cators of adverse events after liver transplantation. In advanced cirrhotics, ADAMTS13:AC was significantly low [24,28], but not all studies have observed this decrease in ADAMTS13:AC [29]. It is unclear why these studies reached different conclusions, but a possible explanation may be related to the differences in the severity and/or etiology of liver diseases [24, 28, 29]. Another may include the difference in the methodology to determine ADAMTS13:AC [24, 28, 29]. It remained to be clarified on the clinical significance of ADAMTS13:AC in advanced liver cirrhosis.

POTENTIAL ROLE OF DECREASED ADAMTS13:AC IN ALCOHOLIC HEPATITIS

The reason why ADAMTS13:AC decreased in AH and SAH patients remains unclear, but we speculate that the following factors are involved: the consumption of ADAMTS13 due to large amounts of UL-VWFM, decreased ADAMTS13:AC production in HSCs, as recently reported in animal models with acute hepatic failure [63], presence of protease inhibitors, which are detected in the majority of patients diagnosed with "idiopathic" pregnancy- or drug-associated TTP [64], and unknown factors such as pro-inflammatory cytokines, which could be involved in the hemostatic abnormalities including thrombotic conditions [65]. On the other hand, the reason why VWF:Ag is increased in AH and SAH patients may be due to decreased ADAMTS13:AC or overproduced VWF from endothelial cells damaged by liver injury and/or inflammatory cytokines, including IL-8 and TNFα [66]. It was recently demonstrated that IL-6 inhibited the action of ADAMTS13 under flow conditions, and that both IL-8 and TNF-α stimulated the release of UL-VWFM from human umbilical vein endothelial cells *in vitro* in a dose-dependent manner [66]. Considering that the plasma cytokines including TNF-α, IL-8 and IL-6 are remarkably high, especially in SAH, and are closely related to a poor AH outcome [2,3, 67], enhanced cytokinemia might contribute to a decrease in ADAMTS13:AC together with an increase in VWF:Ag and UL-VWFM, ultimately resulting in multiorgan failure through microcirculatory disturbances in SAH patients. A recent study demonstrated that severe secondary ADAMTS13 deficiency is associated with sepsis-induced disseminated intravascular coagulation and may contribute to renal failure [68].

Alternatively, another mechanism to reduce ADAMTS13:AC is the presence of a plasma ADAMTS13 inhibitor. We recently encountered two patients with liver diseases who developed TTP: one occurred in the course of hepatitis C virus (HCV)-related advanced liver cirrhosis [69] and the other, after one month of pegylated-interferon alpha-2a therapy for HCV-related chronic hepatitis [70]. In both cases, plasma ADAMTS13:AC was extremely low, and the ADAMTS13 inhibitor was detected in the patient's heated plasma (2.0 BU/ml, 1.6 BU/ml, respectively) and purified IgG (0.19 BU/mg IgG, 0.4 BU/mg IgG, respectively) [69,70]. If the inhibitor would be present in AH patients, it will be necessary to clarify what kind of the inhibitor would be involved in association with inflammatory cytokines.

FUTURE PROSPECTIVE

Ethanol consumption has a complex association with hemostatic and fibrinolytic parameters [71], which may be largely influenced by drinking patterns, nutritional balance, and genetic background. Regular light to moderate alcohol intake protects against ischemic stroke of atherothrombotic origin and peripheral arterial disease [71-73], but both chronic alcoholism and acute ethanol intoxication are increasingly recognized as risk factors for circulatory disorders, including thrombotic complications and hemorrhage [74-76]. Patients with chronic alcoholism show a higher incidence of pulmonary infarction, cerebral infarction, and venous thrombosis in their extremities after abstinence [77-79], indicating that excessive ethanol consumption may lead to platelet hyperaggregability. It will be necessary to clarify the relationship of plasma ADAMTS13:AC to genetic backgrounds, drinking patterns, and nutritional balance [80-82].

CONCLUDING REMARKS

The imbalance between decreased ADAMTS13:AC and increased VWF:Ag in the plasma may contribute to the progression of multiorgan failure through microcirculatory disturbances in both SAH and AH. However, our study has a limited number of cases, and therefore, it will be necessary to investigate the role of plasma ADAMTS13 activity in the development of liver injury and organ failure in a larger number of AH patients. In addition, it will be required to elucidate the mechanism of the decrease in plasma ADAMTS13:AC in association with pro-inflammatory cytokinemia, an ADAMTS13 inhibitor and the production of ADAMTS13 in HSCs. These results raise the possibility for additional supportive therapies for AH patients, such as ADAMTS13 supplementation. The determination of ADAMTS13 and its substrate will give new insights into the pathophysiology and therapeutic approaches of acute alcoholic liver injury.

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ORIGINAL ARTICLE

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Acute myocardial infarction as a systemic prothrombotic condition evidenced by increased von Willebrand factor protein over ADAMTS13 activity in coronary and systemic circulation

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Abstract The aim of the present study is to clarify the roles of circulating ADAMTS13 and von Willebrand factor (VWF) in the formation of coronary artery thrombi in acute myocardial infarction (AMI). Twenty-six AMI patients, 37 age-matched healthy controls, and 20 young controls were studied. Plasma ADAMTS13 activity and levels of VWF antigen (VWF:Ag) and unusually large VWF multimer (UL-VWFM) were measured in the femoral vein (FV), aortic root (Ao), and coronary sinus (Cs) immediately before percutaneous coronary intervention (PCI) during the acute phase of AMI, as well as 6 months later. During the acute phase of AMI, plasma levels of VWF:Ag were similar in FV, Ao, and Cs, and were higher than those of age-matched control. In contrast, ADAMTS13 activity in three sampling points in AMI patients was similar to that of age-matched controls. Thus, the ratio of VWF:Ag to ADAMTS13 activity in the acute phase of AMI was significantly higher in all three sampled sites than that of age-matched controls. In the chronic phase, plasma levels of VWF:Ag, ADAMTS13 activity, and the ratio of VWF:Ag to ADAMTS13 activity were similar to those of age-matched controls. UL-VWFM was detected in the acute phase of AMI but not in the chronic phase. The present study showed that the plasma VWF:Ag levels are increased and ADAMTS13 activity is relatively decreased in both systemic and coronary circulation during the acute phase of AMI, suggesting that an imbalance between the enzyme and its substrate may play a role in the formation of occlusive thrombi in a coronary artery.

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Introduction

The rapid closure of the coronary artery by acutely formed arterial thrombi, which are composed of platelets, fibrin, and inflammatory cells, is the major cause of acute myocardial infarction (AMI).[1,2] Although the exact mechanism of coronary thrombus formation is not fully understood, the binding of von Willebrand factor (VWF) to glycoproteins Ibα and IIb/IIIa on the surface of platelets is known to lead to platelet activation and subsequent aggregation, which is an initial step toward formation of coronary thrombi.[3,4] Earlier reports have shown that circulating levels of VWF antigen (VWF:Ag) is elevated in patients during the acute phase of AMI,[5,6] and increased levels of plasma VWF:Ag can predict primary and secondary coronary events.[7–9] Thus, VWF appears to be involved in the formation of coronary thrombi as a cause of AMI, although blocking of VWF function has not yet been clinically proven to prevent the onset of AMI. It is not clear, however where and how VWF is produced during AMI.

Von Willebrand factor is synthesized in vascular endothelial cells and then released into the plasma as unusually large VWF multimer (UL-VWFM),[4] which has most potent biological activities interacted with platelet, and is rapidly degraded into smaller VWF multimers by ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13),[4,9] a metalloproteinase that specifically cleaves multimeric VWF between Tyr1605 and Met1606 within the VWF A2 domain.[9] Loss-of-function mutation of ADAMTS13 leads to Upshaw–Schulman syndrome, a form of congenital thrombotic thrombocytopenic purpura. Reduction of ADAMTS13 activity keeps circulating UL-VWFM levels high, which leads to platelet clumping and formation of platelet-rich thrombi. Recently, Sakai et al.[6] reported that UL-VWFM was detected in plasma drawn from peripheral veins in patients with AMI. To understand

the mechanism for the formation of coronary arterial thrombi in AMI, we measured plasma ADAMTS13 activity together with circulating levels of its substrate, VWF:Ag, in three sites: the aorta (Ao) near the ostium of the infarction-related coronary artery, the coronary sinus (Cs), and the femoral vein (FV). Samples were taken immediately before the percutaneous transluminal coronary intervention (PCI) during the acute phase of AMI and compared with those taken during the chronic phase.

Materials and methods

Patients

We studied 26 Japanese patients with AMI (5 women and 21 men; mean age 67.8 ± 11.6 years; range 38–89 years) admitted to the Nara Medical University Hospital between August 2004 and February 2005. The diagnosis of AMI was based on sustained chest pain of typical character and location, electrocardiographic ST-T elevation in two or more leads, disrupted regional wall motion on echocardiograms, and plasma levels of cardiac enzymes, including creatine phosphokinase (CK) and its MB fraction, that were greater than twice the normal upper limit. Of the 26 patients, 18 had hypertension, 21 had dyslipidemia, 13 had diabetes mellitus, 5 were obese, and 19 smoked. All of the patients received emergency coronary angiography and PCI within 24 h from the onset of AMI (the first symptoms). Clinical characteristics and drugs used are summarized in Table 1. The culprit lesions were in the right coronary artery in 6 patients, the left anterior descending coronary artery in 18, and the left circumflex coronary artery in 2. The peak CK level in AMI patients averaged 2960 IU/l and ranged from 344 to 12930 IU/l. All of the patients received intracoronary stents, implanted at the culprit lesions, and were subsequently given aspirin (81 mg/day, per os) and ticlopidine (200 mg/day, per os) or cilostazol (200 mg/day, per os) as antiplatelet therapy. An angiotensin-converting enzyme inhibitor and/or angiotensin-II receptor blocker were also administered to all patients. In addition, 10 patients received a β-blocker, 6 a calcium channel blocker, 7 a diuretic, and 15 a statin. Six months after the first onset of AMI, coronary angiography was again carried out in all of the patients. Written informed consent was obtained from all patients and control subjects participating in the study. The protocol was approved by the institutional review board of Nara Medical University (#2002-009).

Young and age-matched healthy control subjects

Study participants included both young and age-matched healthy control subjects. Young healthy subjects consisted of 30 volunteers (15 women and 15 men) aged from 20 to 39 years with a mean age of 30 ± 12.0 years, and age-matched healthy subjects consisted of 37 healthy volunteers (19 women and 18 men) aged from 39 to 93 years with a mean age of 64.2 ± 14.0 years. Both groups had no history of angina, myocardial infarction, coronary artery bypass graft surgery, PCI, or any electrocardiographic abnormalities. Blood samples were collected from the antecubital vein early in the morning, before breakfast. Nine of the age-matched controls (4 women and 5 men, mean age 48.1 ± 4.8 years, range 41–52 years) were also studied to evaluate the circadian variation of VWF:Ag and ADAMTS13 activity in plasma. In those subjects, blood samples were collected from the antecubital vein in the morning (09:30) and in the evening (20:00).

Table 1. Characteristics of patients with acute myocardial infarction

	Patient	Age-matched control subjects	*P* value
Age (years)	67.8 (38–89)	64.2 (39–93)	0.29
Sex (female/male)	5/21	19/18	<0.01
Coronary risk factor (yes/no)			
Hypertension	8/18	0/37	<0.01
Dyslipidemia	21/5	2/35	<0.01
Diabetes mellitus	13/13	3/34	<0.01
Obesity	5/21	7/30	0.41
Smoking	19/7	7/30	<0.01
Peak CK (IU/l) (mean)	2960 (344–12930)		
Location of AMI			
RCA/LAD/LCx	6/18/2		
Medication (yes/no)			
Aspirin	26/0		
Ticlopidine or Cilostazol	26/0		
ACE-I or ARB	26/0		
β-Blocker	10/16		
Calcium-antagonist	6/20		
Diurea	7/19		
Statin	15/11		

Values in parentheses indicate range

CK, creatine phosphokinase; AMI, acute myocardial infarction; RCA, right coronary artery; LAD, left anterior descending artery; LCx, left circumflex artery; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin-II receptor blocker

Blood sampling

In the AMI patients, emergency cardiac catheterization was performed within 90 min of their arrival in our hospital. Blood samples were collected using a 7-F sheath inserted into the patient's femoral vein (FV), a 6-F Cs catheter placed in the Cs through an FV sheath, and a 4-F Judkins catheter placed at the Ao. Unfractionated heparin and contrast medium were not used before pre-PCI blood sampling. Blood was sampled at the femoral vein (FV), the aortic root near the ostium of the infarction-related coronary artery (Ao), and the coronary sinus vein (Cs) immediately before and after emergency PCI. Six months after the onset of AMI, all 26 patients underwent a second round of coronary angiography, at which time blood was again collected from the same three areas. In young healthy and age-matched control subjects, blood samples were drawn from the antecubital vein. Preliminary experiments showed that there was no difference in plasma levels of VWF:Ag and ADAMTS13 activity among the antecubital vein, the FV, and the right atrium.

Blood was collected into plastic tubes with 1/10th volume of 3.8% sodium citrate. Platelet-poor plasma was prepared by centrifugation at 3000× *g* at 4°C for 15 min and stored in aliquots at –80°C until analysis.

Assays of ADAMTS13 activity, VWF:Ag, and UL-VWFM

Plasma ADAMTS13 activity was determined using a highly sensitive enzyme-linked immunosorbent assay (ELISA) recently developed by our laboratory.[10] The assay system includes a recombinant GST-VWF73-His polypeptide as a substrate and a murine monoclonal antibody that specifically recognizes the Tyr1605 residue in the VWF-A2 domain exposed by ADAMTS13 cleavage; it does not recognize the uncleaved form of the peptide. Plasma VWF:Ag was measured by a sandwich enzyme immunoassay using rabbit anti-human VWF polyclonal antibody (Dako, Kyoto, Japan). Plasma ADAMTS13 activity and VWF:Ag levels were expressed as percentages of those of reference peripheral plasma obtained from 20 healthy volunteers aged 20–40 years. The lower detection limit of the ELISA for ADAMTS13 activity was 0.5% of the reference peripheral plasma activity. Plasma UL-VWFM was analyzed by sodium dodecyl sulfate – 0.9% agarose gel electrophoresis using 1 µl samples, after which VWF multimers were visualized by Western blotting and luminography, as described previously.[11]

Statistical analysis

The data are expressed as mean ± SD. Comparison between acute and chronic data was performed using the paired

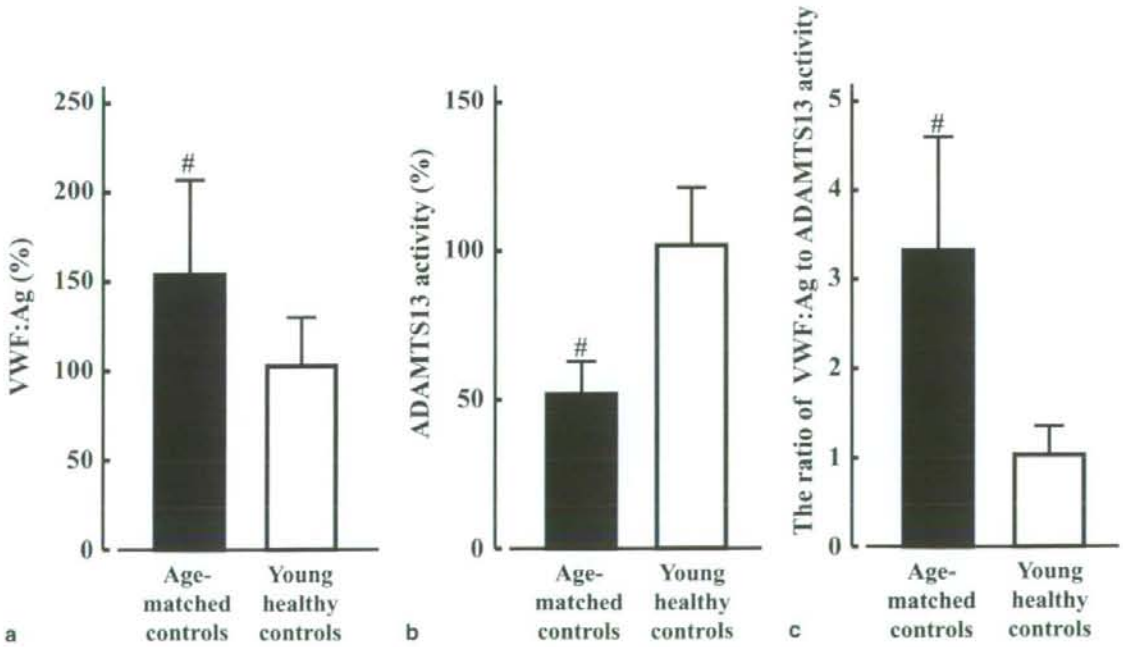

Fig. 1a–c. Comparison of plasma von Willebrand factor antigen (*VWF:Ag*) levels and ADAMTS13 activity between healthy young subjects and age-matched controls. **a** Plasma VWF:Ag levels. **b** Plasma ADAMTS13 activity. **c** Ratios of VWF:Ag to ADAMTS13 activity. Shown are mean ± SD; $^{\#}P < 0.001$ vs young subjects

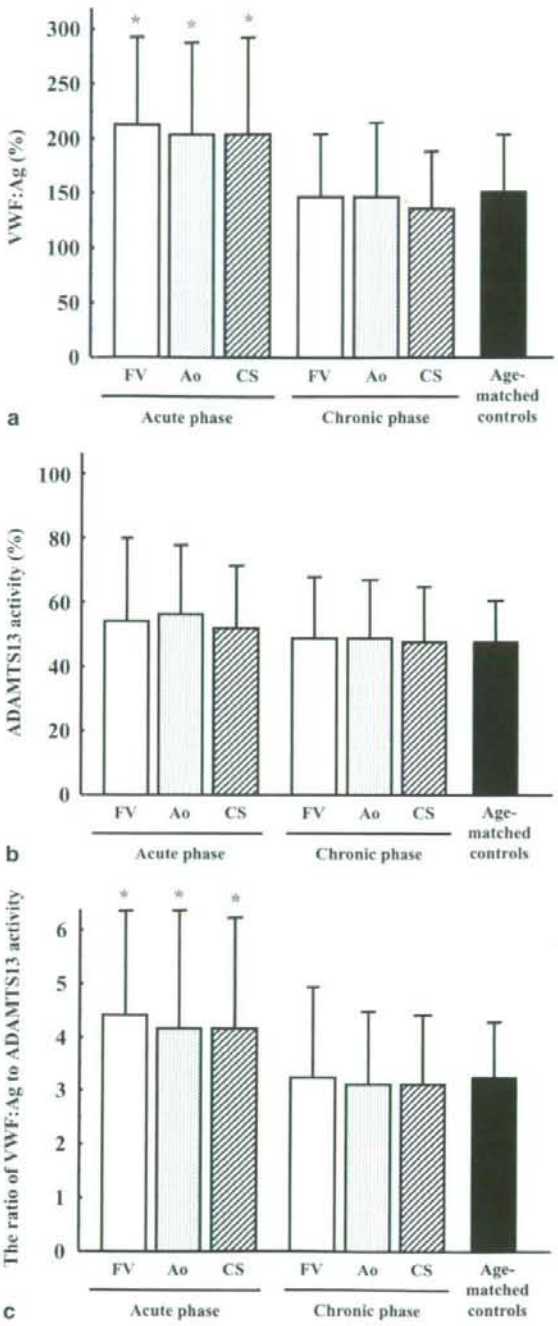

Fig. 2a–c. Plasma von Willebrand factor antigen (*VWF:Ag*) levels and ADAMTS13 activity and the ratio of VWF:Ag to plasma ADAMTS13 activity during the acute and chronic phases of AMI. **a** VWF:Ag levels, **b** ADAMTS13 activity, and **c** ratios of VWF:Ag to ADAMTS13 activity before percutaneous coronary intervention (PCI) during the acute phase and chronic phase of acute myocardial infarction (AMI). Measurements were made using plasma samples collected from the femoral vein (*FV*), aortic root (*Ao*), and coronary sinus (*Cs*) of the AMI patients and peripheral blood samples collected from control subjects. Shown are means ± SD; $^*P < 0.05$ vs age-matched controls

Student's *t*-test or Wilcoxon signed-rank test, when appropriate. Comparison among the three groups of subjects was performed by analysis of variance. The analyses were carried out using the statistical software Statview (version 5.0; SAS Institute, Cary, NC, USA). A P value of less than 0.05 was considered statistically significant.

Results

Differences between healthy young and age-matched controls

Plasma levels of VWF:Ag were significantly higher in healthy age-matched controls than in the young subjects (151% ± 58% vs 102% ± 33%, $P < 0.001$) (Fig. 1). Conversely, the plasma ADAMTS13 activity was lower in the age-matched controls than in the young subjects (51% ± 15% vs 104% ± 22%, $P < 0.001$), resulting in a three-fold higher ratio of VWF:Ag to ADAMTS13 activity in the age-matched controls than in young healthy controls (3.3 ± 1.4 vs 1.0 ± 0.3, $P < 0.001$) (Fig. 1).

VWF:Ag levels

During the acute phase of AMI before PCI, plasma VWF:Ag levels were significantly higher ($P < 0.01$) at the FV (211% ± 75%), Ao (204% ± 78%), and Cs (205% ± 90%) than in peripheral blood samples from the age-matched controls (151% ± 58%) (Fig. 2a). During the chronic phase, these values ($P < 0.05$) fell to levels similar to those seen in the age-matched controls (FV, 149% ± 69%; Ao, 148% ± 73%; and Cs, 133% ± 52%). There also were no differences in VWF:Ag levels among sampling sites (Fig. 2a).

ADAMTS13 activity

Plasma ADAMTS13 activity did not differ among blood samples collected from the FV, Ao, and Cs before PCI during the acute phase of AMI (FV, 55% ± 22%; Ao, 57% ± 22%; Cs, 54% ± 19%), or during the chronic phase of AMI (FV, 51% ± 19%; Ao, 52% ± 17%; Cs, 51% ± 22%). In fact, all of these values were similar to ADAMTS13 activity in peripheral blood from the age-matched controls (51% ± 15%) (Fig. 2b). Moreover, ADAMTS13 activity in the acute phase was similar to that in the chronic phase at each sampling point. There was no significant inverse correlation between ADAMTS13 activity and plasma level of VWF:Ag in the acute phase of AMI.

The ratio of VWF:Ag to ADAMTS13 activity

During the acute phase of AMI, the ratio of VWF:Ag to ADAMTS13 activity before PCI was significantly higher ($P < 0.05$) in the FV (4.5 ± 2.4), Ao (4.2 ± 2.5), and Cs (4.2 ± 2.4) than in the peripheral blood samples from age-matched

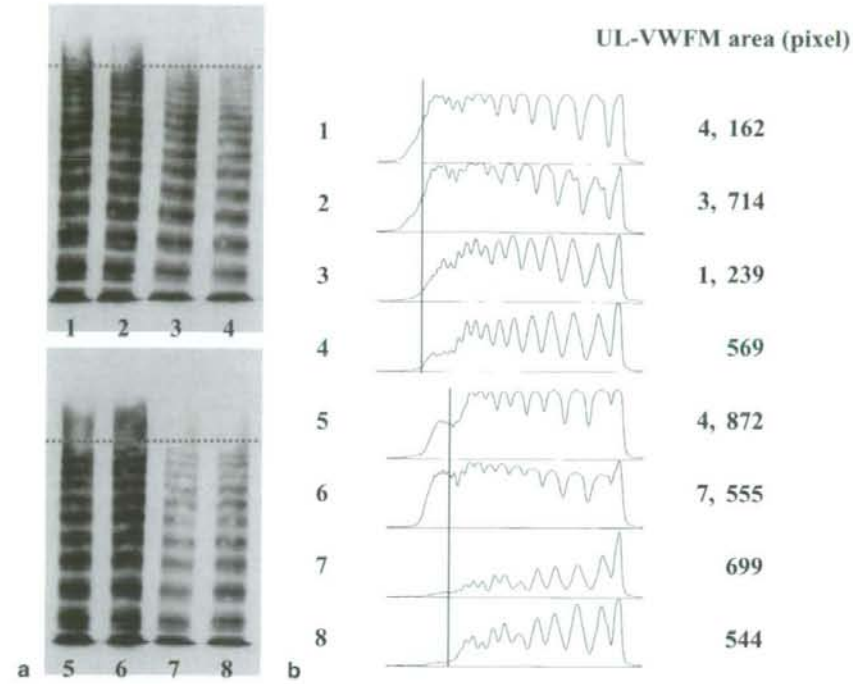

Fig. 3a,b. von Willebrand factor (VWF) multimeric analysis in two representative patients and its densitometric analysis. *1*, patient 1 before PCI in acute phase of AMI; *2*, after PCI of patient 1; *3*, chronic phase of patients 1; *4* and *8*, normal plasma; *5*, before PCI of patient 2; *6*, after PCI of patient 2; *7*, chronic phase of patient 2. **a** von Willebrand factor multimeric analysis was performed using 1% agarose gel electrophoresis. **b** Densitometric analyses of unusually large VWF multimer (*UL-VWFM*) using NIH image J (developed by the National Institute of Health, http://rsb.Info.nih.gov/nih-image/). UL-VWF multimer was detected in samples obtained from the femoral vein before and after PCI in all AMI patients tested, though it was not detected in samples collected during the chronic phase

controls (3.3 ± 1.4), though there was no significant difference in the ratio among sampling points (Fig. 2c). In the chronic phase, those ratios decreased to levels similar to those seen in age-matched controls (FV, 3.2 ± 1.9; Ao, 3.0 ± 1.6; Cs, 3.0 ± 1.5) (Fig. 2c).

Detection of UL-VWFM

We analyzed UL-VWFM in 6 out of 26 AMI patients. UL-VWFM was detected in samples obtained from the FV before and after PCI in all patients (Fig 3a). It was not detected in samples collected during the chronic phase (Fig. 3a). To more easily quantify UL-VWFM levels, we performed densitometric analysis (Fig. 3b).

Circadian variation in plasma VWF:Ag and ADAMTS13 activity

Von Willebrand factor antigen was significantly higher in the morning (100% ± 42%) than in the evening (89% ± 41%) (Fig. 4a); however, for ADAMTS13 activity there was no significant difference between the morning (55% ± 13%) and the evening (61% ± 18%) (Fig. 4b). The ratio of VWF:Ag to ADAMTS13 activity was significantly higher at 09:30 (1.9 ± 0.8) than at 20:00 (1.5 ± 0.8) ($P < 0.05$, Fig. 4c).

Discussion

In the present study we simultaneously measured the activity of ADAMTS13 and the levels of its substrates (VWF:Ag and UL-VWFM) in plasma samples obtained from the FV, Ao, and Cs during the acute and chronic phases of AMI. We demonstrate for the first time that during the acute phase, the ratio of VWF:Ag to ADAMTS13 activity in AMI patients was significantly higher at all three sampling sites than in the peripheral blood of age-matched controls and that during the chronic phase of AMI, these ratios had returned to levels similar to those seen in age-matched controls. Moreover, UL-VWFM was detected during the acute phase but not in the chronic phase of AMI, in agreement with recent reports by Sakai et al.[6] and Goto et al.[12] In agreement with our findings, Kaikita et al.[13] also reported that the ratio of VWF:Ag to ADAMTS13 activity in peripheral venous plasma is higher in AMI patients than in those with stable exertional angina and chest pain syndrome.

During the last decade, evidence has accumulated that release of VWF:Ag from endothelial cells and platelets is a key early step toward occlusive thrombus formation in the coronary circulation. With that in mind, before the beginning of the present study we hypothesized that VWF:Ag would be much higher in the Cs than in the Ao or FV, and that ADAMTS13 activity might be lower in the Cs than in the FV or Ao. However, our study showed that there was

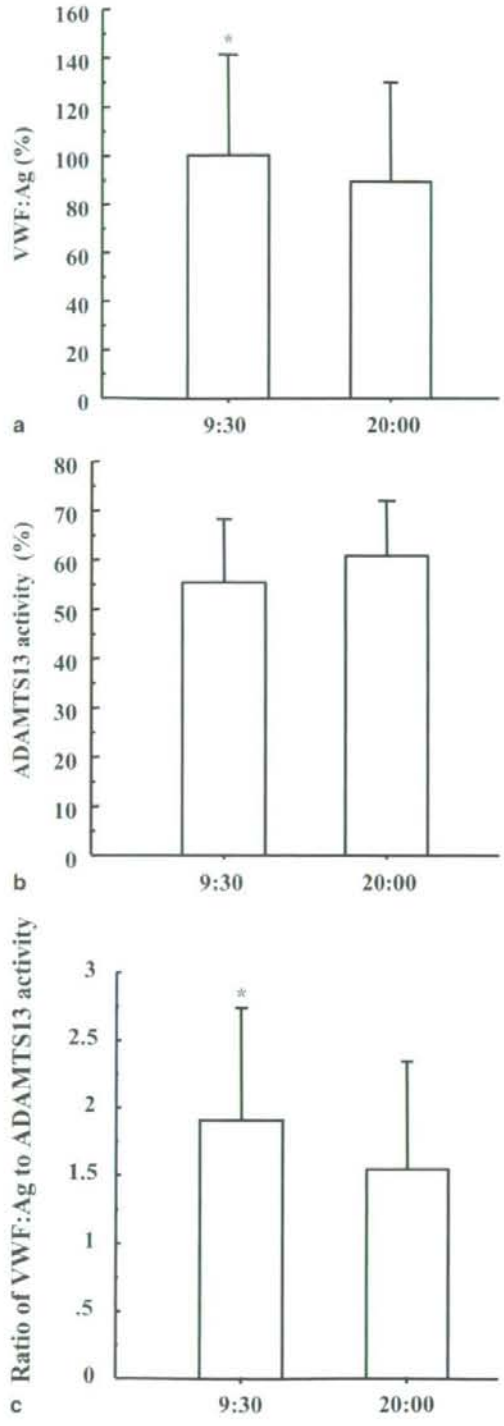

Fig. 4a–c. Comparison with von Willebrand factor antigen (*VWF:Ag*), ADAMTS13, and the ratio of VWF:Ag to ADAMTS13 activity between in the morning and in the evening. **a** VWF:Ag was significantly higher in the morning (100% ± 42%) than in the evening (89% ± 41%). **b** ADAMTS13 activity was no significant difference between in the morning (55% ± 13%) and the evening (61% ± 18%). **c** The ratio of VWF:Ag to ADAMTS13 activity was significantly higher at 09:30 than at 20:00. Shown are mean ± SD. $^*P < 0.05$ vs 20:00

no significant difference in the plasma level of VWF:Ag, ADAMTS activity, and the ratio of VWF:Ag to ADAMTS13 activity among three sampling points, clearly indicating that AMI is not a local but rather a systemic prothrombotic condition. In other words, the present findings support the recent concept that occlusive coronary thrombi develop in vulnerable blood (prone to thrombosis) or in vulnerable patients.[14,15] Further studies are necessary to clarify whether increased VWF may lead to the formation of coronary thrombi or whether coronary thrombi itself may cause elevation of VWF levels in AMI.

It was previously reported that AMI frequently occurs in the morning, between 06:00 and 12:00, a vulnerable period for cardiovascular events,[16] and also frequently occurs after physical exercise, especially in the sun during summer. In that regard, we observed that the ratio of VWF:Ag to ADAMTS13 activity was higher in the morning than in the evening. Acute myocardial infarction is also more frequently observed in aged men than in young men. We previously reported that plasma ADAMTS13 activity declines and plasma VWF:Ag levels increase with increasing age, and that detectable levels of UL-VWFM were circulating in some older people.[11] Here, we confirmed that plasma VWF:Ag is 50% higher while plasma ADAMTS13 activity is nearly 50% lower in healthy age-matched control subjects as compared to young healthy subjects, resulting in a three-fold higher ratio of VWF:Ag to ADAMTS13 activity in the former than in the latter. Interestingly, we have also observed that physical exercise increases the ratio of VWF:Ag to ADAMTS13 activity in healthy men.[17] Thus the simultaneous measurement of ADAMTS13 activity and its substrate, VWF:Ag level, is useful in understanding the pathology of thrombotic diseases.

Although it is not possible to identify the precise site of production of VWF and ADAMTS13, our findings (together with those of others[5,6]) suggest that, during the acute phase of AMI, production of VWF is increased not only in coronary arterial endothelial cells and/or locally activated platelets, but also in systemic vascular beds and/or circulating endothelial cells. ADAMTS13 is mainly produced in hepatic stellate cells,[18] and is also synthesized in both human endothelial cells[19] and platelets,[20] suggesting that this enzyme is produced in the systemic circulation as well as in the liver.

In summary, we observed increased plasma VWF:Ag levels and relatively decreased ADAMTS13 activity in both systemic and coronary circulation during the acute phase of AMI, suggesting that an imbalance between the enzyme and its substrate may play an important predictive role in the formation of occlusive thrombi in a coronary

artery, ultimately leading to AMI. Further analysis of the production–consumption relation between VWF and ADAMTS13 in the coronary and systemic circulations will be necessary to understand the pathophysiological significance of VWF and ADAMTS13 in the formation of occlusive thrombi.

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Comprehensive analysis of ADAMTS13 in patients with liver cirrhosis

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Summary

Decreased plasma ADAMTS13 activity (ADAMTS13:AC) results in the accumulation of unusually large von Willebrand factor multimer (UL-VWFM) and the formation of platelet thrombi. It remains controversial whether or not plasma ADAMTS13:AC decreases in patients with liver cirrhosis (LC), and its relationship to clinical features has not been fully investigated. We measured ADAMTS13:AC and its related parameters in plasma in 33 patients with chronic hepatitis (CH) and in 109 patients with LC. ADAMTS13:AC decreased with increasing severity of liver disease (controls means 100%, CH 87%, Child A-LC 79%, Child B-LC 63%, and Child C-LC 31%), and showed severe deficiency (<3% of controls) in five end-stage LC. Activities measured by act-ELISA strongly correlated with those determined by the VWFM assay and ADAMTS13 antigen. Multivariate analysis showed Child-Pugh score and spleen volume independent factors contributing to ADAMTS13:AC. VWFM patterns were normal in 53% of cases, degraded in 31%, and unusually large in 16%. Patients with unusually large VWFM had the lowest ADAMTS13:AC as well as the highest Child-Pugh score, serum creatinine and blood ammonia levels. Plasma inhibitor against ADAMTS13 detected in 83% of patients with severe to moderate ADAMTS13:AC deficiency mostly showed marginal zone between 0.5 and 1.0 BU/ml. The IgG-type autoantibodies specific to plasma derived-ADAMTS13 was detected by Western blot in only five end-stage LC with severe ADAMTS13:AC deficiency. In conclusion, both plasma ADAMTS13 activity and antigen levels decreased with increasing severity of cirrhosis. An imbalance between the decreased ADAMTS13:AC and its increased substrate may reflect the predisposing state for platelet thrombi formation in patients with advanced LC.

Keywords

ADAMTS13 activity, liver cirrhosis, von Willebrand factor, thrombocytopenia, inhibitor

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Introduction

ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13) is a metalloproteinase that specifically cleaves the Tyr1605-Met1606 bond of von Willebrand factor (VWF) A2 domain (1, 2). VWF is exclusively synthesized in vascular endothelial cells and is secreted into the circulation as unusually large VWF multimers (UL-VWFMs), which are most biologically active to aggregate platelets and form platelet thrombi under high shear stress (3). ADAMTS13 rapidly cleaves UL-VWFMs into smaller VWF multimers, which are less biologically active, and thereby prevents platelet hyperaggregation and thrombi formation (3). Congenital deficiency of ADAMTS13 activity (ADAMTS13:AC) caused by its gene mutations (1, 4), and acquired deficiency due to the development of the neutralizing autoantibodies, ADAMTS13 inhibitors (ADAMTS13:INH) (5, 6), result in thrombotic thrombocytopenic purpura (TTP), a life-threatening generalized disease (7). The *ADAMTS13* gene was originally cloned using liver cell libraries (2), and it was later shown that the enzyme is produced exclusively in hepatic stellate cells (HSCs) (8).

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Variable	Chronic hepatitis (n=33)	Liver cirrhosis		
		Child A (n=35)	Child B (n=33)	Child C (n=41)
Age (years)	56.6 ± 12.4	66.4 ± 7.8[b]	63.6 ± 8.3[a]	64.9 ± 15.8[a]
Sex (male/female)	17/16	25/10	17/16	23/18
Cause of liver disease				
HCV/HBV/Alcohol/PBC/Cryptogenic	29/3/0/0/1	24/4/4/0/3	20/7/2/0/4	23/5/5/4/4
Child-Pugh score	—	5.5 ± 0.5	7.9 ± 1.0[c]	11.6 ± 1.5[e]
Spleen volume (mm^3)	219 ± 123	323 ± 181[a]	399 ± 250[b]	559 ± 248[b, c, d]
Ascites (-/easily mobilized/refractory)	0	35/0/0	21/10/2	8/6/27
Hepatorenal syndrome (+)	0	0	0	10
Encephalopathy (+)	0	0	9	33
Esophageal varices (-/mild/severe)[f]	0	10/12/13	3/7/23	3/6/32
Hepatocellular carcinoma (+)	2	22	16	19
JIS score[g]	0, 0	1.4 ± 0.9	2.8 ± 1.0[c]	3.7 ± 1.1[c, d]

The data are expressed as mean ± SD. HCV, hepatitis C virus; HBV, hepatitis B virus; PBC, primary biliary cirrhosis.
[a]p<0.01 and [b]p<0.001 vs. patients with chronic hepatitis, respectively. [c]p<0.001 vs. cirrhotics with Child A. [d]p<0.01 and [e]p<0.001 vs. cirrhotics with Child B, respectively. [f]Mild or severe esophageal varices indicate lesions without or with endoscopic signs of impending variceal rupture, respectively. [g]The Japan Integrated Staging score obtained via the summation of Child-Pugh score and tumor stage score (32).

Table 1: Clinical data of patients with chronic liver diseases.

Alternatively, increased plasma levels of VWF antigen (VWF:Ag)(9, 10), and thrombocytopenia are commonly seen in patients with advanced LC (11–13). Moreover, cases of advanced LC are often complicated by thrombosis in one or more organs in addition to the portal and hepatic veins (14–16), suggesting a predisposition toward thrombogenesis in advanced LC.

Regarding the possibility of a relationship of ADAMTS13 and various liver diseases, Mannucci et al. (17) originally reported a significant reduction of the ADAMTS13:AC in advanced LC. Subsequently, we have shown that this activity is significantly reduced in patients with hepatic veno-occlusive disease (18), alcoholic hepatitis (19), and those undergoing living-donor related liver transplantation (20). Furthermore, HCV-related cirrhosis patients typically develop TTP with ADAMTS13:INH (21). In cases of advanced LC, however, the recent two studies reported apparently opposite results on plasma levels of ADAMTS13:AC: in one study (22), activity was unchanged, whereas in the other it was reduced (17, 23). The authors of the former study further reported that plasma levels of VWF:Ag were increased, but the higher molecular weight multimer was more degraded than normal controls, thus maintaining normal levels of enzyme-to-substrate (ADAMTS13/UL-VWFMs) ratio to maintain blood fluidity. The study employed the collagen binding assay for ADAMTS13:AC, and the assay has been established, but it is possible that compounds in the tested samples affected the assays in a manner that is not well-understood. In the classic VWFM assay, a gold standard method for ADAMTS13:AC (24), a high concentration of free hemoglobin is well-known to inhibit the enzyme activity (25); likewise, in fluorogenic FRETS-VWF73 assays (26), both bilirubin and chylomicron in tested samples block the activity (27). More recently, however, a chromogenic enzyme-linked immunoassay for ADAMTS13:AC (ADAMTS13-act-ELISA) was shown to be totally insensitive to the presence of such compounds (28).

We have performed comprehensive studies in a large population of patients with chronic liver diseases (n=142), paying particular attention to ADAMTS13 and VWF; plasma levels of ADAMTS13:AC, ADAMTS13 antigen (ADAMTS13:AG), ADAMTS13:INH, VWF:Ag, VWF ristocetin cofactor activity (VWF:RCo), and analysis of plasma UL-VWFMs, in order to explore the relationship between ADAMTS13:AC, the clinical features, and laboratory findings of patients with liver cirrhosis. Furthermore, we examined the presence or absence of immunoglobulin G (IgG)-type autoantibodies specific to plasma derived-ADAMTS13 by Western blot in patients with ADAMTS13:INH in plasma.

Materials and methods

Patients

A total of 142 patients with chronic liver diseases were included in this study, of whom 33 had biopsy-proven chronic hepatitis and 109 had LC, including a case with TTP (21) (Table 1). Patients with a known history of coagulopathies, sepsis, or platelet disorders were excluded. The origin of liver disease was hepatitis C virus (HCV) in 96 cases; hepatitis B virus (HBV) in 19; alcohol abuse in 11; primary biliary cirrhosis (PBC) in four; and cryptogenic in 12. The diagnosis of cirrhosis was based on physical findings, laboratory tests, and in many cases had been confirmed by histological criteria. Of the LC patients, 35 were Child A, 33 were Child B, and 41 were Child C, according to Child-Pugh's criteria (29). Spleen volume, determined by computed tomography scans (30), increased as liver disease progressed. Ascites was easily mobilized in 16 patients and refractory in 29, 10 of whom finally progressed to hepatorenal syndrome according to the criteria described previously (31). Spontaneous bacterial peritonitis (SBP) occurred in 10 patients with refractory ascites, and in seven patients this was complicated by hepatorenal syn-

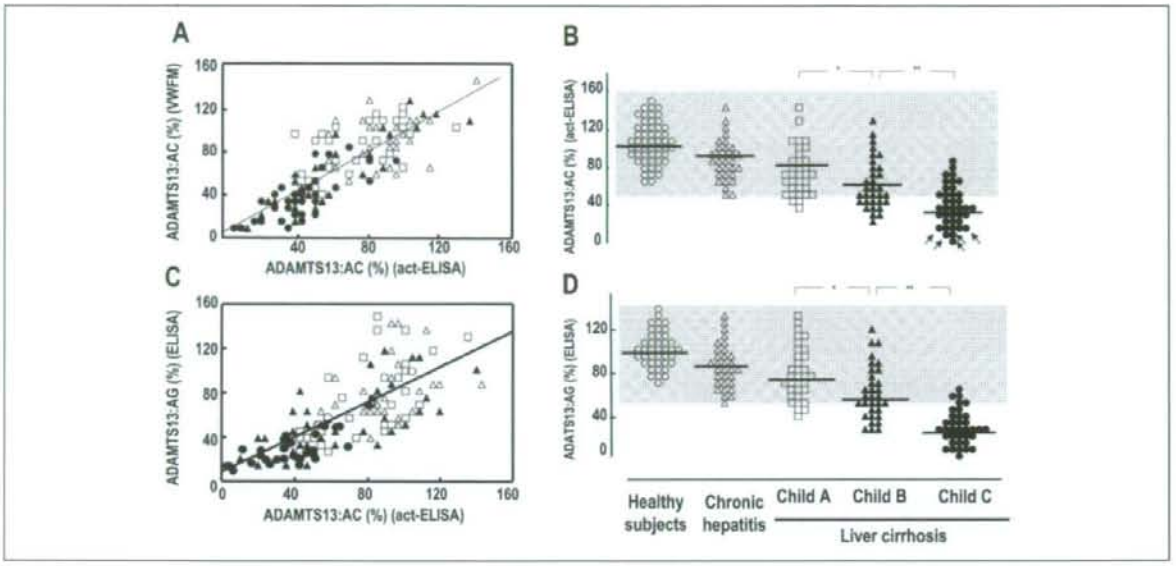

Figure 1: Plasma levels of ADAMTS13:AC and ADAMTS13:AG in patients with chronic liver diseases. The plasma levels of ADAMTS13:AC determined by two methods, the VWFM assay and ADAMTS13-act-ELISA, were in good accordance ($r=0.841$, $p<0.001$), although some discrepant results were observed (A). Severe deficiency in ADAMTS13:AC (<3%) was seen in five LC 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). Plasma ADAMTS13:AC determined by the ELISA progressively decreased with worsening cirrhosis (B). In addition, the plasma levels of ADAMTS13:AG quantified by the ELISA highly correlated with ADAMTS13:AC measured by the act-ELISA ($r=0.715$, $p<0.001$)(C). The ADAMTS13:AG levels also decreased with increasing cirrhosis severity (D). 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, VWFM=von Willebrand factor multimer. *$p<0.05$, and **$p<0.001$ significantly different between the two groups.

drome. Hepatic encephalopathy grade II or greater developed in 42 patients. Sixty-eight LC patients had endoscopic signs of impending variceal rupture. Hepatocellular carcinoma (HCC) was found in two patients with chronic hepatitis and 57 cirrhosis patients. HCC was graded by the Japan Integrated Staging (JIS) score (32) obtained via the summation of tumor stage score and Child-Pugh score (Table 1). All subjects gave informed consent to participate in the study. The study protocol was approved by the Nara Medical University Hospital Ethics Committee.

Determination of ADAMTS13:AC, ADAMTS13:AG, VWF:Ag, VWF:RCo, UL-VWFMs and ADAMTS13:INH

Blood was obtained from the patients at the time of admission or during a hospital stay. Samples were stored in plastic tubes containing $1/10^{th}$ volume of 3.8% sodium citrate. Platelet-poor plasma prepared by centrifugation at 3000 x g at 4°C for 15 minutes was stored in aliquots at – 80°C until analysis. Plasma ADAMTS13:AC was determined by both a classic VWFM assay (24, 33) and a sensitive chromogenic ELISA (ADAMTS13-act-ELISA: Kainos Inc., Tokyo, Japan) (28). The normal value was 102 ± 23% in the VWFM assay (33) and 99 ± 22% in the act-ELISA (28). ADAMTS13:AG was measured by a sandwich ELISA using two murine monoclonal antibodies (mAbs) as previously reported (34), and its normal value was 106 ± 39%. In five LC patients whose ADAMTS13:AC was less than 3% in the VWFM assay, Western blot (WB) analysis was performed to quantify ADAMTS13:AG using the murine anti-ADAMTS13 mAb WH2–11-1 (IgG1-κ) (35). Plasma VWF:Ag was measured by a rabbit polyclonal sandwich ELISA (Dako, Glostrup, Denmark), and its normal level was 100 ± 53% (n=60, 20–39 years of age). VWF:RCo was determined as described (36), and its normal value was 100 ± 15%. In 49 LC patients with lower ADAMTS13:AC (less than 50% of normal control), plasma UL-VWFMs were analyzed by a vertical SDS-1.0% agarose gel electrophoresis system (37), and evaluated using NIH image J. ADAMTS13:INH was evaluated using heat-inactivated plasma at 56°C for 30 minutes (5, 6). One Bethesda unit of inhibitor was defined as the amount of plasma that reduces ADAMTS13:AC to 50% of the control (38), and its titer was defined to be significant at >0.5 Bethesda Units (BU)/ml.

Detection of IgG-type autoantibodies specific to plasma derived-ADAMTS13 by WB

In order to differentiate between IgG associated with autoantibodies against ADAMTS13 and high IgG concentration, we tried to detect IgG-type autoantibodies specific to plasma derived-ADAMTS13 by WB. Plasma-derived (pd)-ADAMTS13 was purified using A10-agarose immunoaffinity chromatography followed by size-exclusion chromatography (39). The A10 was the murine anti-ADAMTS13 monoclonal antibody, which rec-

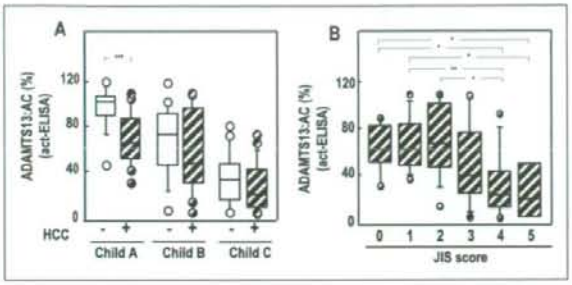

Figure 2: Plasma levels of ADAMTS13:AC with and without hepatocellular carcinoma. Child A LC patients with HCC had reduced ADAMTS13:AC compared to those without HCC, but there were no differences between Child B and C LC patients with and without HCC (A). ADAMTS13:AC was lower in patients with JIS score 4 and score 5 compared to patients with score 0, score 1, and score 2 (B). Open box, cirrhosis patients without HCC; shaded box, with HCC.
ADAMTS13:AC=ADAMTS13 activity, HCC= hepatocellular carcinoma, JIS=The Japan Integrated Staging score obtained via the summation of Child-Pugh score and tumor stage score (32). *p<0.05, **p<0.01, and ***p<0.001 significantly different between the two groups.

ognizes an epitope in the disintegrin domain, totally inhibiting enzyme activity at a final concentration of 50 µg/ml (8). Purified pd-ADAMTS13 had a specific activity of 302 units/mg. SDS-5% polyacrylamide gel electrophoresis (PAGE) analysis revealed a 170 kD-band before and a 190 kD-band after reduction. During storage at –80°C, however, the oxidized 170 kD-band occasionally degraded into fragments with smaller molecular masses of 130~150 kDa. To detect IgG-type autoantibodies specific for purified pd-ADAMTS13, 0.15 µg pd-ADAMTS13 per lane was separated by SDS-5%PAGE under non-reducing conditions, then electrophoretically transferred to polyvinylidine fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After blocking nonspecific binding with 5% skim milk, PVDF membranes were cut longitudinally into small pieces (3 x 800 mm). Each strip was incubated overnight at 4°C with 3 ml 5% skim milk containing 50 µl heat-treated plasma obtained from each patient who had ADAMTS13:INH more than 0.5 Bethesda Units (BU)/ml. The heat-treated plasma was prepared by incubation at 56°C for 30 min. After centrifugation, the supernatant was used in assays. Human IgG bound to the purified pd-ADAMTS13 on PDVF membranes was detected using a horseradish peroxidase (HRP)-conjugated anti-human IgG antibody (ICN Pharmaceuticals Inc., Aurora, OH, USA). Binding was visualized by chemiluminescence with Western Lightning Chemiluminescence reagent (Perkin-Elmer Life Science Inc., Boston, MA, USA) and imaged by X-ray autoradiography (Eastman Kodak, Rochester, NY, USA). Heated plasma from a patient with acquired idiopathic TTP with IgG inhibitors against ADAMTS13 was used as a positive control, while plasma from a normal individual without ADAMTS13:INH was used as a negative control.

Table 2: Correlation coefficients between plasma ADAMTS13:AC and clinical variables in patients with chronic liver diseases.

Variables	Correlation coefficients	P-value
Child-class	–0.597	<0.0001
Child-Pugh score	–0.593	<0.0001
Hemoglobin (g/dl)	0.490	<0.0001
Spleen volume (cm^3)	–0.470	<0.0001
Prothrombin time (%)	0.451	<0.0001
Serum albumin (g/dl)	0.418	<0.0001
Platelet count ($x10^4/mm^3$)	0.413	<0.0001
C-reactive protein (mg/dl)	–0.395	<0.0001
Blood ammonia (µg/dl)	–0.382	0.0005
Total cholesterol (mg/dl)	0.361	<0.0001
Blood urea nitrogen (mg/dl)	–0.353	<0.0001
Serum indirect bilirubin (mg/dl)	–0.329	<0.0001
Serum creatinine (mg/dl)	–0.326	0.0001
Interleukin-6 (pg/ml)	–0.267	0.002
Serum total bilirubin (mg/dl)	–0.256	<0.005
Lactate dehydrogenase (IU/l)	–0.240	<0.005
White blood cell count ($/mm^3$)	–0.237	<0.005

ADAMTS13:AC, ADAMTS13 activity.

Measurements of cytokines

Plasma concentrations of cytokines, tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and interleukin 8 (IL-8), were determined with commercially available kits (Immunoassay Kits, BioSource International, Camarillo, CA, USA).

Statistical analysis

Differences between paired and unpaired groups were analyzed using Student's *t* test. Correlations were calculated by Spearman rank test. To determine which clinical parameters independently correlated with plasma ADAMTS13:AC, we applied a stepwise selection procedure based on multiple regression analysis. The analyses were carried out using Statview statistical software (version 5.0; SAS Institute, Cary, NC, USA). The data are expressed as the mean ± SD. A two-tailed p-value less than 0.05 was considered significant.

Results

Plasma levels of ADAMTS13:AC and ADMTS13:AG

Plasma levels of ADAMTS13:AC determined by VWFM assay were 56 ± 34% in LC patients, which was significantly lower than those of both healthy subjects ($p<0.001$) and patients with chronic hepatitis (87 ± 27%, $p<0.001$). The ADAMTS13:AC levels progressively decreased with worsening cirrhosis; 79 ± 25% in Child A, 63 ± 34% ($p<0.05$) in Child B, and 31 ± 22% ($p<0.001$) in Child C. The levels of ADAMTS13:AC determined by the act-ELISA were in good accordance with those measured by the VWFM assay ($r=0.841$, $p<0.001$) (Fig. 1A); these values were 91 ± 22% in chronic hepatitis, 80 ± 24% in Child A, 65 ±

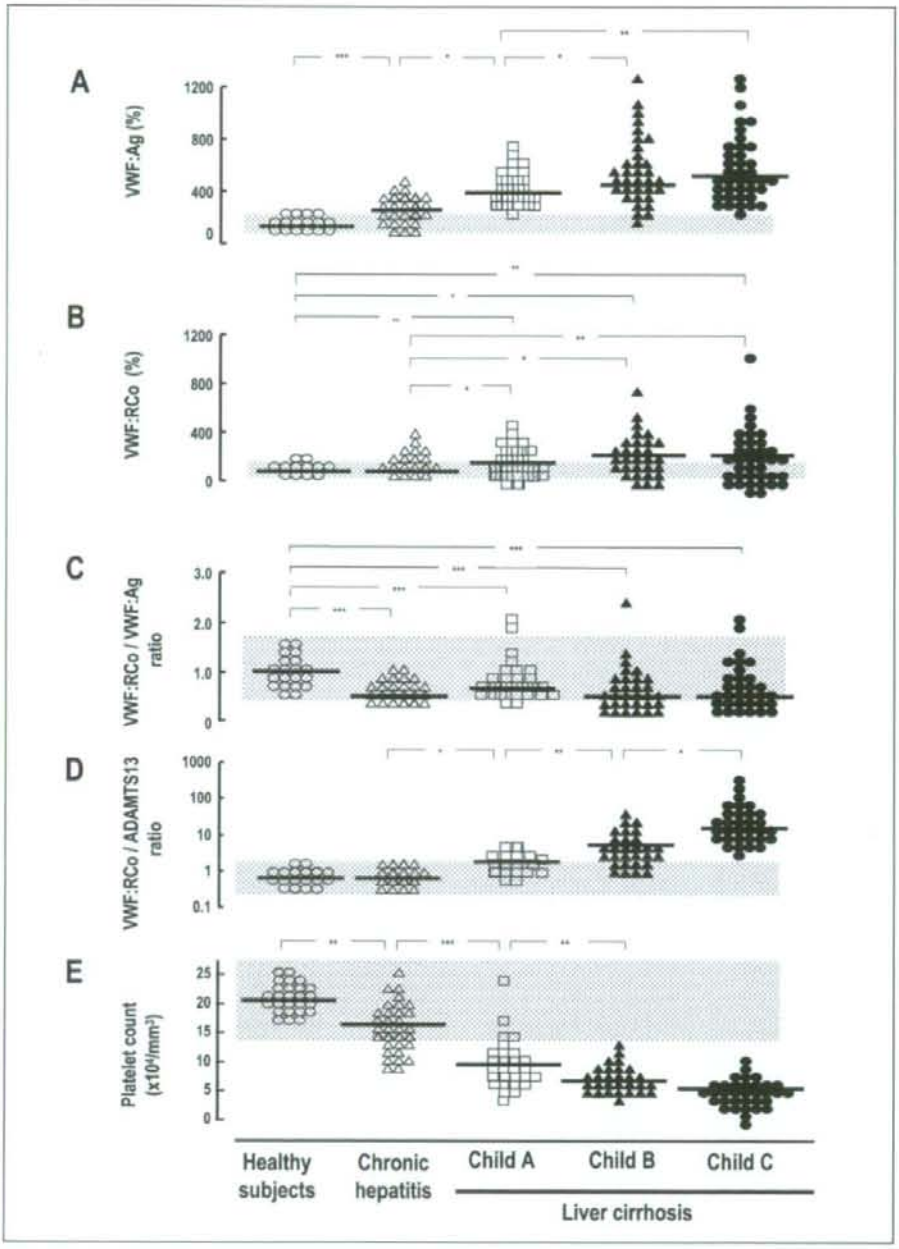

Figure 3: Plasma levels of VWF:Ag, VWF:RCo, VWF:RCo/VWF:Ag ratio, and VWF:RCo/ ADAMTS13:AC ratio, and platelet count in patients with chronic liver disease. The VWF:Ag increased with the progression of chronic liver diseases, but the difference between Child B and C did not reach statistical significance (A). The VWF:RCo is higher in LC patients than in patients with chronic hepatitis and healthy subjects, but it did not differ among subgroups within LC (B). The VWF:RCo relative to VWF:Ag was lower in patients with chronic hepatitis and LC than in healthy subjects, but no differences were found among subgroups within LC (C). Furthermore, the VWF:RCo relative to ADAMTS13:AC progressively increased with worsening chronic liver disease (D). Platelet count decreased with the severity of chronic liver diseases, but no difference was found between Child B and 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.

31% in Child B, and 40 ± 22% in Child C (Fig. 1B). Some discrepancies were observed: for example, deficiency in ADAMTS13:AC (<3%) by the VWFM assay was seen in five LC patients with Child C, but by the act-ELISA these patients ranged from <0.5 to 15.9% of the normal control (Fig. 1B, shown by arrows). Because the two types of assays were in good agreement, plasma levels of ADAMTS13:AC described in this paper are the values determined by act-ELISA unless otherwise noted.

Furthermore, the plasma levels of ADAMTS13:AG were highly correlated with ADAMTS13:AC (r=0.715, p<0.001) (Fig. 1C). The levels of ADAMTS13:AG were significantly lower in LC patients (52 ± 33%), than in healthy subjects (p<0.001) and patients with chronic hepatitis (81 ± 28%, p<0.001). These values also decreased with increasing cirrhosis severity (Child A, 74 ± 36%; Child B, 55 ± 29%, p<0.05; Child C, 30 ± 15%, p<0.001) (Fig. 1D).

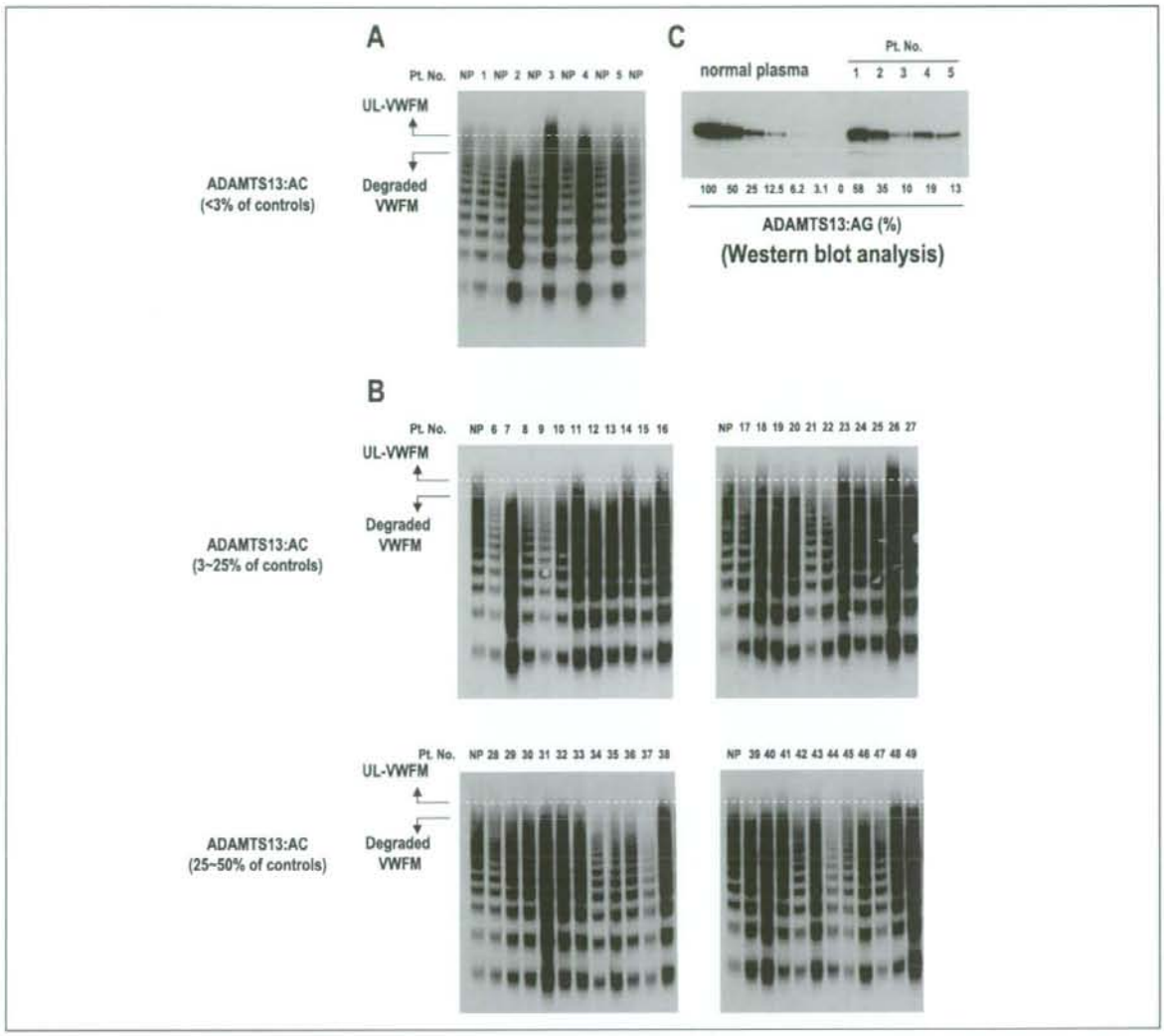

Figure 4: Plasma VWF multimer in 49 LC patients with severe to mild deficiency of ADAMTS13:AC and Western blot analysis of ADAMTS13 in five LC patients with severe deficiency of ADAMTS13:AC. The VWF multimer was analyzed by a vertical SDS-1.0% agarose gel electrophoresis system (A, B). Five patients (patients no. 1–5) (A) were originally identified as a severe deficiency of plasma ADAMTS13:AC by the VWFM assay. Twenty-two patients (patients nos. 6–27) showed a moderate deficiency (3–25% of the control), and the remaining 22 patients (nos. 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 nos. 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. Panel C showed the Western blot analysis of ADAMTS13:AG, which was performed using a murine anti-ADAMTS13 monoclonal antibody. ADAMTS13:AG was quantified from 10% to 58% in five LC patients according to the intensity of blotting using NIH image J. The left part of panel C shows a standard curve using diluted normal plasma.

Plasma levels of ADAMTS13:AC versus clinical variables

In order to investigate the effects of HCC complicated with LC on plasma levels of ADAMTS13:AC, the patients were divided into two groups, with and without HCC. As shown in Figure 2A, patients of Child A LC patients with HCC had a significantly lower ADAMTS13:AC than those without HCC (69 ± 22% versus 103 ± 11%, $p<0.001$), but there were no differences between Child B and Child C LC patients with and without HCC. Furthermore, as shown in Figure 2B, plasma ADAMTS13:AC levels were lower in patients with JIS score 4 (32 ± 28%, $p<0.05$) and score 5 (26 ± 29%, $p<0.05$) than in patients with score 0 (67 ±

Table 3: Comparison of clinical parameters among cirrhotic patients according to VWF multimer patterns.

Variables	VWF multimer pattern			A vs. B	A vs. C	B vs. C
	degraded[a]	normal[b]	unusually large[c]			
	n=15	n=26	n=8			
ADAMTS13:AC (%) (act-ELISA)	47 ± 24	44 ± 13	26 ± 14	n.s.	p<0.05	p<0.01
VWF:RCo (%)	110 ± 92	196 ± 134	216 ± 110	p<0.05	p<0.05	n.s.
Child-Pugh score	8.6 ± 2.5	10.9 ± 2.1	12.4 ± 1.7	p<0.01	p<0.005	n.s.
Serum albumin (g/dl)	3.07 ± 0.54	2.85 ± 0.54	2.59 ± 0.25	n.s.	p<0.05	n.s.
Cholinesterase (IU/l)	126 ± 62	78 ± 64	60 ± 36	p<0.05	p<0.02	n.s.
Total cholesterol (mg/dl)	142 ± 51	93 ± 45	88 ± 40	p<0.01	p<0.03	n.s.
Hemoglobin (g/dl)	11.0 ± 1.7	9.3 ± 2.0	8.9 ± 1.7	p<0.02	p<0.02	n.s.
Serum creatinine (mg/dl)	1.06 ± 0.72	1.11 ± 0.79	2.43 ± 2.16	n.s.	p<0.05	p<0.03
Blood urea nitrogen (mg/dl)	22 ± 17	30 ± 21	74 ± 62	n.s.	p<0.01	p<0.01
Blood ammonia (μg/dl)	87 ± 50	100 ± 39	144 ± 53	n.s.	p<0.05	p<0.05

VWF, von Willebrand factor; ADAMTS13:AC, ADAMTS13 activity; ELISA, enzyme-linked immunosorbent assay; VWF:RCo, VWF ristocetin cofactor activity; n.s., not significant.

24%), score 1 (65 ± 37%), and score 2 (69 ± 22%). In addition, the levels of ADAMTS13:AC were significantly lower in LC patients with the following clinical conditions than those without; hepatic encephalopathy (28 ± 24% versus 71 ± 29%, p<0.001), hepatorenal syndrome (13 ± 12% versus 61 ± 33%, p<0.001), and severe esophageal varices (46 ± 33% versus 75 ± 34%, p<0.05). Moreover, patients with refractory ascites had lower ADAMTS13:AC (25 ± 17%) than those without ascites (70 ± 30%, p<0.001) or those with easily mobilized ascites (56 ± 37%, p<0.001).

We next performed a univariate analysis using all patients with chronic hepatitis and liver cirrhosis, excluding a patient with TTP, and the 17 examined variables significantly correlated with ADAMTS13:AC (Table 2). We further performed a multivariate analysis using all significant baseline parameters determined by the univariate analysis except Child-Pugh score. Spleen volume, blood ammonia, and serum creatinine independently correlated with ADAMTS13:AC (r=0.690, p<0.0001). As a second step, the three parameters that contribute to the Child-Pugh classification (total bilirubin, albumin, and prothrombin time) were replaced by the Child-Pugh score. As a result, the Child-Pugh score and spleen volume were independently selected (r=0.548, p<0.0001).

Plasma levels of VWF:Ag and VWF:RCo, the ratios of VWF:RCo/VWF:Ag and VWF:RCo/ADAMTS13:AC, and platelet count

Plasma levels of VWF:Ag were significantly higher in LC patients (421±249%) than in patients with chronic hepatitis (245 ± 135%, p<0.001) and healthy subjects (p<0.001) (Fig. 3A); in subgroups of LC patients, it was 320 ± 174% in Child A, 436 ± 267% (p<0.05) in Child B, and 486 ± 254% (p<0.01) in Child C. Like VWF:Ag, plasma levels of VWF:RCo were higher in LC patients (205 ± 168%) than in patients with chronic hepatitis (101 ± 86%, p<0.005) and healthy subjects (100 ± 15%, p<0.01), but did not differ among subgroups of LC patients: 186 ± 137% in Child A, 198 ± 172% in Child B, and 227 ± 187% in Child C (Fig. 3B).

The ratio of VWF:RCo to VWF:Ag was lower in patients with chronic hepatitis (0.45 ± 0.27, p<0.001) and LC (0.54 ± 0.45, p<0.001) than in healthy subjects (1.1±0.42), but no differences were found among LC patients with Child A (0.63±0.49), Child B (0.50±0.46) and Child C (0.51±0.40) (Fig. 3C). In contrast, the ratio of VWF:RCo to ADAMTS13:AC significantly increased with the progression of liver diseases (0.9 ± 0.2 in healthy subjects; 0.7 ± 0.5 in chronic hepatitis; 1.6 ± 1.7 in Child A; 5.0 ± 5.7 in Child B; and 16.8 ± 28.2 in Child C) (Fig. 3D). On the other hand, platelet count decreased with the severity of chronic liver diseases: 15.9 ± 4.8 x10^4/mm^3 in chronic hepatitis; 9.6 ± 4.6 x10^4/mm^3 in Child A; 6.9 ± 2.4 x10^4/mm^3 in Child B; and 6.0 ± 2.3 x10^4/mm^3 in Child C.

Clinical and laboratory characteristics of 49 LC patients with a severe to mild reduction of plasma ADAMTS13:AC

Five patients (patients no. 1–5, Fig. 4A) were originally identified as being severely deficient in plasma ADAMTS13:AC by the VWFM assay. Twenty-two patients (patient no. 6–27, Fig. 4B) showed a moderate deficiency (3–25% of the control), and the remaining 22 patients (no. 28–49, Fig. 4B) exhibited a mild reduction (25–50% of the control) of plasma ADAMTS13:AC by both the methods of VWFM assay and the act-ELISA, without discordant results. Out of these 49 patients, the VWFM analysis (Fig. 4A, B) revealed that 26 (53.1%) had normal VWFMs, 15 (30.6%) had degraded-VWFMs, and the remaining eight (patients no. 3, 4, 11, 14, 16, 18, 23, and 26) (16.3%) had UL-VWFMs. With respect to the comparison of clinical parameters according to VWFM patterns, UL-VWFMs-positive patients showed the lowest ADAMTS13:AC, and the highest values of serum creatinine, blood urea nitrogen, and blood ammonia (Table 3). In addition, LC patients with UL- and normal-VWFMs showed higher levels of VWF:RCo and Child-Pugh score, and lower values of cholinesterase, total cholesterol, and hemoglobin than those with degraded-VWFMs (Table 3).

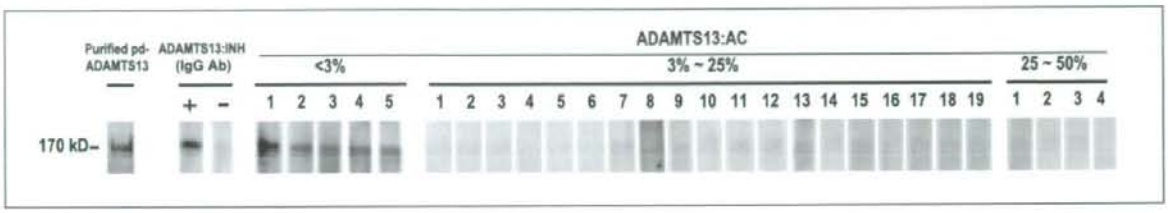

Figure 5: Detection of IgG-type autoantibodies specific to plasma derived-ADAMTS13 by Western blot. To detect IgG-type autoantibodies against ADAMTS13, we performed Western blot analysis using plasma derived (pd)-ADAMTS13 purified by A10-agarose immunoaffinity chromatography as described in *Materials and methods*. Heated plasma from a patient with acquired idiopathic TTP with IgG inhibitors against ADAMTS13 was used as a positive control, while that from a normal individual without ADAMTS13:INH was used as a negative control. Of the 28 LC patients with ADAMTS13:INH, five LC patients with severe ADAMTS13:AC deficiency (<3% of controls) exhibited in plasma a 170 kD-band, indicating the presence of IgG autoantibodies specific for purified pd-ADAMTS13 under non-reducing conditions. However, the IgG inhibitor against ADAMTS13 was not detected in the remaining 23 LC patients with moderate or mild deficiency of ADAMTS13:AC. pd=plasma derived, ADAMTS13:INH=ADAMTS13 inhibitor, IgG=immunoglobulin G, Ab=antibody, ADAMTS13:AC=ADAMTS13 activity.

ADAMTS13:INH and detection of IgG-type autoantibodies specific to pd-ADAMTS13 by WB

ADAMTS13:INH was detected in all five patients with severe ADAMTS13:AC deficiency (<3%), in 19 (86.4%) of 22 LC patients with moderate deficiency (3–25%), and in four (18.2%) of 22 LC patients with mild ADAMTS13:AC deficiency (25–50%). The inhibitory activity was 2.0 BU/ml (21) in a LC patient with TTP, and 3.0 BU/ml in a patient with severe ADAMTS13:AC deficiency, but in remaining patients the inhibitory activity showed marginal zone between 0.5 and 1.0 BU/ml. Of the 28 LC patients with ADAMTS13:INH, five LC patients with severe ADAMTS13:AC deficiency (<3%) exhibited in plasma a 170 kD-band, indicating the presence of IgG autoantibodies specific for purified pd-ADAMTS13 under non-reducing conditions (Fig. 5). However, the IgG inhibitor against ADAMTS13 was not detected in the remaining 23 LC patients with moderate or mild deficiency of ADAMTS13:AC (Fig. 5).

Clinical and laboratory characteristics of five LC patients with extremely low levels of plasma ADAMTS13:AC determined by the VWFM assay

All five patients with extremely low levels of plasma ADAMTS13:AC displayed signs of end stage liver disease (Table 4). Case 1 was associated with TTP (21) and case 2 had HRS and liver abscess. Case 3 was complicated by SBP and HRS, case 4 with direct invasion of HCC to transverse colon, and case 5 with SBP, HRS and portal thrombus. Cases 2, 4, and 5 had a marked inflammatory reaction, and Cases 2 and 4 showed a high concentration of IL-6. Plasma ADAMTS13:AC ranged from <0.5% to 15.9% as determined by act-ELISA. The plasma levels of ADAMTS13:AC determined by act-ELISA were in good accordance with those of plasma ADAMTS13:AG quantified both by the antigen-ELISA and by the Western blot analysis in cases 3, 4, and 5 (Table 4, Fig. 4C), but there was some discrepancy between the activity and antigen of ADAMTS13 in cases 1 and 2. UL-VWFM was detected in cases 3 and 4, whose VWF:RCo and the ratio of VWF:RCo to VWF:Ag was high (1.0, 0.8), whereas in the other three patients without UL-VWFM (cases 1, 2, and 5), VWF:RCo did not increase and the ratios were low (0.2–0.3). In contrast, cases 1 and 2 showed degraded high-molecular-VWF multimers, which are present in normal plasmas. The ADAMTS13:INH levels in these five patients ranged from 0.5 to 3.0 BU/ml, and the inhibitor showed the IgG-type autoantibodies specific to pd-ADAMTS13 (Fig. 5).

Plasma cytokine levels

Plasma IL-6 concentrations were the highest in patients with Child C (51 ± 78 pg/ml) as compared with Child A (10 ± 5 pg/ml, $p<0.05$), Child B (10 ± 6 pg/ml, $p<0.05$), and chronic hepatitis (<7.8 pg/ml, $p<0.05$), but IL-8 and TNF-α were below the detection limits in all patients. The levels of IL-6 in the 10 LC patients with SBP were among the highest (189 ± 403 pg/ml). In addition, there was a weak negative correlation between plasma levels of ADAMTS13:AC and IL-6 concentration in patients with chronic liver diseases ($r=-0.267$, $p<0.002$) (Table 2).

Discussion

Until now, it has not been clear whether plasma ADAMTS13:AC would decrease in cirrhosis patients (17, 22, 23). In this study, we clearly demonstrated that plasma ADAMTS13:AC decreased with increasing cirrhosis severity in a large number of patients (Fig. 1B). We used two different techniques to measure ADAMTS13:AC, and values determined by the act-ELISA correlated well with those quantified by the VWFM assay (Fig. 1A). Additionally, plasma ADAMTS13:AC was closely correlated with plasma ADAMTS13:AG determined by the antigen-ELISA (Fig. 1C, D) as described in TTP patients (40, 41), confirming that both ADAMTS13 activity and antigen decreased with increasing cirrhosis severity. In contrast, Lisman et al. reported that both ADAMTS13 activity and antigen levels were highly variable; they did not distinguish between patients with varying degrees of cirrhosis (22). It is unclear why these studies reached differing conclusions, but one possible explanation relates to differences in disease etiology: a majority of our patients developed cirrhosis secondary to HCV infection, whereas in the Lisman et al. study, half of the patients suffered from alcohol abuse related cirrhosis. Alternatively, the techniques used to determine ADAMTS13:AC differed between our study (24, 28, 33) and

Table 4: Clinical characteristics in five cirrhotic patients with severe deficiency of ADAMTS13 determined by VWFM assay.

Pt.	Age/ Sex	Etiology	Child-Pugh Class/ Score	ADAMTS13:AC (%)		ADAMTS13:AG (%)		ADAMTS13: INH		VWF: Ag (%)	VWF: RCo (%)	VWF: RCo/ VWF:Ag	VWF: RCo/ ADAMTS13:AC	VWFM pattern
				VWFM assay	act-ELISA	ELISA	WB	act-ELISA (BU/ml)	WB (IgG)					
1	65/M	HCV	C/14	<3	6.4	13	58	2.0	+	155	25	0.2	3.9	degraded
2	64/M	Crypto-genic	C/10	<3	<0.5	28	35	3.0	+	332	75	0.2	>150	degraded
3	70/M	PBC	C/13	<3	6.3	10	10	1.0	+	277	280	1.0	44.4	unusually large
4	75/M	HCV	C/11	3	10.3	14	19	0.8	+	528	420	0.8	40.8	unusually large
5	70/M	HBV	C/14	3	15.9	17	13	0.5	+	305	84	0.3	5.3	normal

Pt.	Hb (g/dl)	WBC (/mm^3)	Plat. (10^4/ mm^3)	CRP (mg/dl)	IL-6 (pg/ml)	IL-8 (pg/ml)	TNFα (pg/ml)	SBP	HRS	JIS score	Remarks
1	5.1	7800	0.4	0.3	17	<15.6	<15.6	-	-	-	thrombotic thrombocytopenic purpura
2	11.0	18600	8.3	23.4	455	18.2	<15.6	-	+	3	liver abscess, diabetes mellitus, hypertension
3	9.6	3800	8.8	1.5	54	<15.6	<15.6	+	+	-	
4	9.2	8300	7.7	4.6	110	<15.6	<15.6	-	-	5	direct invasion of HCC to transverse colon
5	7.1	10300	4.0	11.7	12	<15.6	<15.6	+	+	4	portal thrombus, diabetes mellitus

HCV, hepatitis C virus; HBV, hepatitis B virus; PBC, primary biliary cirrhosis; ADAMTS13:AC, ADAMTS13 activity; ADAMTS13:AG, ADAMTS13 antigen; ADAMTS13:INH, ADAMTS13 inhibitor; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity; VWFM, von Willebrand factor multimer; ELISA, enzyme-linked immunosorbent assay; WB, Western blot; BU, Bethesda Units; Hb, hemoglobin; WBC, white blood cell count; Plat, platelet count; CRP, C-reactive protein; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrosis factor-α; SBP, spontaneous bacterial peritonitis; HRS, hepatorenal syndrome; JIS, The Japan Integrated Staging score obtained via the summation of Child-Pugh score and tumor stage score (32); HCC, hepatocellular carcinoma.

theirs, which might explain the high variability they observed. They used the collagen binding assay, which can be influenced by the increase in plasma VWF:Ag usually found in LC patients (23), whereas our act-ELISA is neither influenced by a large amount of VWF:AG nor by hyperbilirubinemia (28). Our present data indicating that ADAMTS13:AC decreases with increasing cirrhosis severity are consistent with those recently reported by Feys et al. (23) who analyzed ADAMTS13:AC using modified collagen binding methods.

Additionally, in our study plasma ADAMTS13:AC was remarkably low in LC patients with hepatic encephalopathy, hepatorenal syndrome and refractory ascites. Furthermore, the decreased activity was also seen in patients with higher JIS scores (4 to 5), indicating that ADAMTS13:AC was markedly reduced when cirrhosis was complicated by diffuse HCC (Fig. 2). It was recently reported that disseminated malignancies are associated with a moderately decreased ADAMTS13:AC (15% of the control), implying that a mechanism regulating the primary platelet-tumor adhesive interactions is involved in the metastatic process (42). In addition, using univariate analysis, we showed that ADAMTS13:AC significantly correlated with 17 clinical variables (Table 2). Among these factors excluding Child-Pugh score, multivariate analysis identified spleen volume, blood ammonia and serum creatinine as the independent factors contributing to the changes in ADAMTS13:AC. When Child-Pugh score was incorporated into the analysis instead of the three parameters used to generate the score, the Child-Pugh score and spleen volume were independently selected, indicating that ADAMTS13:AC is closely related to the severity of liver disease and splenomegaly in cirrhotic patients.

Alternatively, the plasma levels of VWF:Ag were increased by 2.5-fold in patients with chronic hepatitis, and 3– to 5-fold in both early and advanced LC (Fig. 3A), as previously reported (9, 10). The marked elevation of plasma VWF:Ag in LC patients may be partly attributable to increased endothelial production induced by endotoxin (9, 43) and/or increased synthesis by extrahepatic endothelial cells (44). The VWF:RCo was higher (Fig. 3B), but the VWF:RCo relative to VWF:Ag was lower in LC patients (Fig. 3C) than in healthy subjects, indicating that increased VWF:Ag appears less functional in cirrhosis patients. These findings are consistent with previous reports (22). Nevertheless, it is interesting to note that the VWF:RCo relative to ADAMTS13:AC progressively increased with worsening chronic liver diseases (Fig. 3D), suggesting an enhanced thrombogenesis as liver dysfunction and thrombocytopenia progresses (Fig. 3E). We found that decreased platelet counts were coincident with decreased plasma ADAMTS13:AC (Table 2), which suggests an additional mechanism for thrombocytopenia related to hypercoagulability in patients with advanced cirrhosis, distinct from hypersplenism (11) and decreased production of thrombopoietin (12, 13).

There were three different VWFM patterns in 49 LC patients with lower ADAMTS13:AC (<50 % of controls): normal-VWFM was detected in 53%, degraded-VWFM in 31%, and

UL-VWFM in 16% (Fig. 4). UL-VWFM-positive patients showed the lowest ADAMTS13:AC, and the highest levels of serum creatinine, blood urea nitrogen, and blood ammonia (Table 3). Furthermore, LC patients with UL- and normal-VWFM showed higher levels of VWF:RCo, lower functional liver capacity, and a higher prevalence of anemia relative to those with degraded-VWFM (Table 3). From these results, plasma VWFM appears to shift from degraded- to normal-VWFM, and finally to the UL-VWFM as functional liver capacity and renal function deteriorates. ADAMTS13 may be gradually consumed and decreased, resulting in increased VWF:Ag concomitant with decreased ADAMTS13:AC, leading to an appearance of UL-VWFM in advanced cirrhosis. Taken together, advanced cirrhosis may be a predisposing state for platelet microthrombi formation, even in the absence of clinically overt thrombotic events. In fact, portal or hepatic vein thrombosis is often observed in advanced LC patients routinely screened with Doppler ultrasound (14), as well as in cirrhotic liver tissue removed at transplantation (15). Moreover, microthrombi were found in one or more organs in half of cirrhotic livers at autopsy (16), which is consistent with our hypothesis.

Remarkably, using the VWFM assay we found severe deficiency of ADAMTS13:AC (<3% of controls) corresponding to the level of TTP in five LC patients with end-stage liver disease. One of them showed apparent TTP (21), and others were complicated by HRS, SBP, a marked inflammation together with cytokinemia, and advanced HCC (Table 4). In general, various clinical conditions, including infection, malignancies, and certain drugs, can lead to acquired TTP (45). In advanced cirrhosis, endotoxemia is frequently detected (9), and SBP sometimes occurs (31). HCC is highly complicated as the cirrhotic stage progresses (46), indicating a high risk state for platelet microthrombi formation. Furthermore, in cases 3, 4 and 5, the ADAMTS13:AC by the act-ELISA were in good accordance with ADAMTS13:AG (Table 4, Fig. 4C), but in cases 1 and 2, there was some discrepancy between them, indicating that ADAMTS13:AG was considerably present in these cases, even if the activity was extremely low.

Plasma ADAMTS13:AC may decrease in advanced cirrhosis due to decreased production of ADAMTS13 in HSCs (47), enhanced consumption to degrade large quantities of VWF:Ag, and/or its plasma inhibitor (5, 6), the binding site of which is considered to be cysteine-rich/spacer domains (48). We observed plasma ADAMTS13:INH in 83% of patients with severe to moderate ADAMTS13:AC deficiency, but its inhibitory activity showed marginal zone between 0.5 and 1.0 BU/ml in most cases except a TTP patient (2.0 BU/ml) (Table 4, case 1) (21) and a patient with severe ADAMTS13:AC deficiency (3.0 BU/ml) (Table 4, case 2). Remarkably, we could detect the IgG-type autoantibodies specific to purified pd-ADAMTS13 by Western blot in five end-stage LC patients with severe ADAMTS13:AC deficiency (<3%) (Fig. 5), as described in TTP (5, 6, 49), but not in those with moderate or mild deficiency of the protease (Fig. 5). One of them (case 1, Table 4) certainly had characteristic clinical features of TTP with the IgG inhibitor against ADAMTS13 (0.4 BU/mg IgG using purified IgG from the patient's plasma) as previously reported (21). The remaining four patients did not show any apparent clinical features of TTP, but seem to be indistinguishable from the typical TTP patient (Case 1, Table 4) from the points of ADAMTS13:AC and its inhibitor. These results indicate that some end-stage LC patients who have extremely low ADAMTS13:AC with the IgG inhibitor against ADAMTS13 might be under the condition similar to TTP, or might reflect "subclinical" TTP. 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 are significantly higher in patients with HCV (about one third of all cases) than in both healthy subjects and patients with HBV (50–52). The etiology of our five end-stage LC patients with IgG-type autoantibodies was HCV in two, HBV in one, PBC in one, and cryptogenic in one, indicating that the presence of the autoantibodies against ADAMTS13 might be more associated with the disease progression. The decrease in ADAMTS13:AC would mainly be attributable to a decreased synthesis of ADAMTS13 due to liver failure and/or enhanced consumption to degrade large quantities of VWF:Ag, but further studies are warranted in order to clarify which kind of inhibitor other than the IgG inhibitor would be involved in patients with lower ADAMTS13:AC.

In addition, ADAMTS13:AC negatively correlated with plasma IL-6 concentrations, which is thought to promote the decrease in the ADAMTS13:AC (53) *in vitro*. The inflammation caused by SBP, endotoxemia, or other processes may thus be an important factor precipitating the decreased plasma ADAMTS13:AC in advanced cirrhosis (54). A recent study suggested that in patients with sepsis-induced disseminated intravascular coagulation, decreased ADAMTS13:AC could act together with UL-VWFM to contribute to the development of renal failure (55). In our patients with hepatorenal syndrome frequently complicated with SBP, the ADAMTS13:AC was extremely low, suggesting that markedly decreased ADAMTS13:AC could be a precipitating for the development of hepatorenal syndrome.

In summary, both plasma ADAMTS13 activity and antigen levels decreased with increasing severity of liver cirrhosis. An imbalance between the decreased ADAMTS13:AC and its increased substrate may reflect the predisposing state for platelet thrombi formation in patients with advanced liver cirrhosis.

Abbreviations

ADAMTS, a disintegrin-like and metalloproteinase domain with thrombospondin type-1 motif; ADAMTS13:AC, ADAMTS13 activity; ADAMTS13:AG, ADAMTS13 antigen; ADAMTS13:INH, ADAMTS13 inhibitors; ELISA, enzyme-linked immunosorbent assay; HCC, hepatocellular carcinoma; IL-6, interleukin 6; IL-8, interleukin 8; JIS, Japan Integrated Staging; SBP, spontaneous bacterial peritonitis; TNF-α, tumor necrosis factor-α; TTP, thrombotic thrombocytopenic purpura; UL-VWFM, unusually large von Willebrand factor multimer; VWF, von Willebrand factor; VWF:AG, von Willebrand factor antigen; VWFM, VWF multimer; VWF:RCo, VWF ristocetin cofactor activity.