference was found between Child B and C (**a**). Plasma ADAMTS13:AC determined by the ELISA progressively decreased with worsening cirrhosis (**b**). Severe deficiency in ADAMTS13:AC (<3%) was seen in five liver cirrhosis patients with Child C by the VWFM assay, but by the act-ELISA they ranged from <0.5 to 15.9% of the normal control (**b**, shown by *arrows*). The ADAMTS13:AG levels determined by ELISA also decreased with increasing cirrhosis severity (**c**), which highly correlated with ADAMTS13:AC measured by the act-ELISA ($r = 0.715$, $p < 0.001$). *Open circles* normal controls, *open triangles* chronic hepatitis, *open squares* cirrhosis with Child A, *closed triangles* cirrhosis with Child B, *closed circles* cirrhosis with Child C. *Shaded area* shows normal range. *ADAMTS13:AC* ADAMTS13 activity, *ADAMTS13:AG* ADAMTS13 antigen. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ significantly different between the two groups (partially modified from [36])

patients developed cirrhosis secondary to HCV infection, whereas in the study of Lisman et al. a half of the patients suffered from alcohol abuse-related cirrhosis. Further, the techniques used to determine ADAMTS13:AC differed between our study and theirs. It is assumed that the collagen-binding assay they used can be highly influenced by the increased amount of VWF:Ag in tested cirrhotic plasmas [38], because the substrate in this assay is intact multimeric VWF. In this regard, our act-ELISA is performed using VWF73-based fusion protein, termed GST-VWF73-His, which is readily cleaved by ADAMTS13

without any protein denaturant, and therefore the increased amount of VWF:Ag in tested plasmas does not interfere the assays [36].

Obviously, plasma levels of VWF:Ag substantially increase as liver diseases progress (Fig. 2a) [36], as previously indicated [22, 23]. This is presumably attributed to sinusoidal and/or extrahepatic endothelial damage induced by endotoxin and cytokines [22, 23, 40, 41]. The VWF:RCo was higher (Fig. 2b) [36], but the ratio of VWF:RCo/VWF:Ag was lower in cirrhotic patients than in healthy subjects, suggesting that increased VWF:Ag appears less functional in cirrhosis patients [39]. Nevertheless, our study has clearly shown that the ratio of VWF:RCo/ADAMTS13:AC progressively increases with the worsening of chronic liver diseases (Fig. 2c), more strengthening an enhanced thrombogenesis with the progresses of liver dysfunction and thrombocytopenia [36]. As a part of reflection in our scenario, the decreased platelet counts paralleled to the plasma levels of ADAMTS13:AC [36].

Regarding VWF multimers, the higher molecular weight multimer showed greater degradation than in healthy controls, thus maintaining normal enzyme-to-substrate (ADAMTS13/UL-VWFMs) ratio to maintain blood fluidity [39]. Our recent study showed that there were three different VWFM patterns in cirrhotic patients with lower ADAMTS13:AC (<50% of controls): normal-VWFM was detected in 53%, degraded-VWFM in 31%, and UL-VWFM in 16% (Fig. 3) [36]. UL-VWFM-positive patients showed the lowest ADAMTS13:AC, and the highest values of serum creatinine, blood urea nitrogen, and blood ammonia. In addition, cirrhotic patients with UL- and normal-VWFM had higher levels of VWF:RCo and Child-Pugh score, and lower values of cholinesterase and hemoglobin than those with degraded-VWFM [36]. The pattern, therefore, appears to shift from degraded- to normal-VWFM, and finally to UL-VWFM as functional liver capacity and renal function deteriorate, indicating that advanced cirrhosis may be a predisposing state toward platelet microthrombi formation, even in the absence of clinically overt thrombotic events [36]. In fact, portal or hepatic vein thrombosis is often observed in advanced liver cirrhosis patients routinely screened with Doppler ultrasound [27] and in cirrhotic liver tissue removed at transplantation [28] and at autopsy [29], consistent with our hypothesis.

The mechanism responsible for the decrease in ADAMTS13:AC in advanced cirrhotics may include enhanced consumption due to the degradation of large quantities of VWF:AG [37], inflammatory cytokines [42, 43], and/or ADAMTS13 plasma inhibitor [7, 8]. It is controversial whether ADAMTS13 deficiency is caused by decreased production in the liver; Kume et al. [44] reported that HSC apoptosis plays an essential role in decreased

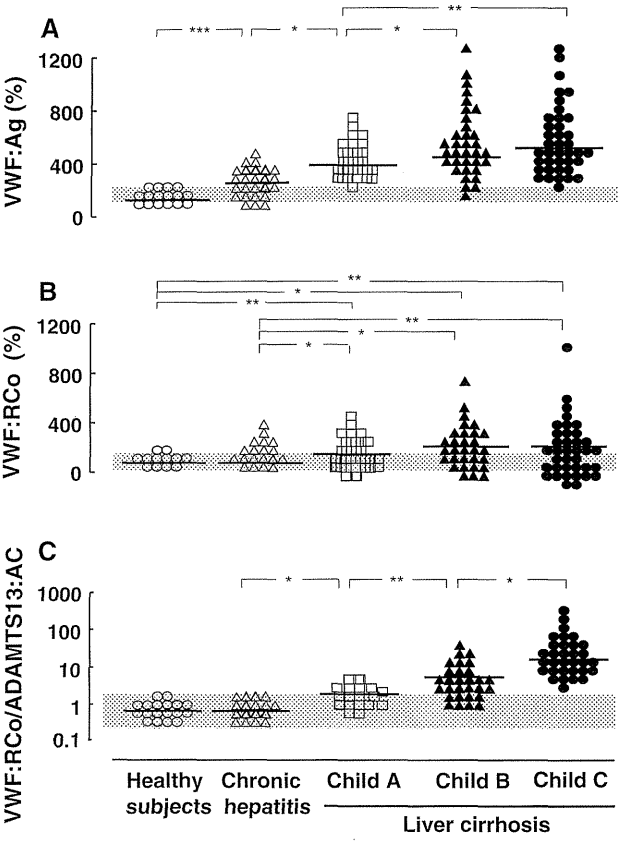

Fig. 2 Plasma levels of VWF:Ag, VWF:RCo, and VWF:RCo/ADAMTS13:AC ratio in patients with chronic liver disease. The VWF:Ag increased with the progression of chronic liver diseases, but the difference between Child B and C did not reach statistical significance (**a**). The VWF:RCo is higher in liver cirrhosis patients than in patients with chronic hepatitis and healthy subjects, but it did not differ among subgroups within liver cirrhosis (**b**). The VWF:RCo relative to ADAMTS13:AC progressively increased with worsening chronic liver disease (**c**). *VWF:Ag* von Willebrand factor antigen, *VWF:RCo* von Willebrand factor ristocetin cofactor activity, *ADAMTS13:AC* ADAMTS13 activity. *Shaded area* shows normal range. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ significantly different between the two groups (partially modified from [36])

ADAMTS13:AC using dimethylnitrosamine-treated rats, but not carbon tetrachloride (CCl_4)-treated animals, whereas Niiya et al. [45] found up-regulation of ADAMTS13 antigen and proteolytic activity in liver tissue using rats with CCl_4-induced liver fibrosis. We observed the inhibitor of ADAMTS13 in 83% of patients with severe to moderate ADAMTS13 deficiency, but its inhibitory activity was in a marginal zone between 0.5 and 1.0 BU/ml in most cases except a TTP patient (2.0 BU/ml) and a patient with severe ADAMTS13 deficiency (3.0 BU/ml) [36]. Interestingly, IgG-type autoantibodies specific to purified plasma derived-ADAMTS13 were detected by western blotting only in five end-stage cirrhotics with severe ADAMTS13 deficiency (<3%) corresponding to TTP [36]. One patient showed an apparent TTP [46], while the other

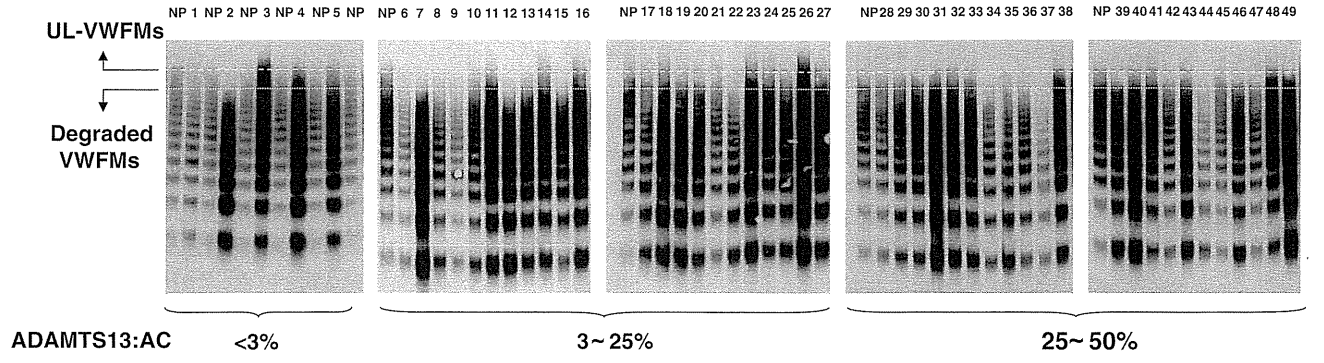

Fig. 3 Plasma VWF multimer in 49 liver cirrhosis patients with severe to mild deficiency of ADAMTS13:AC. The VWF multimer was analyzed by a vertical SDS–1.0% agarose gel electrophoresis system. Five patients (patients no. 1–5) were originally identified as a severe deficiency of plasma ADAMTS13:AC by the von Willebrand factor multimer (VWFM) assay. Twenty-two patients (patients no. 6–27) showed a moderate deficiency (3–25% of the control), and remaining 22 patients (no. 28–49) mild deficiency (25–50% of the control) of plasma ADAMTS13:AC by both methods of VWFM assay and the act-ELISA, without discordant results. There were three different patterns including degraded-, normal-, and UL-VWFM. Out of these 49 patients, 26 (53.1%) showed normal VWFMs, 15 (30.6%) degraded-VWFMs, and the remaining eight (patients no. 3, 4, 11, 14, 16, 18, 23, and 26) (16.3%) UL-VWFMs. *ADAMTS13:AC* ADAMTS13 activity, *VWFM* von Willebrand factor multimer, *UL-VWFM* unusually large von Willebrand factor multimer, *NP* normal control plasma (partially modified from [36])

four cirrhotics did not show apparent clinical features of TTP, but had complications of hepatorenal syndrome (HRS), spontaneous bacterial peritonitis (SBP), marked inflammation together with cytokinemia, and advanced hepatocellular carcinoma (HCC) [36]. Various clinical conditions, including infection, malignancies, and certain drugs, can lead to acquired TTP [47]. In advanced cirrhotics, endotoxemia is frequently detected [23], and SBP sometimes occurs [48]. HCC is highly complicated as the cirrhotic stage progresses [49], suggesting a high-risk state of platelet microthrombi formation. Some end-stage cirrhotics who have extremely low ADAMTS13:AC as well as its IgG inhibitor might be under conditions similar to TTP, or might reflect "subclinical TTP" [36].

With respect to the autoantibodies in patients with HCV-associated liver diseases, there is a general consensus that the overall prevalence of serum non-organ-specific autoantibodies is significantly higher in patients with HCV (about one-third of all cases) than in both healthy subjects and patients with HBV [50–52], but not alcoholic liver injury. That might be additional reason why ADAMTS13:AC significantly decreased in our most patients with HCV-related cirrhosis, but its activity seemed to be highly variable in most patients with alcohol abuse-related cirrhosis as shown by Lisman et al. [39]. Indeed, of our five end-stage LC patients with IgG-type autoantibodies, two were related to HCV, and each one to HBV, PBC and cryptogenic, but none of patients with alcohol abuse-related cirrhosis were found. Further studies will be necessary to clarify whether inhibitors other than the IgG inhibitor might be involved in cirrhotics with lower ADAMTS13:AC.

4.2 Alcoholic hepatitis (AH)

In alcoholic liver diseases, sinusoidal microcirculatory disturbance is thought to play an important pathogenic role [53, 54]. This includes narrowing of the sinusoidal space due to ballooned hepatocytes and perisinusoidal fibrosis, imbalances between endothelin and nitric oxide, and contraction of HSC [53, 54]. AH is a potentially life-threatening complication of alcohol abuse. The severe form of AH, severe alcoholic hepatitis (SAH), is characterized by multiorgan failure with manifestations of acute hepatic failure [55, 56]. In the pathogenesis of SAH, endotoxemia due to hepatic reticuloendothelial dysfunction and increased intestinal permeability may trigger enhanced proinflammatory cytokine production, which potentially causes systemic inflammatory response syndrome together with microcirculatory disturbances, and subsequent multiorgan failure [55, 56].

In our study, plasma ADAMTS13:AC was markedly decreased in the non-survivors of SAH with multiorgan failure; in contrast, mild to moderate decrease was observed in survivors of SAH and those with AH [57]. The VWF:AG was remarkably high in the non-survivors of SAH [58]. At the recovery stage, ADAMTS13:AC returned to the normal range, and the VWF:AG decreased in the survivors, whereas in a non-survivor with SAH, ADAMTS13:AC remained extremely low, and the VWF:AG was still high [57, 58]. UL-VWFM was detected in four of five SAH patients and in five of nine AH patients [58]. The findings of enhanced UL-VWFM production and deficient ADAMTS13:AC may, in part, contribute not only to the development of multiorgan failure but also to the

progression of liver injury through microcirculatory disturbances [57, 58].

Potential mechanism for decreased ADAMTS13:AC may include cytokinemia [42, 43, 59], endotoxemia [59, 60], the inhibitor of ADAMTS13 [7, 8, 59], and the consumption of the protease [37]. Recent investigations demonstrated that IL-6 inhibited the action of ADAMTS13 under flow conditions, and both IL-8 and TNF-α stimulated the release of UL-VWFM in human umbilical vein endothelial cells in vitro [42]. It remains to be clarified whether the IL-6 directly would hamper the cleavage of UL-VWFM or IL-6 would down-regulate gene expression of ADAMTS13 with modifying the promoter activity. IFN-γ, IL-4, and TNF-α also inhibit ADAMTS13 synthesis and activity in rat primary HSC [43]. In addition, inflammation-associated ADAMTS13 deficiency promotes formation of UL-VWFM [61], and intravenous infusion of endotoxin to healthy volunteers caused a decrease in plasma ADAMTS13:AC together with the appearance of UL-VWFM [60]. From these results as well as our own, marked endotoxemia may be closely related to decreased ADAMTS13:AC and the appearance of UL-VWFM through enhanced cytokinemia in AH patients [59]. It will be necessary to clarify what types of inhibitor may be involved in the association with inflammatory cytokines and endotoxin.

4.3 Hepatic veno-occlusive disease (VOD)

Hepatic VOD is a life-threatening complication of patients undergoing allogenic stem cell transplantation (SCT), and occurs at frequencies of 1–54% [62, 63]. Clinically, hepatic VOD is characterized by hyperbilirubinemia, painful hepatomegaly, and fluid retention [63]. Histologically, VOD features sinusoidal fibrosis, necrosis of pericentral hepatocytes, and consequent narrowing of central veins [62, 63]. In these patients, the SEC is the primary site of toxic injury caused by chemotherapy and/or radiation in the setting of SCT, and this initial insult may ultimately lead to the circulatory compromise of centrilobular hepatocytes [62, 63].

Our recent study demonstrated that plasma ADAMTS13:AC is reduced in hepatic VOD patients after SCT (12–32% of normal) compared to non-VOD patients (57–78% of normal), even before any conditioning regimen and throughout SCT, and that the activity might thus be a predictor for the development of hepatic VOD [64]. A multicenter, prospective, randomized controlled study revealed that prophylactic fresh frozen plasma (FFP) infusion as a source of ADAMTS13 may be instrumental in preventing the development of hepatic VOD after SCT [65]. In two typical cases with hepatic VOD, plasma levels of VWF:AG progressively increased and ADAMTS13:AC gradually decreased from preconditioning or the early period after the SCT to the later period at the occurrence of hepatic VOD [65].

Interestingly, in VOD patients, VWFM corresponding to high and intermediate molecular weight, which is usually seen in normal plasma, were lacking at preconditioning or the early period after SCT, and thereafter gradually appeared [65]. Furthermore, in the group without prophylactic FFP infusion, high and/or intermediate molecular weight VWFM was also lacking in the early stage and even in the later stage after SCT. In contrast, in the group with FFP infusion, no apparent changes in VWFM patterns were found throughout SCT [65]. It remains unclear why such a phenomenon occurred, but one possible explanation may be the SEC injury caused by intensive chemotherapy and/or total body irradiation in the setting of SCT. Indeed, chemotherapy before SCT is a regimen with a high incidence of hepatic VOD, and total body irradiation causes radiation-induced liver disease [62, 63]. The amount of VWF released from injured SEC may be increased at first, but may thereafter decrease because the endothelial cells are extensively damaged [65]. After SCT, as damaged endothelial cells gradually regenerate, the release of VWF may increase, resulting in the appearance of high and intermediate VWFM. Under these circumstances, plasma ADAMTS13 may be consumed to degrade the large amounts of VWF. The imbalance caused by decreased ADAMTS13:AC versus increased production of VWF:AG before and during the early stage after SCT would contribute to a microcirculatory disturbance that could ultimately lead to VOD, especially in zone 3 of the hepatic lobule where hepatocytes are susceptible to damage induced by hypoxia [65]. The supplementation of ADAMTS13 by prophylactic FFP infusion may suppress the increase in VWF:AG that is extensively released from damaged SEC.

4.4 Liver transplantation

One of the serious complications in solid organ transplantation is the occurrence of sporadic thrombotic microangiopathies (TMAs) at an estimated frequency of 0.5–3.0% [66–68]. For instance, various degrees of thrombocytopenia are commonly observed after liver transplantation, especially during the first postoperative week, and some clinical studies have demonstrated that thrombocytopenia was significantly associated with poor prognosis [69]. The imbalance between endothelin and nitric oxide produced by the SEC may lead to active vasoconstriction, narrowing of the sinusoidal lumen, and subsequent sinusoidal microcirculatory disturbance [70]. During the past decade, the measurement of plasma ADAMTS13:AC was utilized as a differential diagnostic tool for TMAs [68], but its relevance to organ transplantation itself was not well evaluated.

In this regard, we first reported in 2006 that a significant reduction of ADAMTS13:AC with a concomitant appearance of UL-VWFM was consistently observed in patient plasma soon after liver transplantation [71]. Consecutive analysis of ADAMTS13:AC indicated that these changes reflected liver graft dysfunction, including ischemia–reperfusion injury and acute rejection. The ADAMTS13:AC in these patients often decreased to less than 10% of normal controls, concurrent with severe thrombocytopenia. These clinical and laboratory features appeared to be similar to TMAs, and more specifically to TTP, which is typically defined by severe deficiency of plasma ADAMTS13 with or without neutralizing autoantibodies to this enzyme. However, different from TTP, the liver transplant recipients in our study had no additional clinical signs of TTP, such as neurological manifestation, fever, or renal dysfunction. Thus, the organ dysfunction appeared to be restricted to the liver graft. From these observations, we suggested that a decrease of plasma ADAMTS13:AC coupled with the appearance of UL-VWFM in liver transplant recipients was caused by the mechanism of "local TTP" within the liver graft [71]. It is assumed that the primary target is vascular endothelial cells within the liver graft in both ischemia–reperfusion injury and acute rejection after liver transplantation [72–74]. Indeed, depositions of activated platelets on the sinusoidal endothelium with a concomitant increase of VWF expression have been found in the liver immediately after reperfusion or cold preservation [73, 74]. In addition, the up-regulated VWF expression has been observed in liver allografts during acute rejection [74]. Thus, newly released UL-VWFM from vascular endothelial cells [71], together with consumption of ADAMTS13, induces platelet aggregation or thrombi formation at the hepatic sinusoid, and results in microcirculatory disturbance. This hypothesis might address why organ dysfunction restricts in the graft liver in liver transplantation-associated 'subclinical' TMA, distinct from systemic organ involvements found in "classical TTP".

Recently, two groups of investigators from Japan [75] and the Netherlands [76] reported interesting results as compared with ours. The report by Kobayashi et al. [75] appeared to be in good agreement with ours, because by examining a large number of liver transplant patients ($n = 81$) they provided solid data showing decreased platelet counts and plasma ADAMTS13:AC levels in the early stage of transplantation. Further, they were able to show increased plasma levels of VWF with the appearance of UL-VWFMs, as a reflection of the reduced plasma ADAMTS13:AC. On the other hand, Pereboom et al. [76] reported that a reduction of ADAMTS13:AC occurred within 1 day after liver transplantation, and was followed by an increased plasma level of fully functional VWF; however, they did not address platelet count in their patients ($n = 20$). One of their patients with severe deficiency of ADAMTS13 indeed had thrombotic complications after transplantation, but the patient did not have UL-VWFMs in the plasma. As a partial explanation for this reason, the authors suggested that plasmin activity was increased in these patients by demonstrating increased plasma levels of tissue plasminogen activator. But, if this hypothesis is true, these patients should have severe bleeding symptoms rather than thrombotic complications, or the investigators might be able to demonstrate the presence of VWF fragments specifically generated by plasmin cleavage in patient plasmas [77]. If not, it will be necessary that the presence of UL-VWFMs is carefully re-examined.

Through our experience, we would like to emphasize here that it is extremely important to monitor plasma ADAMTS13:AC in the treatment of thrombocytopenia associated with allograft dysfunction after liver transplantation. This is because the infusions of platelet concentrate under an imbalance of decreased ADAMTS13:AC to enhanced UL-VWFM production might further exacerbate the formation of platelet aggregates mediated by uncleaved UL-VWFM, leading to graft failure via the "local TTP" mechanism [71]. To date, FFP is a unique source of ADAMTS13 replacement therapy, and may improve both liver dysfunction and thrombocytopenia in liver transplant patients. From this point of view, we are particularly interested in the start of clinical trials on recombinant ADAMTS13 preparations.

5 Conclusion and future perspectives

The introduction of ADAMTS13 to the field of hepatology not only enabled us to confirm the diagnosis of TTP early, but also provided novel insight into the pathophysiology of liver diseases. Some diseases were shown to be TTP itself, but others did not show any apparent clinical features of TTP, even in the presence of extremely decreased ADAMTS13:AC and increased UL-VWFM corresponding to TTP. Such TTP-like states, but without disseminated intravascular coagulation, might be "subclinical TTP" as seen in advanced liver cirrhotics [36] and SAH patients [57, 58], or "local TTP" as shown in patients with hepatic VOD after SCT [64, 65] and patients with adverse events after living donor liver transplantation [71]. One would essentially be unable to detect such TTP-like phenomena without the determination of ADAMTS13:AC, because the interaction of ADAMTS13 and UL-VWFM is the initial step in hemostasis, and their abnormalities do occur in the absence of apparent imbalance in other hemostatic factors and/or irrespective of the presence or absence of abnormal