antiphospholipid antibody syndrome, and IgM autoantibodies are also detected in 18% of patients with systemic lupus erythematosus or antiphospholipid antibody syndrome. Moreover, 4% of healthy individuals are reported to have anti-ADAMTS13 IgG autoantibodies [22].

The pivotal epitopes of anti-ADAMTS13 autoantibodies reside in the spacer domain [23–28]. In the present study, to clarify the precise peptide sequences recognized by anti-ADAMTS13 IgG autoantibodies, we constructed a random cDNA fragment library expressing various peptides of ADAMTS13 on the surface of the lambda phage (ADAMTS13 phage library). We then screened the library using purified IgG immobilized on a microtiter plate or protein G beads in solution, and detected the specific peptide sequence in the spacer domain in 5 of 13 TTP patients. We next assessed the association of the specific autoantibody with the plasma levels of the ADAMTS13 antigen or activity, and the amounts of anti-ADAMTS13 IgG autoantibodies or the inhibitory autoantibody titer.

Materials and methods

Patient samples

Plasma IgG from 13 patients with acquired TTP were used for this screening. These samples were collected at the Mie University (P1 ~ 10) and Nara Medical University (P11 ~ 13) according to the guidelines of the Ethics Committees of each facility. The clinical features of the TTP patients are described in Table 1.

Construction of ADAMTS13 phage library

The ADAMTS13 phage library was constructed according to previously described methods [29]. Briefly, human wild-type ADAMTS13 cDNA cloned in pcDNA3.1/ myc-His (Invitrogen, Carlsbad, CA) was digested with DNase I, blunted with T4 DNA polymerase and attached to SfiI adaptors. The ligated fragments were fractionated by 3% agarose gel electrophoresis and agarose-containing cDNA fragments from 80 to 160 base pairs were excised, purified, and inserted into the lambda fooDc phage vector digested with SfiI. The phage particles were then created with packaging mixtures (MaxPlax™ Lambda Packaging Extracts; EPICENTRE Biotechnologies, Madison, WI). The phage library was amplified using the *Escherichia coli* strain Q447 to approximately 1×10^7 plaque forming units and stored at 4 °C until screening was performed.

The library was grown with the *E. coli* strain TG1 until complete lysis. After centrifugation, the supernatant was incubated with DNase I and RNase. After another centrifugation, the phage particles were precipitated with polyethylene glycol solution on ice, collected by centrifugation, and resuspended in blocking buffer #1 (0.25% bovine serum albumin, 5% skim milk, 0.1% Tween20) or blocking buffer #2 (2.5% bovine serum albumin, 0.1% Tween20).

Library screening using IgG purified from TTP patients

The ADAMTS13 phage library screening and DNA sequence analysis were performed according to previously described methods [29]. The library was screened using IgG purified from TTP patients, either immobilized or in solution. For screening in the immobilized condition, serially diluted IgG was immobilized on the wells of a microtiter plate overnight at 4 °C. The wells were preblocked with blocking buffer #1 for 1 h at room temperature, then 50 μl of the ADAMTS13 phage library was added to the wells and incubated overnight at 4 °C. The wells were then washed three times with blocking buffer #1, twice with washing buffer #1 (5% skim milk, 0.5% Tween20), and once with washing buffer #2 (10 mM Tris-HCl pH7.4, 5 mM $MgSO_4$, 0.2 M NaCl, 10 mM $CaCl_2$). Bound phages were eluted with 50 μl of washing buffer #2 containing collagenase for 1 h at 37 °C. After the panning procedure was repeated five times, phages were randomly selected and subjected to DNA sequence analysis.

For the screening in solution, 10 μg of purified IgG from TTP patients was mixed with 25 μl of protein G beads (Dynabead® Protein G; Invitrogen) for 40 min at room temperature. The beads were then preblocked with blocking buffer #2 for 1 h at room temperature, mixed with 50 μl of the phage library, and incubated overnight at 4 °C. The beads were washed three times with blocking buffer #2, twice with washing buffer #1 (2.5% bovine serum albumin, 0.5% Tween20), and once with washing buffer #2 (10 mM Tris-HCl pH7.4, 5 mM $MgSO_4$, 0.2 M NaCl, 10 mM $CaCl_2$). The same procedure was performed for DNA sequence analysis as above.

ADAMTS13 antigen and activity levels, and IgG autoantibody titer in TTP plasma

The ADAMTS13 antigen level in the plasma of patients with TTP was measured using an enzyme linked immunosorbent assay (ELISA) kit (IMUBIND® ADAMTS13 ELISA; American Diagnostica, Stamford, CT) according to the manufacturer's protocol.

ADAMTS13 activity was measured using a FRETS-VWF73 assay (Peptide Institute, Inc., Osaka, Japan) [30] for P1 ~ 10 or an ADAMTS13 act-ELISA (Kainos Inc., Tokyo, Japan) [31] for P11 ~ 13.

The level of anti-ADAMTS13 IgG autoantibody was examined using an ELISA kit (IMUBIND® ADAMTS13 Autoantibody ELISA; American Diagnostica) according to the manufacturer's protocol. The inhibitory effect of the autoantibody was titrated using Bethesda units (BU), where one BU was defined to reduce the ADAMTS13 activity to 50% that in normal human plasma. Patient plasma was serially diluted and mixed with the same volume of normal human plasma. After incubation for 2 h

Table 1
Clinical characteristics and laboratory data of TTP patients. ADAMTS13 antigen and anti-ADAMTS13 IgG autoantibody titer are indicated as mean ± SD (n = 3). M, male; F, female; SLE, systemic lupus erythematosus; ND, not done.

Patient	Age	Sex	Context	ADAMTS13 antigen (ng/ml)	ADAMTS13 activity (%)	Anti-ADAMTS13 IgG autoantibody titer (μg/ml)	Inhibitory autoantibody titer (BU/ml)
1	28	M	SLE	203.4 ± 21.6	45.3	30.1 ± 5.0	not detected
2	61	M	SLE	86.7 ± 3.4	<3.0	40.5 ± 5.3	1.6
3	16	F	Idiopathic	12.6 ± 2.5	<3.0	43.3 ± 5.3	6.6
4	34	F	Idiopathic	51.9 ± 9.0	<3.0	30.4 ± 8.3	3.5
5	43	F	Idiopathic	4.3 ± 6.6	<3.0	41.4 ± 10.0	2.1
6	59	M	Idiopathic	24.1 ± 12.4	10.5	21.9 ± 5.7	2.5
7	79	M	Idiopathic	13.1 ± 5.6	<3.0	25.9 ± 4.6	0.6
8	45	F	SLE	1.9 ± 6.9	<3.0	37.4 ± 9.0	2.2
9	75	M	Idiopathic	11.6 ± 4.0	<3.0	25.6 ± 6.8	8.2
10	34	F	Idiopathic	76.0 ± 19.4	<3.0	27.1 ± 7.0	2.2
11	21	F	Idiopathic	ND	<3.0	ND	14
12	15	M	Idiopathic	ND	<3.0	ND	64
13	25	M	Idiopathic	ND	<3.0	ND	1.4

Table 2
Summary of results from the screening for ADAMTS13 peptide sequences binding to IgG from TTP patients. Functional domains of ADAMTS13 are shown with numbers of the first and last amino acid residue of each domain on top. Peptide sequences encoded by phage clones are listed with residue numbers of the N- and C-termini. The number in parenthesis indicates the number of identical phage clones obtained independently from one screening.

	Signal peptide, Propeptide	Metalloprotease	Dis-integrin	TSP1-1	Cysteine-rich	Spacer	TSP1-2-8	CUB1-2
	(1–74)	(75–289)	(290–385)	(386–439)	(440–555)	(556–685)	(686–1191)	(1192–1427)
P1				389-402	491-519	662-687 (2)	1023-1062	
P2			286-322		438-472	662-687 (2)	1067-1080	
					503-538			
P3		96-121					722-735	
P4			332-364			681-688		
P5								
P6						620-659	754-773	
						662-687 (4)		
P7		252-259				662-687 (4)	856-873	
							923-930	
P8		281-299					815-837 (2)	1215-1233
							1159-1182	
P9	1-11					662-687		
P10		96-121						
P11	1-9					617-657	690-709	
							927-943 (2)	
P12								
P13		202-218						
		224-244						

at 37 °C, residual ADAMTS13 activity in the mixture was measured using a FRETS-VWF73 assay.

Results

Binding sites of anti-ADAMTS13 autoantibodies

To define the epitopes of anti-ADAMTS13 IgG autoantibodies, the phage library expressing approximately 30 to 50 amino acids of the ADAMTS13 peptide sequence on the surface, was screened with IgG purified from 13 patients with acquired TTP. After 5 rounds of panning, 40 phage clones were picked from each screening and subjected to DNA sequence analysis. Results of the epitope mapping are summarized in Table 2. We detected various ADAMTS13 peptide sequences possibly recognized by IgG from 11 of the 13 TTP patients. The sequences came from almost entire domains except TSP1-6 and CUB2, and there seemed to be at least 2 to 4 recognition sites in each TTP patient. In the case of P1, for example, we obtained 4 peptide sequences, Ser389-Gly402 (TSP1-1), Gly491-Leu519 (cysteine-rich), Gly662-Val687 (spacer) and Pro1023-Glu1062 (TSP1-7). In particular, Gly662-Val687 in the spacer domain (designated as sp662-687) was detected independently from two different phage clones in the screening (the numbers of clones obtained are shown in parenthesis in Table 2). Moreover, the identical peptide sequence was repeatedly obtained from 4 other patients (P2, 6, 7, and 9), suggesting that sp662-687, the carboxyl-terminal sequence of the spacer domain, is one of the specific sites recognized by IgG obtained from patients with acquired TTP.

ADAMTS13 antigen and activity levels, and IgG titer in TTP plasma

Plasma ADAMTS13 antigen level and the anti-ADAMTS13 IgG autoantibody titer were measured using ELISA (Table 1). The antigen levels were all markedly low (1.9 to 86.7 ng/ml) except P1 (203.4 ng/ml). Of 13 samples, 11 had severely low levels of ADAMTS13 activity (< 3%), whereas P1 had 45.3% and P6 had 10.5%.

All the samples were anti-ADAMTS13 IgG titer positive (cut off: 9.6 μg/ml), with values ranging from 21.9 to 43.3 μg/ml (mean 32.4 μg/ml). The inhibitor assay revealed that 12 samples had positive inhibitory autoantibody titers, ranging from 0.6 to 64 BU/ml (mean 9.1 BU/ml), and P1 was negative.

Association of autoantibody to sp662-687 with ADAMTS13

The results of screening indicated an autoantibody bound to Gly662-Val687 in the C-terminus of spacer domain (sp662-687) was detected repeatedly in 5 of 13 patients (P1, P2, P6, P7, and P9). We thus speculated that sp662-687 would affect ADAMTS13 activity. Accordingly, we compared the antigen or activity levels and anti-ADAMTS13 IgG autoantibody or inhibitory autoantibody titers in sp662-687 positive or negative samples. The mean ADAMTS13 antigen, and anti-ADAMTS13 IgG autoantibody and inhibitory autoantibody titers of positive and negative samples were, respectively, 67.8 vs. 29.3 (ng/ml), 28.8 vs. 35.9 (μg/ml) and 2.6 vs. 12 (BU/ml) (Table 3). No significant differences were detected by Mann-Whitney's U-test ($p>0.05$; SPSS version 17.0, SPSS Inc, Chicago, IL). We could not evaluate the association of the ADAMTS13 activity and the sp662-687 autoantibody because 11 of 13 samples showed severely decreased ADAMTS13 activity (<3%).

Discussion

Both inhibitory and non-inhibitory autoantibodies to ADAMTS13 are associated with the pathogenesis of TTP [15–17,22]. The cysteine-rich/spacer domains are the main and common targets of the autoantibodies [9,23,28]. The spacer domain contains major antigenic sites, such as amino acid regions 572–579 and 657–666 [27], and the sites are recognized by specific autoantibodies produced by B cell clones [26]. Furthermore, one autoantibody type binds an epitope comprising

Table 3
Comparison of laboratory findings between autoantibody to sp662-687 positive and negative samples in TTP patients. ADAMTS13 antigen level or anti-ADAMTS13 IgG autoantibody titer were compared in 10 patients, while ADAMTS13 activity or inhibitory autoantibody titer were compared in all patients. The range of values is indicated in parenthesis.

Autoantibody to sp662-687	Positive	Negative
Mean value of ADAMTS13 antigen level (ng/ml)	67.8 (11.6-203.4)	29.3 (1.9-76.0)
Severe ADAMTS13 activity (<3%)	3/5	8/8
Mean value of anti-ADAMTS13 IgG autoantibody titer (μg/ml)	28.8 (21.9-40.5)	35.9 (27.1-43.4)
Mean value of inhibitory autoantibody titer (BU/ml)	2.6 (0–8.2)	12 (1.4-64)

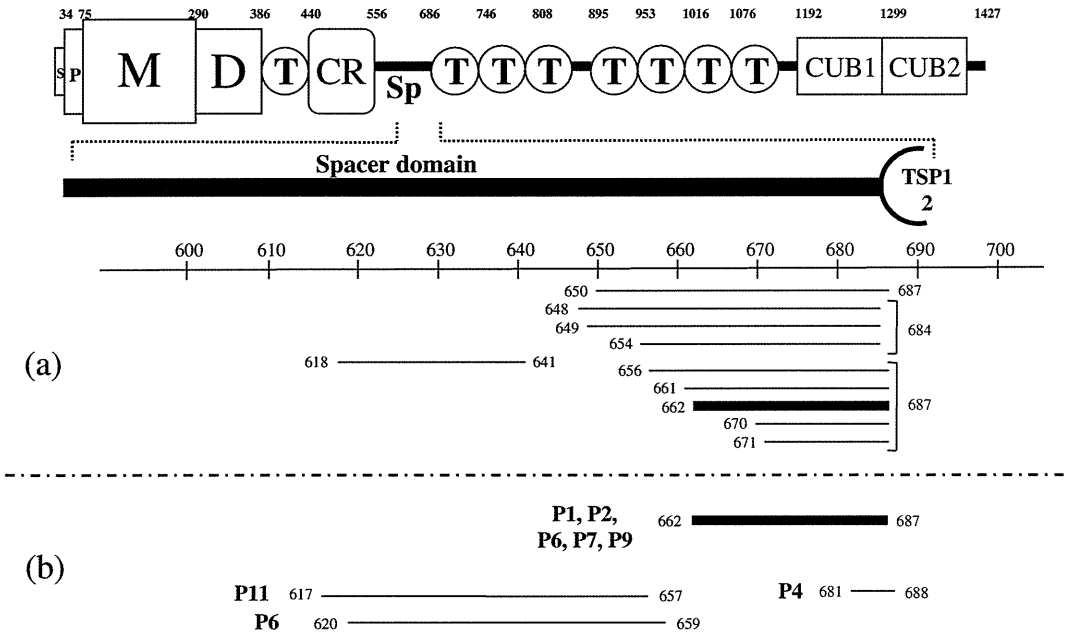

Fig. 1. Overview of peptide sequences in the ADAMTS13 spacer domain encoded by phage clones obtained from screenings for VWF binding sites and the current study. Molecular structure of ADAMTS13 is depicted as a chain of domains with the number of the first amino acid residue of each domain on top. Peptide sequences obtained from the screenings are indicated by horizontal lines with residue numbers of the N- and C-termini. Bold lines indicate an identical peptide sequence. The following abbreviations are used: S, signal peptide; P, propeptide; M, metalloprotease domain; D, disintegrin-like domain; T, thrombospondin-1 repeat; CR, cysteine-rich domain; Sp, spacer domain; and the CUB domain. (a) ADAMTS13 peptide sequences in the spacer domain binding to immobilized VWF [29]. (b) Results of the current screening for peptide sequences in the spacer domain binding to IgG from TTP patients. Of 13 TTP patients, 8 harboured IgG that bound to a peptide sequence that overlapped with VWF binding sequences. Of note, an identical sequence from Gly662 to Val687 (sp662-687) included in the VWF binding sequences was repeatedly detected in 5 patients (P1, P2, P6, P7, and P9).

Arg660, Tyr661, and Tyr665, which interacts with the A2 domain of VWF [32].

In the present study, we aimed to find major ADAMTS13 peptide sequences recognized by IgG autoantibodies in patients with acquired TTP. For this purpose, we constructed a lambda phage library expressing various peptide sequences of ADAMTS13 on its surface and screened it with IgG purified from 13 patients with acquired TTP. Several short peptide sequences of ADAMTS13 were detected from 11 patients. Screening IgG from P5 and P12 revealed no significant peptide sequences (Table 2), however, because other unrelated peptide sequences that were derived from plasmid, frame-shifted, or reversed DNA sequences bound predominantly to the patient IgG, resulting in a loss of targeted peptide sequences.

Multiple autoantibody binding sites were detected in almost all of the domains obtained from 10 TTP samples. Most of the sites were between the metalloprotease and spacer domains, which are the essential regions for the recognition and catalysis of VWF [9–12]. In particular, the peptide sequence from Gly662 to Val687 in the C-terminus of the spacer domain (sp662-687) was repeatedly detected in 5 patients (P1, P2, P6, P7, and P9, Table 2). Interestingly, sp662-687 was included in one of the VWF-binding epitope sequences that we reported previously [29] (Fig. 1). The spacer domain is considered essential for the specific binding of VWF. The recently published crystal structure of ADAMTS13 from the disintegrin-like domain to the spacer domain suggests that the peptide sequence from Tyr661 to Leu668 between the β9 and β10 sheets forms one of the loop structures that interact with the C-terminal α6 helix of the VWF A2 domain [33]. Arg659, Arg660, and Tyr661 are also critical for the cleavage of VWF [34], and Arg660, Tyr661, and Tyr665 are recognized by an autoantibody derived from patients with TTP [32]. Taken together, these findings indicate that the C-terminal portion of the spacer domain, especially the peptide sequences comprising the β9-β10 loop, is a major antigenic site for the production of autoantibodies. It is uncertain why, in the present study, sp662-687 contained the structure of the following β10 sheet and the initial peptide sequences of the TSP1-2 domain in addition to the β9-β10 loop. We speculate that the structure subsequent to the β9-β10 loop is concealed under the steady state, although exposed to the surface by a flexible conformation, leading to its recognition as an antigenic site.

Therefore, we assessed the impact of an autoantibody to sp662-687 on the plasma levels of ADAMTS13 antigens, ADAMTS13 activity, anti-ADAMTS13 IgG autoantibody and inhibitory autoantibody titers. Unfortunately, none of these measurements was associated with the presence or absence of a specific autoantibody. This result may indicates that the autoantibody itself does not affect the function of ADAMTS13, although it is produced as a result of ADAMTS13 degradation in the antigen-presenting cells in patients with TTP, suggesting that this autoantibody is a feature of TTP; however, other autoantibodies contribute to the inhibitory effect on the catalytic function. Because the phage surface display system used in this study could detect only limited peptide sequences recognized by the autoantibodies, there are likely more peptide sequences that are blocked by other autoantibodies, resulting in inhibition of the protein function.

In conclusion, we identified multiple binding sites of autoantibodies to ADAMTS13 in 11 of 13 patients with acquired TTP. In particular, an autoantibody to the C-terminal sequence of the spacer domain was repeatedly detected from 5 TTP patients, although the autoantibody was not likely associated with the inhibitory effect on the catalytic function. Further studies are required to determine other crucial binding sites recognized by the autoantibodies that directly block the protease function.

Conflict of interest statement

All authors have no conflict of interest.

Acknowledgements

This study was supported in part by a grant from Keio Gijuku Academic Development Funds (T.M.) and a grant from the Ministry of Health, Labour, and Welfare of Japan (M.M).

References

[1] Moake JL, Rudy CK, Troll JH, Weinstein MJ, Colannino NM, Azocar J, et al. Unusually large plasma factor VIII:von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. N Engl J Med 1982;307:1432–5.

[2] Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, et al. von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. N Engl J Med 1998;339:1578–84.

[3] Tsai HM, Lian EC. Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. N Engl J Med 1998;339:1585–94.

[4] Dong JF, Moake JL, Nolasco L, Bernardo A, Arceneaux W, Shrimpton CN, et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. Blood 2002;100: 4033–9.

[5] Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. Blood 2001;98:1662–6.

[6] Gerritsen HE, Robles R, Lammle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. Blood 2001;98:1654–61.

[7] Soejima K, Mimura N, Hirashima M, Maeda H, Hamamoto T, Nakagaki T, et al. A novel human metalloprotease synthesized in the liver and secreted into the Blood: possibly, the von Willebrand factor-cleaving protease? J Biochem 2001;130:475–80.

[8] Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. J Biol Chem 2001;276: 41059–63.

[9] Soejima K, Matsumoto M, Kokame K, Yagi H, Ishizashi H, Maeda H, et al. ADAMTS-13 cysteine-rich/spacer domains are functionally essential for von Willebrand factor cleavage. Blood 2003;102:3232–7.

[10] Zheng X, Nishio K, Majerus EM, Sadler JE. Cleavage of von Willebrand factor requires the spacer domain of the metalloprotease ADAMTS13. J Biol Chem 2003;278:30136–41.

[11] Ai J, Smith P, Wang S, Zhang P, Zheng XL. The proximal carboxyl-terminal domains of ADAMTS13 determine substrate specificity and are all required for cleavage of von Willebrand factor. J Biol Chem 2005;280:29428–34.

[12] Majerus EM, Anderson PJ, Sadler JE. Binding of ADAMTS13 to von Willebrand factor. J Biol Chem 2005;280:21773–8.

[13] Tao Z, Peng Y, Nolasco L, Cal S, Lopez-Otin C, Li R, et al. Recombinant CUB-1 domain polypeptide inhibits the cleavage of ULVWF strings by ADAMTS13 under flow conditions. Blood 2005;106:4139–45.

[14] Zhang P, Pan W, Rux AH, Sachais BS, Zheng XL. The cooperative activity between the carboxyl-terminal TSP1 repeats and the CUB domains of ADAMTS13 is crucial for recognition of von Willebrand factor under flow. Blood 2007;110:1887–94.

[15] Scheiflinger F, Knobl P, Trattner B, Plaimauer B, Mohr G, Dockal M, et al. Nonneutralizing IgM and IgG antibodies to von Willebrand factor-cleaving protease (ADAMTS-13) in a patient with thrombotic thrombocytopenic purpura. Blood 2003;102:3241–3.

[16] Shelat SG, Smith P, Ai J, Zheng XL. Inhibitory autoantibodies against ADAMTS-13 in patients with thrombotic thrombocytopenic purpura bind ADAMTS-13 protease and may accelerate its clearance in vivo. J Thromb Haemost 2006;4:1707–17.

[17] Ferrari S, Scheiflinger F, Rieger M, Mudde G, Wolf M, Coppo P, et al. Prognostic value of anti-ADAMTS 13 antibody features (Ig isotype, titer, and inhibitory effect) in a cohort of 35 adult French patients undergoing a first episode of thrombotic microangiopathy with undetectable ADAMTS 13 activity. Blood 2007;109:2815–22.

[18] Ferrari S, Mudde GC, Rieger M, Veyradier A, Hovinga JA, Scheiflinger F. IgG-subclass distribution of anti-ADAMTS13 antibodies in patients with acquired thrombotic thrombocytopenic purpura. J Thromb Haemost 2009;7:1703–10.

[19] Zheng XL, Kaufman RM, Goodnough LT, Sadler JE. Effect of plasma exchange on plasma ADAMTS13 metalloprotease activity, inhibitor level, and clinical outcome in patients with idiopathic and nonidiopathic thrombotic thrombocytopenic purpura. Blood 2004;103:4043–9.

[20] Coppo P, Wolf M, Veyradier A, Bussel A, Malot S, Millot GA, et al. Prognostic value of inhibitory anti-ADAMTS13 antibodies in adult-acquired thrombotic thrombocytopenic purpura. Br J Haematol 2006;132:66–74.

[21] Hovinga JA, Vesely SK, Terrell DR, Lammle B, George JN. Survival and relapse in patients with thrombotic thrombocytopenic purpura. Blood 2010;115:1500–11 quiz 1662.

[22] Rieger M, Mannucci PM, Hovinga JA, Herzog A, Gerstenbauer G, Konetschny C, et al. ADAMTS13 autoantibodies in patients with thrombotic microangiopathies and other immunomediated diseases. Blood 2005;106:1262–7.

[23] Klaus C, Plaimauer B, Studt JD, Dorner F, Lammle B, Mannucci PM, et al. Epitope mapping of ADAMTS13 autoantibodies in acquired thrombotic thrombocytopenic purpura. Blood 2004;103:4514–9.

[24] Luken BM, Turenhout EA, Hulstein JJ, Mourik JA, Fijnheer R, Voorberg J. The spacer domain of ADAMTS13 contains a major binding site for antibodies in patients with thrombotic thrombocytopenic purpura. Thromb Haemost 2005;93:267–74.

[25] Zhou W, Dong L, Ginsburg D, Bouhassira EE, Tsai HM. Enzymatically active ADAMTS13 variants are not inhibited by anti-ADAMTS13 autoantibodies: a novel therapeutic strategy? J Biol Chem 2005;280:39934–41.

[26] Luken BM, Kaijen PH, Turenhout EA, Hovinga JA, Mourik JA, Fijnheer R, et al. Multiple B-cell clones producing antibodies directed to the spacer and disintegrin/thrombospondin type-1 repeat 1 (TSP1) of ADAMTS13 in a patient with acquired thrombotic thrombocytopenic purpura. J Thromb Haemost 2006;4:2355–64.

[27] Luken BM, Turenhout EA, Kaijen PH, Greuter MJ, Pos W, Mourik JA, et al. Amino acid regions 572–579 and 657–666 of the spacer domain of ADAMTS13 provide a common antigenic core required for binding of antibodies in patients with acquired TTP. Thromb Haemost 2006;96:295–301.

[28] Zheng XL, Wu HM, Shang D, Falls E, Skipwith CG, Cataland SR, et al. Multiple domains of ADAMTS13 are targeted by autoantibodies against ADAMTS13 in patients with acquired idiopathic thrombotic thrombocytopenic purpura. Haematologica 2010;95:1555–61.

[29] Moriki T, Maruyama IN, Igari A, Ikeda Y, Murata M. Identification of ADAMTS13 peptide sequences binding to von Willebrand factor. Biochem Biophys Res Commun 2010;391:783–8.

[30] Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. Br J Haematol 2005;29:93–100.

[31] Kato S, Matsumoto M, Matsuyama T, Isonishi A, Hiura H, Fujimura Y. Novel monoclonal antibody-based enzyme immunoassay for determining plasma levels of ADAMTS13 activity. Transfusion 2006;46:1444–52.

[32] Pos W, Crawley JT, Fijnheer R, Voorberg J, Lane DA, Luken BM. An autoantibody epitope comprising residues R660, Y661, and Y665 in the ADAMTS13 spacer domain identifies a binding site for the A2 domain of VWF. Blood 2010;115: 1640–9.

[33] Akiyama M, Takeda S, Kokame K, Takagi J, Miyata T. Crystal structures of the noncatalytic domains of ADAMTS13 reveal multiple discontinuous exosites for von Willebrand factor. Proc Natl Acad Sci USA 2009;106:19274–9.

[34] Jin SY, Skipwith CG, Zheng XL. Amino acid residues Arg(659), Arg(660), and Tyr (661) in the spacer domain of ADAMTS13 are critical for cleavage of von Willebrand factor. Blood 2010;115:2300–10.

SAGE-Hindawi Access to Research
International Journal of Hepatology
Volume 2011, Article ID 759047, 12 pages
doi:10.4061/2011/759047

Review Article

Determination of ADAMTS13 and Its Clinical Significance for ADAMTS13 Supplementation Therapy to Improve the Survival of Patients with Decompensated Liver Cirrhosis

Masahito Uemura,[1] Yoshihiro Fujimura,[2] Saiho Ko,[3] Masanori Matsumoto,[2] Yoshiyuki Nakajima,[3] and Hiroshi Fukui[1]

[1] *Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan*
[2] *Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Nara 634-8522, Japan*
[3] *Department of Surgery, Nara Medical University, Kashihara, Nara 634-8522, Japan*

Correspondence should be addressed to Masahito Uemura, muemura@naramed-u.ac.jp

Received 25 February 2011; Accepted 8 April 2011

Academic Editor: Deepak Amarapurkar

The liver plays a central role in hemostasis by synthesizing clotting factors, coagulation inhibitors, and fibrinolytic proteins. Liver cirrhosis (LC), therefore, impacts on both primary and secondary hemostatic mechanisms. ADAMTS13 is a metalloproteinase, produced exclusively in hepatic stellate cells, and specifically cleaves unusually large von Willebrand factor multimers (UL-VWFM). Deficiency of ADAMTS13 results in accumulation of UL-VWFM, which induces platelet clumping or thrombi under high shear stress, followed by sinusoidal microcirculatory disturbances and subsequent progression of liver injuries, eventually leading to multiorgan failure. The marked imbalance between decreased ADAMTS13 activity (ADAMTS13 : AC) and increased production of UL-VWFM indicating a high-risk state of platelet microthrombi formation was closely related to functional liver capacity, hepatic encephalopathy, hepatorenal syndrome, and intractable ascites in advanced LC. Some end-stage LC patients with extremely low ADAMTS13 : AC and its IgG inhibitor may reflect conditions similar to thrombotic thrombocytopenic purpura (TTP) or may reflect "subclinical TTP." Hence, cirrhotic patients with severe to moderate deficiency of ADAMTS13 : AC may be candidates for FFP infusion as a source of ADAMTS13 or for recombinant ADAMTS13 supplementation. Such treatments may improve the survival of patients with decompensated LC.

1. Introduction

The liver is a major source of clotting and fibrinolytic proteins and plays a central role in thromboregulation [1–4]. Liver diseases, hence, impact on both primary and secondary hemostatic mechanisms. Because the hemostatic system is normally in a delicate balance between pro-hemostatic and antihemostatic processes, advanced liver cirrhosis (LC) patients experience multiple changes in the hemostatic system that may lead to either bleeding or thrombosis [1–4]. Despite clinical evidence of increasing bleeding tendency in LC patients, many facts indicate local and systemic hypercoagulability including portal or hepatic vein thrombosis, pulmonary embolism, and deep vein thrombosis, which are closely related to microcirculatory disturbances [4]. Deficiency of anticoagulant proteins and high levels of several procoagulant factors may favor hypercoagulability [4], but the mechanisms underlying this disorder have not been fully elucidated.

ADAMTS13 (**a** **d**isintegrin-like **a**nd **m**etalloproteinase with **th**rombo**s**pondin type-1 motifs **13**) is a metalloproteinase that specifically cleaves multimeric von Willebrand factor (VWF) between Tyr1605 and Met1606 residues in the A2 domain [5, 6]. In the absence of ADAMTS13 activity (ADAMTS13 : AC), unusually large VWF multimers (UL-VWFMs) are released from vascular endothelial cells (ECs) and improperly cleaved, causing them to accumulate and to induce the formation of platelet thrombi in the microvasculature under conditions of high shear stress. Currently, a severe deficiency in ADAMTS13 : AC, which results either

from genetic mutations in the *ADAMTS13* gene (Upshaw-Schulman syndrome, (USS)) [5–8] or acquired autoantibodies against ADAMTS13 [9, 10], is thought to be a specific feature of thrombotic thrombocytopenic purpura (TTP) [5–12].

In 2000, we demonstrated that a decreased plasma ADAMTS13 : AC in patients with cirrhotic biliary atresia can be fully restored after liver transplantation, indicating that the liver is the main organ producing ADAMTS13 [13]. One year later, northern blot analysis showed that the 4.6-kilobase ADAMTS13 mRNA was highly expressed in the liver [7, 14, 15], and subsequently both *in situ* hybridization and immunohistochemistry clearly indicated that ADAMTS13 is produced exclusively in hepatic stellate cells (HSCs) [16]. Platelets [17], vascular ECs [18], and kidney podocytes [19] have also been implicated as ADAMTS13-producing cells, but the amount produced by these cell types in the liver appears to be far less than that produced by HSC.

Mannucci et al. [20] originally reported a reduction of the ADAMTS13 : AC in advanced LC. Since HSCs were shown to be the major producing cells in the liver [16], much attention has been paid to the potential role of ADAMTS13 in the pathophysiology of liver diseases associated with sinusoidal and/or systemic microcirculatory disturbance [21–35]. ADAMTS13 : AC significantly decreased in patients with hepatic veno-occlusive disease (VOD) [22, 23], alcoholic hepatitis [24–27], liver cirrhosis [29, 30], and those undergoing living-donor-related liver transplantation [31–33] and partial hepatectomy [34]. Furthermore, hepatitis C virus- (HCV-) related LC patients with ADAMTS13 inhibitor (ADAMTS13 : INH) typically developed TTP [35]. Once patients with LC develop a decompensated condition, the risk of early mortality sharply increases for specific life-threatening complications such as ascites, hepatic encephalopathy, sepsis, hepatorenal syndrome, or hepatopulmonary syndrome [36].

In this paper, we will focus on the importance of ADAMTS13 determination for a better understanding of pathophysiology and/or for possible therapeutic approaches of ADAMTS13 supplementation to improve survival in patients with advanced LC.

2. Hepatic Microcirculation and Hypercoagulable State in LC

Hepatic microcirculation compromises a unique system of capillaries, called sinusoids, which are lined by three different cell types: sinusoidal endothelial cells (SECs), HSC, and Kupffer cells [37]. The SEC modulates microcirculation between hepatocytes and the sinusoidal space through the sinusoidal endothelial fenestration. The SEC has tremendous endocytic capacity, including VWF and the extracellular matrix, and secretes many vasoactive substances [37]. 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 [38]. Kupffer cells are intrasinusoidally located tissue macrophages and secrete potent inflammatory mediators during the early phase of liver inflammation [37]. Intimate cell-to-cell interaction has been found between these sinusoidal cells and hepatocytes [37, 38]. In LC, a sinusoidal microcirculatory disturbance occurs when the normal hepatic structure is disrupted by fibrin deposition [39] or by impaired balance between the action of vasoconstrictors and vasodilators in hepatic vascular circulation [37]. Studies have shown that cirrhotic liver exhibits a hyperresponse to vasoconstrictors, including catecholamine, endothelin, and leukotrienes D_4 [37].

Vascular endothelial cells play a pivotal role in hemostasis and thrombosis [5, 6]. VWF is a marker of endothelial cell activation (damage) and plays an essential role in hemostasis [5, 6]. In the normal state, VWF immunostaining is usually positive in large vessels but negative in the SEC [40]. 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 [39]. Subsequently, platelets adhere to subendothelial tissue mediated by UL-VWFM [5, 6]. ADAMTS13 then cleaves UL-VWFM into smaller VWF multimers [5, 6]. This interaction of ADAMTS13 and UL-VWFM is, indeed, the initial step in hemostasis [5, 6].

In patients with LC, circulating plasma VWF levels are extremely high [41, 42]. In liver tissue from cirrhotics [43] and even from the early stages of alcoholic liver diseases [44], VWF immunostaining shows positive cells predominantly at the scar-parenchyma interface, within the septum, and in the sinusoidal lining cells. Actually, portal or hepatic vein thrombosis is often observed in advanced LC routinely screened with Doppler ultrasound [45], and, in cirrhotic liver removed at transplantation, intimal fibrosis suggesting hepatic and portal vein thrombosis was frequently observed [46]. An autopsy series revealed microthrombi in one or multiple organs in one-half of cirrhotics [47]. Such a hypercoagulable state in liver diseases may be involved in hepatic parenchymal destruction, the acceleration of liver fibrosis and disease progression [4], leading to hepatorenal syndrome, portopulmonary hypertension, and spontaneous bacterial peritonitis [48].

Systemically, deficiency of anticoagulant proteins (antithrombin, protein C, and protein S) and the high levels of several procoagulant factors (factor VIII and VWF) may contribute to hypercoagulability in patients with LC [4]. Locally, the SEC dysfunction could lead to the development of a hypercoagulable state at the hepatic sinusoids corresponding to the site of liver injury, even in the face of a systemic hypocoagulable state [4]. Considering that ADAMTS13 is synthesized in HSC and its substrate, UL-VWFM, is produced in transformed SEC during liver injury, decreased plasma ADAMTS13 : AC may involve not only sinusoidal microcirculatory disturbances, but also subsequent progression of liver diseases, finally leading to multiorgan failure. Based on these findings, it is of particular interest to evaluate the activity of plasma ADAMTS13 : AC in LC patients.

3. Cleavage of UL-VWFM by ADAMTS13

Although the mechanism by which TTP develops in the absence of ADAMTS13 : AC has not been fully elucidated,

accumulating evidence has provided a hypothesis as illustrated in Figure 1 [49]. UL-VWFMs are produced exclusively in vascular ECs and stored in an intracellular organelle termed Weidel-palade bodies (WPBs) and then released into the circulation upon stimulation. Under physiological conditions, epinephrine acts as an endogenous stimulus, but under nonphysiological conditions, DDAVP (1-deamino-8-D-arginine vasopressin), hypoxia, and several cytokines such as interleukin IL-2, IL-6, IL-8, and tumor necrosis factor- (TNF-) α act as stimuli that upregulate VWF release. Once ECs are stimulated, UL-VWFMs and P-selectin, both stored in WPBs, move to the membrane surface of ECs, where P-selectin anchors UL-VWFMs on the ECs surface [50]. Under these circumstances, high shear stress generated in the microvasculature induces a change in the UL-VWFM from a globular to an extended form [51]. The ADAMTS13 protease efficiently cleaves the active extended form of UL-VWFM between the Tyr1605 and Met1606 residues in the A2 domain [52]. In this context, it has been postulated that multiple exocites within the disintegrin-like/TSP1/cysteine-rich/spacer (DTCS) domains of ADAMTS13 play an important role in interacting with the unfolded VWF-A2 domain [53]. ADAMTS13 may more efficiently cleave newly released UL-VWFMs that exist as solid-phase enzymes anchored to the vascular EC surface by binding to CD36, because CD36 is a receptor for TSP1, which is a repeated domain within the ADAMTS13 molecule [54]. When ADAMTS13 activity is reduced, UL-VWFM interacts more intensively with platelet GPIb and generates signals that further accelerate platelet activation [5, 6]. A series of these reactions leads to platelet microaggregates and thrombocytopenia. However, little information has been available on the cleavage of the UL-VWFMs by ADAMTS13 in the sinusoidal microcirculation in LC.

4. Assays for Plasma ADAMTS13 : AC and ADAMTS13 : INH

ADAMTS13 : AC was determined with a classic VWFM assay in the presence of 1.5 mol/L urea using purified plasma-derived VWF as a substrate according to the method described by Furlan et al. [55], and the detection limit of this assay was 3% of the normal control in our laboratory [56]. In 2005, we developed a novel chromogenic ADAMTS13-act-ELISA using both an N- and C-terminal tagged recombinant VWF substrate (termed GST-VWF73-His). This assay was highly sensitive, and the detection limit was 0.5% of the normal control [57]. Plasma ADAMTS13 : AC levels highly correlated between VWFM assay and ADAMTS13-act-ELISA (mean $\pm$ SD, 102 $\pm$ 23% versus 99.1 $\pm$ 21.5%, $r^2 = 0.72$, $P < .01$) [57]. No interference of the ADAMTS13-act-ELISA occurred even in the presence of hemoglobin, bilirubin, or chylomicrons in the samples, thus enabling distinction from the FRETS-VWF73 assay [58]. Because of its high sensitivity, easy handling, and lack of interference from plasma components, the ADAMTS13-act-ELISA would be recommended for routine laboratory use.

The ADAMTS13 : INH has also been evaluated with the chromogenic act-ELISA by means of the Bethesda method [59]. Prior to the assay, the test samples were heat-treated at 56°C for 60 min to eliminate endogenous enzyme activity, mixed with an equal volume of intact normal pooled plasma, and incubated for 2 hours at 37°C. The residual enzyme activity is measured after incubation. One Bethesda unit is defined as the amount of inhibitor that reduces activity by 50% of the control value, and values greater than 0.5 U/mL are significant.

5. Thrombocytopenia, Determination of ADAMTS13 : AC, and Its Clinical Significance in LC

5.1. Thrombocytopenia. It is well accepted that thrombocytopenia gradually progresses as functional liver capacity decreases [30, 60] (Figure 2(a)). The pathogenesis of thrombocytopenia in LC includes splenic sequestration in portal hypertension [61], impaired platelet production due to decreased synthesis of thrombopoietin in the liver [62] or due to myelosupression resulting from HCV infection [63], folic acid deficiency, or ethanol chronic consumption [64], which has a negative effect on megacaryocytopoiesis. However, our recent studies have provided evidence that in patients with advanced LC, elevated plasma levels of UL-VWFM enhance high-shear stress-induced platelet aggregation, resulting in thrombocytopenia [30].

5.2. ADAMTS13 : AC. Our study showed that ADAMTS13 : AC decreased with increasing severity of cirrhosis [30] (Figure 2(b)). The values determined by act-ELISA correlated well with those of the classical VWFM assay and also closely correlated with ADAMTS13 antigen de-termined by the antigen-ELISA. These results confirmed that both ADAMTS13 activity and antigen decreased with increasing cirrhosis severity [30] (Figures 2(b) and 2(c)), which are consistent with findings described by Feys et al. [29]. In contrast, Lisman et al. showed that both ADAMTS13 activity and antigen levels were highly variable; however, they did not distinguish between patients with varying degrees of cirrhosis [28]. It is unclear why they reached different conclusions from ours. One possible explanation relates to different etiologies: a majority of our patients developed cirrhosis secondary to HCV infection, whereas in their study one-half of the patients suffered from alcohol abuse-related cirrhosis. Further, the techniques used to determine ADAMTS13 : AC differed between our study [55–57] and theirs [65]. 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 [29], 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 with the assays [57].

As shown in Figure 3, ADAMTS13 : ACs were significantly lower in LC patients with hepatic encephalopathy (Figure 3(a)), hepatorenal syndrome (Figure 3(b)), and

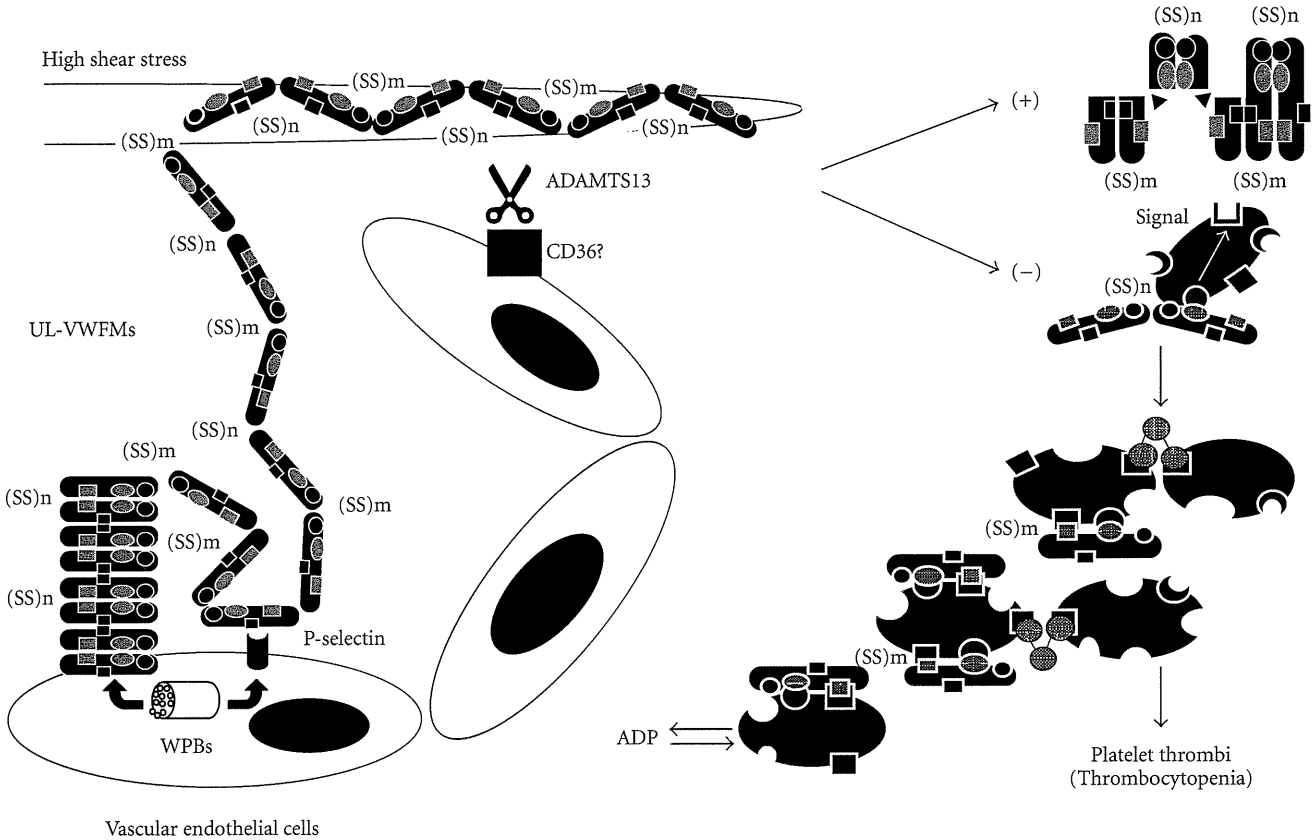

FIGURE 1: Proposed mechanism of platelet thrombi under high shear stress in the absence of ADAMTS13 : AC. Unusually large von Willebrand factor multimers (UL-VWFMs) are produced in vascular endothelial cells (ECs) and stored in Weidel-palade bodies (WPBs). UL-VWFMs are released from WPBs into the circulation upon stimulation by cytokines, hypoxia, DDAVP, and epinephrine. P-selectin that comigrates from WPBs anchors UL-VWFMs on the vascular EC surface. Under these circumstances, high shear stress changed the molecular conformation of UL-VWFMs from a globular to an extended form, allowing ADAMTS13 to access this molecule. In the absence of ADAMTS13 : AC, UL-VWFMs remain uncleaved, allowing them to excessively interact with platelet glycoprotein (GP)Ibα and activate platelets via intraplatelet signaling, which result in the formation of platelet thrombi. (Partially modified from Fujimura et al., [49]).

severe esophageal varices than those without [30]. Moreover, patients with refractory ascites had lower ADAMTS13 : AC levels than patients without ascites or those with easily mobilized ascites (Figure 3(c)). A multivariate analysis using all significant baseline parameters determined by the univariate analysis, excluding the Child-Pugh score, showed spleen volume, blood ammonia, and serum creatinine independently correlated with ADAMTS13 : AC. 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, indicating that ADAMTS13 : AC is closely related to the severity of liver disease and splenomegaly in cirrhotic patients [30].

5.3. VWF : Ag and VWF Multimer Patterns. Plasma levels of VWF : Ag substantially increase as liver diseases progress (Figure 2(d)) [30], as previously reported [41, 42]. This is presumably attributed to sinusoidal and/or extrahepatic endothelial damage induced by endotoxin and cytokines [41, 42, 66, 67]. The VWF : RCo was higher (Figure 2(e)) [30], but the ratio of VWF : RCo/VWF : Ag was lower in LC patients than that in healthy subjects. These findings suggest that increased VWF : Ag appears less functional in LC patients [30], which are consistent with previous reports [28]. Nevertheless, our study has clearly shown that the ratio of VWF : RCo/ADAMTS13 : AC progressively increases with the worsening of chronic liver diseases (Figure 2(f)), further intensifying an enhanced thrombogenesis with the progression of liver dysfunction and thrombocytopenia [30].

With regard to 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 [29]. We showed that there were three different VWFM patterns in LC patients with lower ADAMTS13 : AC (<50 % of controls): normal-VWFM was detected in 53%, degraded-VWFM in 31%, and UL-VWFM in 16% (Table 1) [30]. UL-VWFM-positive patients showed the lowest ADAMTS13 : AC and the highest values of serum creatinine, blood urea nitrogen, and blood ammonia. In addition, LC patients with UL- and normal-VWFM had higher levels of VWF : RCo

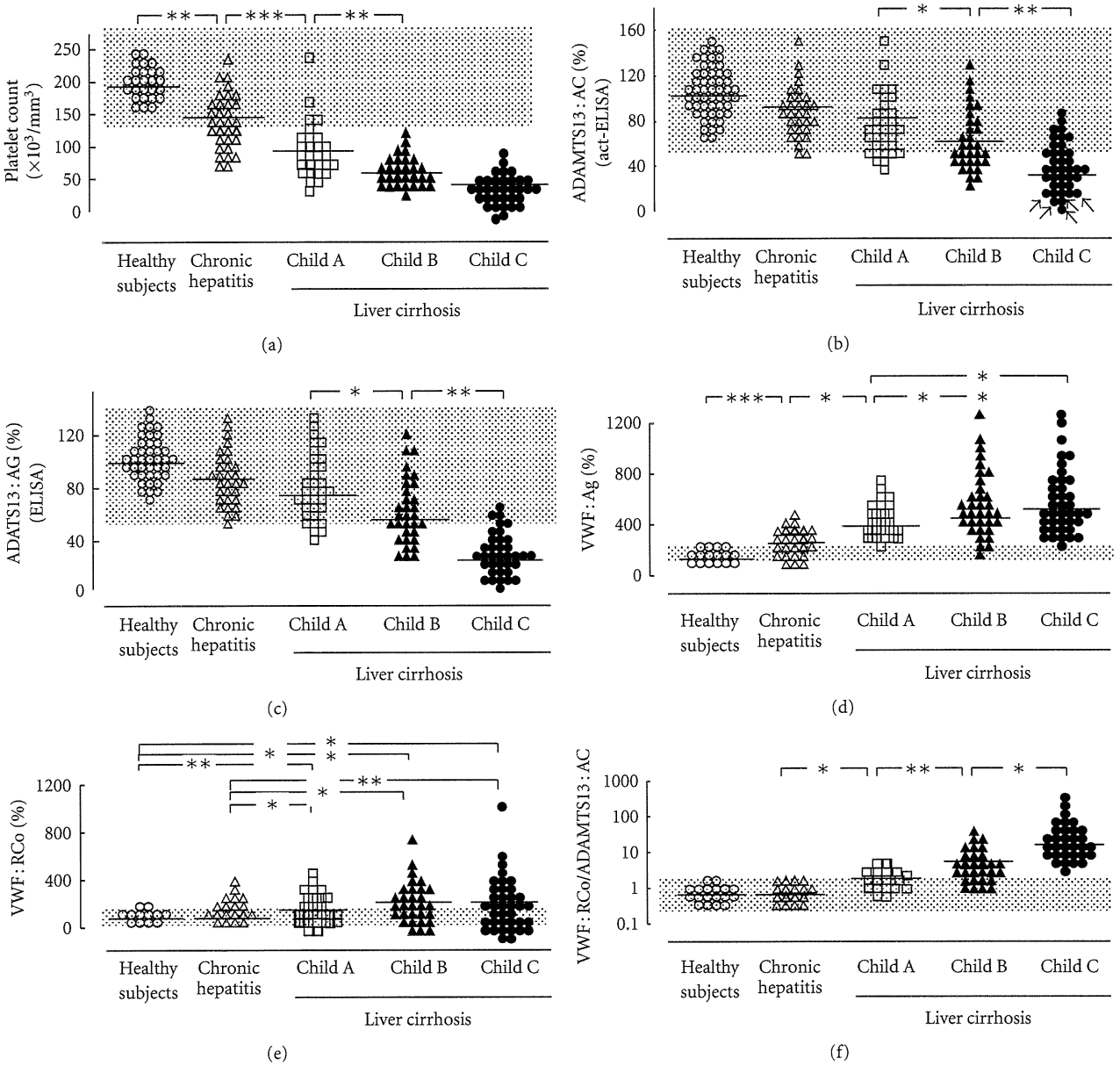

FIGURE 2: Platelet counts and plasma levels of ADAMTS13 : AC and its related parameters in patients with chronic liver diseases. Platelet counts decreased with the severity of chronic liver diseases, but no difference was found between Child B and C (a). Plasma ADAMTS13 : AC determined by ELISA progressively decreased with worsening cirrhosis (b). Arrows indicate patients whose plasma ADAMTS13 : AC was extremely low (< 3% of normal control by VWFM assay). 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 ($r = 0.715$, $P < .001$). The VWF : Ag increased with the progression of chronic liver diseases, but the difference between Child B and C did not reach statistical significance (d). The VWF : RCo is higher in liver cirrhosis patients than that in patients with chronic hepatitis and healthy subjects, but it did not differ among subgroups within liver cirrhosis (e). The VWF : RCo relative to ADAMTS13 : AC progressively increased with worsening chronic liver disease (f). 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. VWF : Ag = von Willebrand factor antigen, VWF : RCo = von Willebrand factor ristocetin cofactor activity; $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$ significantly different between the two groups. (Partially modified from Uemura et al., [30]).

and Child-Pugh score and lower values of cholinesterase and hemoglobin than those with degraded-VWFM [30] (Table 1). The pattern, therefore, appears to shift from degraded- to normal-VWFM, and finally to UL-VWFM as functional liver capacity and renal function deteriorates, indicating that advanced LC may be a predisposing state toward platelet microthrombi formation, even in the absence of clinically overt thrombotic events [30].

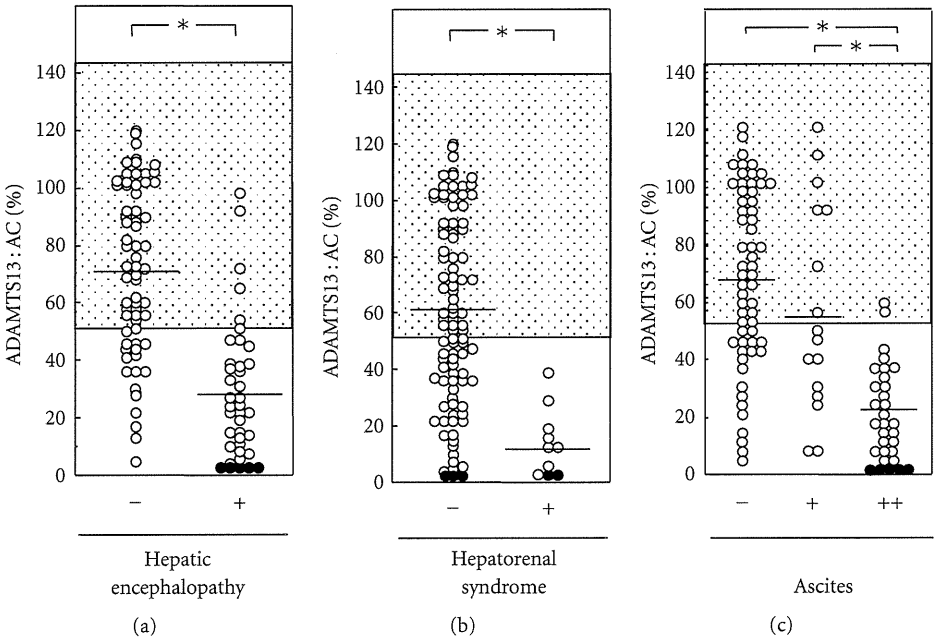

FIGURE 3: Relationship of ADAMTS13 : AC to the presence or absence of hepatic encephalopathy, hepatorenal syndrome, and ascites in patients with liver cirrhosis. The ADAMTS13 : AC was significantly lower in LC patients with hepatic encephalopathy (a) and hepatorenal syndrome (b) than that those without. Moreover, patients with refractory ascites had lower ADAMTS13 : AC than those without ascites or those with easily mobilized ascites (c). Closed circles indicate patients whose plasma ADAMTS13 : AC was extremely low (< 3% of normal control by VWFM assay). ADAMTS13 : AC: ADAMTS13 activity; $^*P < .001$ significantly different between the two groups. (Partially modified from Uemura et al., [30]).

TABLE 1: Comparison of clinical parameters among cirrhotic patients according to VWF multimer patterns.

Variables	VWF multimer pattrens			a versus b	a versus c	b versus c
	Degraded[a] ($n = 15$)	Normal[b] ($n = 26$)	Unusually large[c] ($n = 8$)			
ADAMTS13 : AC (%) (ELISA)	47 ± 24	44 ± 13	26 ± 14	n.s.	$P < .05$	$P < .01$
VWF : RCo (%)	110 ± 92	196 ± 134	216 ± 110	$P < .05$	$P < .05$	n.s.
Child-Pugh score	8.6 ± 2.5	10.9 ± 2.1	12.4 ± 1.7	$P < .01$	$P < .005$	n.s.
Serum albumin (g/dL)	3.07 ± 0.54	2.85 ± 0.54	2.59 ± 0.25	n.s.	$P < .05$	n.s.
Cholinesterase (IU/L)	126 ± 62	78 ± 64	60 ± 36	$P < .05$	$P < .02$	n.s.
Total cholesterol (mg/dL)	142 ± 51	93 ± 45	88 ± 40	$P < .01$	$P < .03$	n.s.
Hemoglobin (g/dL)	11.0 ± 1.7	9.3 ± 2.0	8.9 ± 1.7	$P < .02$	$P < .02$	n.s.
Serum creatinine (mg/dL)	1.06 ± 0.72	1.11 ± 0.79	2.43 ± 2.16	n.s.	$P < .05$	$P < .03$
Blood urea nitrogen (mg/dL)	22 ± 17	30 ± 21	74 ± 62	n.s.	$P < .01$	$P < .01$
Blood ammonia (μg/dL)	87 ± 50	100 ± 39	144 ± 53	n.s.	$P < .05$	$P < .05$

VWF: von Willebrand factor; ADAMTS13 : AC : ADAMTS13 activity; ELISA : enzyme-linked immunosorbent assay; VWF : RCo: VWF ristocetin cofactor activity; n.s.: not significant. (Partially modified from Uemura et al., [30]).

6. Mechanism of Decreased ADAMTS13 : AC in LC Patients

The mechanism responsible for the decrease in ADAMTS13 : AC in advanced LC may include enhanced consumption due to the degradation of large quantities of VWF : AG [20], inflammatory cytokines [68, 69], and/or ADAMTS13 plasma inhibitor [9, 10]. It is controversial whether ADAMTS13 deficiency is caused by decreased production in the liver; Kume et al. reported that HSC apoptosis plays an essential role in decreased ADAMTS13 : AC using dimethylnitrosamine-treated rats, but not carbon tetrachloride- (CCl_4-) treated

animals [70], whereas Niiya et al. found upregulation of ADAMTS13 antigen and proteolytic activity in liver tissue using rats with CCl_4-induced liver fibrosis [71]. 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 in cases of a TTP patient (2.0 BU/mL) and a patient with severe ADAMTS13 deficiency (3.0 BU/mL) [30]. 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 [30]. One patient showed an apparent TTP [35], while the other four cirrhotics did not show apparent clinical features of TTP but had complications of hepatorenal syndrome, spontaneous bacterial peritonitis (SBP), marked inflammation together with cytokinemia, and advanced hepatocellular carcinoma (HCC) [30]. Various clinical conditions, including infection, malignancies, and certain drugs, can lead to acquired TTP [72]. In advanced LC patients, endotoxemia is frequently detected [42, 73], and SBP sometimes occurs [74]. HCC is highly complicated as the cirrhotic stage progresses [75], suggesting a high-risk state of platelet microthrombi formation. Some end-stage LC patients with extremely low ADAMTS13 : AC and its IgG inhibitor may reflect conditions similar to TTP or may reflect "subclinical TTP" [21]. Further studies will be necessary to clarify whether inhibitors other than the IgG inhibitor might be involved in cirrhotics with lower ADAMTS13 : AC.

Alternatively, cytokinemia [25, 68, 69, 76] and endotoxemia [25, 77] are additional potential candidates for decreasing plasma ADAMTS13 : AC. 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* [68]. It remains to be clarified whether IL-6 directly hampers the cleavage of UL-VWFM or downregulates gene expression of ADAMTS13 with modification of promoter activity. IFN-γ, IL-4, and TNF-α also inhibit ADAMTS13 synthesis and activity in rat primary HSC [69]. In addition, ADAMTS13 deficiency associated with inflammation promoted formation of UL-VWFM [78], and intravenous infusion of endotoxin to healthy volunteers caused a decrease in plasma ADAMTS13 : AC together with the appearance of UL-VWFM [77]. In patients with alcoholic hepatitis, especially in severe cases complicated by LC, ADAMTS13 : AC concomitantly decreased, and VWF : Ag progressively increased with increasing concentrations of these cytokines from normal range to over 100 pg/mL [25]. Plasma endotoxin concentration inversely correlated with ADAMTS13 activity and was higher in patients with UL-VWFM than that those without [25]. From these results as well as our own, marked cytokinemia and/or enhanced endotoxemia may be closely related to decreased ADAMTS13 : AC and the appearance of UL-VWFM [25]. It will be necessary to clarify what types of inhibitor may be involved in association with inflammatory cytokines and endotoxin.

7. Typical TTP in Patients with Liver Diseases

We previously encountered a patient with HCV-related LC who was compromised by fatal TTP [35]. This case showed advanced LC and rigid ascites. As reported in the literature, since 1979, there have been 13 patients with liver diseases who developed TTP [35, 79–90]. Five of them were treated with IFN therapy, but the remaining 8 were not. Three of them showed evidence of autoimmune hepatitis, one of which was complicated by systemic lupus erythematosus (SLE). The remaining 4 patients had HCV-related LC, hepatitis B virus- (HBV-) related LC, alcoholic LC, or haemochromatosis. IFN may be able to induce autoimmune reactions, resulting in the generation of autoantibodies against ADAMTS13, although this phenomenon has yet to be confirmed. On the other hand, irrespective of IFN therapy, HCV infection and/or advanced LC itself may contribute to the development of TTP.

There is 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 that in both healthy subjects and patients with HBV [91–93], but not alcoholic liver injury. In addition, HCV infection was confirmed in five of 10 patients (50%) who developed thrombotic microangiopathy (TMA) after living-donor liver transplantation [94]. In our study, the etiology of our five end-stage LC patients with IgG-type autoantibodies was HCV in 2, HBV in 1, PBC in 1, and cryptogenic in 1, but none of the patients displayed alcohol-abuse-related cirrhosis [30]. Nevertheless, the diagnosis of TTP may be hampered by clinical features accompanying hepatic failure similar to the pentad of typical TTP (fever, thrombocytopenia, renal failure, fluctuating neurological signs, and microangiopathic hemolytic anemia) [11, 12].

8. Possible Therapeutic Approaches of ADAMTS13 Supplementation for Patients with Decompensated LC

Fresh frozen plasma (FFP) infusion is commonly used to correct the prolonged prothrombin time in patients with advanced chronic liver disease, but exact indication for its use has not been clearly defined [95]. The aim of FFP infusions is usually either to improve the coagulopathy before invasive procedures or to control ongoing bleeding from various sites in patients with vitamin K-unresponsiveness prolonged prothrombin time. The mean prothrombin time was improved by the infusion of 2–6 units of FFP, but only 12.5% of the retrospective study group and 10% of the prospective study groups showed reversal of their coagulopathy, and higher volume (6 or more units) may be more effective but rarely is employed [96]. However, attention should be directed to complications including the risk of infection, allergic reaction, and acute volume expansion leading to heart failure or pulmonary edema [95, 96].

With regard to FFP infusion as a unique source of ADAMTS13, we clearly showed that preexisting UL-VWFMs

in the plasma of USS patients began to diminish within 1 hour and completely diminished 24 hours after ADAMTS13 was replenished with infusions of FFP [97]. Retrospectively, these results indicated that exogenous ADAMTS13 could efficiently cleave both UL-VWFMs that preexisted in the circulation and the newly produced molecules at the ECs surface. Advanced LC is known to be a predisposing state toward platelet microthrombi formation, even in the absence of clinically overt thrombi [30]. In our study, UL-VWFM-positive patients showed the lowest ADAMTS13 : AC and the highest values of serum creatinine, blood urea nitrogen, and blood ammonia, and the VWFM patterns appeared to shift from degraded to normal VWFM and finally to UL-VWFM as functional liver capacity and renal function deteriorated (Table 1). From these results, it may be reasonable to assume that advanced LC patients with severe to moderate deficiency of ADAMTS13 : AC (<3% to ~25% of normal control) could be candidates for FFP infusion as a source of ADAMTS13. It is necessary to evaluate the effectiveness of FFP administration to patients with ADAMTS13 : AC levels from 25% to 50%.

Alternatively, our recent study demonstrated that plasma ADAMTS13 : AC is reduced in VOD patients after stem cell transplantation (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 [22]. A multicenter, prospective, randomized controlled study revealed that prophylactic FFP infusion may be instrumental in preventing the development of hepatic VOD after SCT [23]. 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 [23]. The supplementation of ADAMTS13 by prophylactic FFP infusion may suppress the increase in VWF : AG that is extensively released from damaged SEC. Furthermore, 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 [31]. These changes were closely related to liver-graft dysfunction, ischemia-reperfusion injury, and acute rejection. The ADAMTS13 : AC often decreased to less than 10% of normal controls, concurrent with severe thrombocytopenia. The organ dysfunction appeared to be restricted to the liver graft, indicating that a decrease of plasma ADAMTS13 : AC coupled with the appearance of UL-VWFM was attributed to a mechanism of "local TTP" within the liver graft [21, 31]. It is, therefore, 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 conditions of 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 [21, 31]. FFP infusion as ADAMTS13 replacement therapy may improve both liver dysfunction and thrombocytopenia in liver transplant patients. From this point of view, we are particularly interested in conducting clinical trials with recombinant ADAMTS13 preparations not only in patients with advanced LC but also in patients with VOD and liver transplantations.

9. 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 [30] and SAH patients [24–27] or "local TTP" as shown in patients with hepatic VOD after SCT [22, 23] and patients with adverse events after living-donor liver transplantation [31, 32]. Essentially, one would 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 conventional hemostatic factors. The origin of VWF, the substrate of ADAMS13, indeed may be transformed hepatic sinusoidal and/or extrahepatic endothelial cells, but not hepatocytes. The procoagulant and anticoagulant proteins synthesized in hepatocytes decrease as liver disease progresses, whereas VWF markedly increases. Under such circumstances, ADAMTS13 deficiency may lead to a microcirculatory disturbance not only in the liver, but also in the systemic circulation. The determination of ADAMTS13 and its related parameters thus will be quite useful for improved understanding of the pathophysiology and for providing appropriate treatments especially in severe liver disease patients. It will be necessary to measure ADAMTS13 : AC when patients with unexplained thrombocytopenia are encountered in the course of liver disease. When "subclinical or local TTP" status would be confirmed, FFP infusion as ADAMTS13 replacement therapy may improve both liver dysfunction and thrombocytopenia. Further investigation will be necessary to define candidates for ADAMTS13 supplementation therapy and to evaluate its potential therapeutic efficacy in advanced LC patients.

Acknowledgments

The authors sincerely thank Hiromichi Ishizashi, Ayami Isonishi, Seiji Kato, Tomomi Matsuyama, Chie Morioka, and Masatoshi Ishikawa for their great help in the assay of ADAMTS13 activity, VWF antigen, and UL-VWFM. This work was supported in part by research grants from the Japanese Ministry of Education, Culture, Science (to M. Uemura, Y. Fujimura, S. Ko, and M. Matsumoto) and from

the Ministry of Health, Labour and Welfare of Japan for Blood Coagulation Abnormalities (to Y. Fujimura).

References

[1] L. N. Roberts, R. K. Patel, and R. Arya, "Haemostasis and thrombosis in liver disease," *British Journal of Haematology*, vol. 148, no. 4, pp. 507–521, 2010.

[2] A. Tripodi, M. Primignani, and P. M. Mannucci, "Abnormalities of hemostasis and bleeding in chronic liver disease: the paradigm is challenged," *Internal and Emergency Medicine*, vol. 5, no. 1, pp. 7–12, 2010.

[3] T. Lisman and R. J. Porte, "Rebalanced hemostasis in patients with liver disease: evidence and clinical consequences," *Blood*, vol. 116, no. 6, pp. 878–885, 2010.

[4] P. G. Northup, V. Sundaram, M. B. Fallon et al., "Hypercoagulation and thrombophilia in liver disease," *Journal of Thrombosis and Haemostasis*, vol. 6, pp. 2–9, 2008.

[5] J. L. Moake, "Thrombotic microangiopathies," *New England Journal of Medicine*, vol. 347, no. 8, pp. 589–600, 2002.

[6] Y. Fujimura, M. Matsumoto, H. Yagi, A. Yoshioka, T. Matsui, and K. Titani, "Von Willebrand factor-cleaving protease and Upshaw-Schulman syndrome," *International Journal of Hematology*, vol. 75, no. 1, pp. 25–34, 2002.

[7] G. G. Levy, W. C. Nichols, E. C. Lian et al., "Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura," *Nature*, vol. 413, pp. 488–494, 2001.

[8] K. Kokame, M. Matsumoto, K. Soejima et al., "Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 18, pp. 11902–11907, 2002.

[9] M. Furlan, R. Robles, M. Galbusera et al., "Von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome," *New England Journal of Medicine*, vol. 339, no. 22, pp. 1578–1584, 1998.

[10] H. M. Tsai and E. C. Lian, "Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura," *New England Journal of Medicine*, vol. 339, no. 22, pp. 1585–1594, 1998.

[11] E. Moschcowitz, "Hyaline thrombosis of the terminal arterioles and capillaries: a hitherto undescribed disease," *Proceeding New York Pathological Society*, vol. 24, pp. 21–24, 1924.

[12] E. L. Amorosi and J. E. Ultmann, "Thrombotic thrombocytopenic purpura: report of 16 cases and review of the literature," *Medicine*, vol. 45, pp. 139–159, 1966.

[13] M. Matsumoto, H. Chisuwa, Y. Nakazawa et al., "Living-related liver transplantation rescues reduced vWF-cleaving protease activity in patients with cirrhotic biliary atresia," *Blood*, vol. 96, p. 636a, 2000.

[14] X. Zheng, D. Chung, T. K. Takayama, E. M. Majerus, J. E. Sadler, and K. Fujikawa, "Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura," *Journal of Biological Chemistry*, vol. 276, no. 44, pp. 41059–41063, 2001.

[15] K. Soejima, N. Mimura, M. Hirashima et al., "A novel human metalloprotease synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease?" *Journal of Biochemistry*, vol. 130, no. 4, pp. 475–480, 2001.

[16] M. Uemura, K. Tatsumi, M. Matsumoto et al., "Localization of ADAMTS13 to the stellate cells of human liver," *Blood*, vol. 106, no. 3, pp. 922–924, 2005.

[17] M. Suzuki, M. Murata, Y. Matsubara et al., "von Willebrand factor-cleaving protease (ADAMTS-13) in human platelets," *Biochemical and Biophysical Research Communications*, vol. 313, no. 1, pp. 212–216, 2004.

[18] N. Turner, L. Nolasco, Z. Tao, J. F. Dong, and J. Moake, "Human endothelial cells synthesize and release ADAMTS-13," *Journal of Thrombosis and Haemostasis*, vol. 4, no. 6, pp. 1396–1404, 2006.

[19] M. Manea, A. Kristoffersson, R. Schneppenheim et al., "Podocytes express ADAMTS13 in normal renal cortex and in patients with thrombotic thrombocytopenic purpura," *British Journal of Haematology*, vol. 138, no. 5, pp. 651–662, 2007.

[20] P. M. Mannucci, M. T. Canciani, I. Forza, F. Lussana, A. Lattuada, and E. Rossi, "Changes in health and disease of the metalloprotease that cleaves von Willebrand factor," *Blood*, vol. 98, no. 9, pp. 2730–2735, 2001.

[21] M. Uemura, Y. Fujimura, S. Ko, M. Matsumoto, Y. Nakajima, and H. Fukui, "Pivotal role of ADAMTS13 function in liver diseases," *International Journal of Hematology*, vol. 91, no. 1, pp. 20–29, 2010.

[22] Y. D. Park, A. Yoshioka, K. Kawa et al., "Impaired activity of plasma von Willebrand factor-cleaving protease may predict the occurrence of hepatic veno-occlusive disease after stem cell transplantation," *Bone Marrow Transplantation*, vol. 29, no. 9, pp. 789–794, 2002.

[23] M. Matsumoto, K. Kawa, M. Uemura et al., "Prophylactic fresh frozen plasma may prevent development of hepatic VOD after stem cell transplantation via ADAMTS13-mediated restoration of von Willebrand factor plasma levels," *Bone Marrow Transplantation*, vol. 40, pp. 251–259, 2007.

[24] M. Uemura, Y. Fujimura, T. Matsuyama et al., "Potential role of ADAMTS13 in the progression of alcoholic hepatitis," *Current Drug Abuse Reviews*, vol. 1, no. 2, pp. 188–196, 2008.

[25] M. Ishikawa, M. Uemura, T. Matsuyama et al., "Potential role of enhanced cytokinemia and plasma inhibitor on the decreased activity of plasma ADAMTS13 in patients with alcoholic hepatitis: relationship to endotoxemia," *Alcoholism: Clinical and Experimental Research*, vol. 34, supplement 1, pp. S25–S33, 2010.

[26] T. Matsuyama, M. Uemura, M. Ishikawa et al., "Increased von Willebrand factor over decreased ADAMTS13 activity may contribute to the development of liver disturbance and multiorgan failure in patients with alcoholic hepatitis," *Alcoholism: Clinical and Experimental Research*, vol. 31, supplement 1, pp. S27–S35, 2007.

[27] M. Uemura, T. Matsuyama, M. Ishikawa et al., "Decreased activity of plasma ADAMTS13 may contribute to the development of liver disturbance and multiorgan failure in patients with alcoholic hepatitis," *Alcoholism: Clinical and Experimental Research*, vol. 29, no. 12, supplement 3, pp. 264S–271S, 2005.

[28] T. Lisman, T. N. Bongers, J. Adelmeijer et al., "Elevated levels of von Willebrand factor in cirrhosis support platelet adhesion despite reduced functional capacity," *Hepatology*, vol. 44, no. 1, pp. 53–61, 2006.

[29] H. B. Feys, M. T. Canciani, F. Peyvandi, H. Deckmyn, K. Vanhoorelbeke, and P. M. Mannucci, "ADAMTS13 activity to antigen ratio in physiological and pathological conditions associated with an increased risk of thrombosis," *British Journal of Haematology*, vol. 138, no. 4, pp. 534–540, 2007.

[30] M. Uemura, Y. Fujimura, M. Matsumoto et al., "Comprehensive analysis of ADAMTS13 in patients with liver cirrhosis," *Thrombosis and Haemostasis*, vol. 99, no. 6, pp. 1019–1029, 2008.

[31] S. Ko, E. Okano, H. Kanehiro et al., "Plasma ADAMTS13 activity may predict early adverse events in living donor liver transplantation: observations in 3 cases," *Liver Transplantation*, vol. 12, no. 5, pp. 859–869, 2006.

[32] T. Kobayashi, H. Wada, M. Usui et al., "Decreased ADAMTS13 levels in patients after living donor liver transplantation," *Thrombosis Research*, vol. 124, no. 5, pp. 541–545, 2009.

[33] I. T. A. Pereboom, J. Adelmeijer, Y. Van Leeuwen, H. G. D. Hendriks, R. J. Porte, and T. Lisman, "Development of a severe von Willebrand factor/ADAMTS13 dysbalance during orthotopic liver transplantation," *American Journal of Transplantation*, vol. 9, no. 5, pp. 1189–1196, 2009.

[34] E. Okano, S. Ko, H. Kanehiro, M. Matsumoto, Y. Fujimura, and Y. Nakajima, "ADAMTS13 activity decreases after hepatectomy, reflecting a postoperative liver dysfunction," *Hepato-Gastroenterology*, vol. 57, no. 98, pp. 316–320, 2010.

[35] M. Yagita, M. Uemura, T. Nakamura, A. Kunitomi, M. Matsumoto, and Y. Fujimura, "Development of ADAMTS13 inhibitor in a patient with hepatitis C virus-related liver cirrhosis causes thrombotic thrombocytopenic purpura," *Journal of Hepatology*, vol. 42, no. 3, pp. 420–421, 2005.

[36] F. Durand and D. Valla, "Assessment of prognosis of cirrhosis," *Seminars in Liver Disease*, vol. 28, no. 1, pp. 110–122, 2008.

[37] Z. Kmieć, "Cooperation of liver cells in health and disease," *Advances in Anatomy, Embryology, and Cell Biology*, vol. 161, pp. 1–151, 2001.

[38] D. C. Rockey, "Hepatic blood flow regulation by stellate cells in normal and injured liver," *Seminars in Liver Disease*, vol. 21, no. 3, pp. 337–350, 2001.

[39] F. Schaffner and H. Popper, "Capillarization of hepatic sinusoids in man," *Gastroenterology*, vol. 44, pp. 239–242, 1963.

[40] M. Hattori, Y. Fukuda, M. Imoto, Y. Koyama, I. Nakano, and F. Urano, "Histochemical properties of vascular and sinusoidal endothelial cells in liver diseases," *Gastroenterologia Japonica*, vol. 26, no. 3, pp. 336–343, 1991.

[41] L. Albornoz, D. Alvarez, J. C. Otaso et al., "Von Willebrand factor could be an index of endothelial dysfunction in patients with cirrhosis: relationship to degree of liver failure and nitric oxide levels," *Journal of Hepatology*, vol. 30, no. 3, pp. 451–455, 1999.

[42] D. Ferro, C. Quintarelli, A. Lattuada et al., "High plasma levels of von Willebrand factor as a marker of endothelial perturbation in cirrhosis: relationship to endotoxemia," *Hepatology*, vol. 23, no. 6, pp. 1377–1383, 1996.

[43] T. Knittel, K. Neubauer, T. Armbrust, and G. Ramadori, "Expression of von Willebrand factor in normal and diseased rat livers and in cultivated liver cells," *Hepatology*, vol. 21, no. 2, pp. 470–476, 1995.

[44] S. Urashima, M. Tsutsumi, K. Nakase, J. S. Wang, and A. Takada, "Studies on capillarization of the hepatic sinusoids in alcoholic liver disease," *Alcohol and Alcoholism*, vol. 1B, pp. 77–84, 1993.

[45] L. Amitrano, M. A. Guardascione, V. Brancaccio et al., "Risk factors and clinical presentation of portal vein thrombosis in patients with liver cirrhosis," *Journal of Hepatology*, vol. 40, no. 5, pp. 736–741, 2004.

[46] I. R. Wanless, F. Wong, L. M. Blendis, P. Greig, E. J. Heathcote, and G. Levy, "Hepatic and portal vein thrombosis in cirrhosis: possible role in development of parenchymal extinction and portal hypertension," *Hepatology*, vol. 21, no. 5, pp. 1238–1247, 1995.

[47] K. Oka and K. Tanaka, "Intravascular coagulation in autopsy cases with liver diseases," *Thrombosis and Haemostasis*, vol. 42, no. 2, pp. 564–570, 1979.

[48] A. Pluta, K. Gutkowski, and M. Hartleb, "Coagulopathy in liver diseases," *Advanced Medical Science*, vol. 55, pp. 16–21, 2010.

[49] Y. Fujimura, M. Matsumoto, and H. Yagi, *Thrombotic Microangiopathy*, Springer, Tokyo, Japan, 2008.

[50] A. Padilla, J. L. Moake, A. Bernardo et al., "P-selectin anchors newly released ultralarge von Willebrand factor multimers to the endothelial cell surface," *Blood*, vol. 103, no. 6, pp. 2150–2156, 2004.

[51] C. A. Siedlecki, B. J. Lestini, K. K. Kottke-Marchant, S. J. Eppell, D. L. Wilson, and R. E. Marchant, "Shear-dependent changes in the three-dimensional structure of human von Willebrand factor," *Blood*, vol. 88, no. 8, pp. 2939–2950, 1996.

[52] J. A. Dent, S. D. Berkowitz, J. Ware, C. K. Kasper, and Z. M. Ruggeri, "Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 16, pp. 6306–6310, 1990.

[53] M. Akiyama, S. Takeda, K. Kokame, J. Takagi, and T. Miyata, "Crystal structures of the noncatalytic domains of ADAMTS13 reveal multiple discontinuous exosites for von Willebrand factor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 46, pp. 19274–19279, 2009.

[54] A. K. Davis, R. S. Makar, C. P. Stowell, D. J. Kuter, and W. H. Dzik, "ADAMTS13 binds to CD36: a potential mechanism for platelet and endothelial localization of ADAMTS13," *Transfusion*, vol. 49, no. 2, pp. 206–213, 2009.

[55] M. Furlan, R. Robles, and B. Lämmle, "Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis," *Blood*, vol. 87, no. 10, pp. 4223–4234, 1996.

[56] S. Kinoshita, A. Yoshioka, Y. D. Park et al., "Upshaw-Schulman syndrome revisited: a concept of congenital thrombotic thrombocytopenic purpura," *International Journal of Hematology*, vol. 74, pp. 101–108, 2001.

[57] S. Kato, M. Matsumoto, T. Matsuyama, A. Isonishi, H. Hiura, and Y. Fujimura, "Novel monoclonal antibody-based enzyme immunoassay for determining plasma levels of ADAMTS13 activity," *Transfusion*, vol. 46, no. 8, pp. 1444–1452, 2006.

[58] S. C. Meyer, I. Sulzer, B. Lämmle, and J. A. Kremer Hovinga, "Hyperbilirubinemia interferes with ADAMTS-13 activity measurement by FRETS-VWF73 assay: diagnostic relevance in patients suffering from acute thrombotic microangiopathies," *Journal of Thrombosis and Haemostasis*, vol. 5, no. 4, pp. 866–867, 2007.

[59] C. K. Kasper, L. Aledort, D. Aronson et al., "A more uniform measurement of factor VIII inhibitors," *Thrombosis et Diathesis Haemorrhagica*, vol. 34, no. 2, pp. 869–872, 1975.

[60] N. Afdhal, J. McHutchison, R. Brown et al., "Thrombocytopenia associated with chronic liver disease," *Journal of Hepatology*, vol. 48, no. 6, pp. 1000–1007, 2008.

[61] R. H. Aster, "Pooling of platelets in the spleen: role in the pathogenesis of "hypersplenic" thrombocytopenia," *Journal of Clinical Investigation*, vol. 45, no. 5, pp. 645–657, 1966.

[62] M. Peck-Radosavljevic, M. Wichlas, J. Zacherl et al., "Thrombopoietin induces rapid resolution of thrombocytopenia after orthotopic liver transplantation through increased platelet production," *Blood*, vol. 95, no. 3, pp. 795–801, 2000.

[63] J. García-Suárez, C. Burgaleta, N. Hernanz, F. Albarran, P. Tobaruela, and M. Alvarez-Mon, "HCV-associated thrombocytopenia: clinical characteristics and platelet response after recombinant alpha2b-interferon therapy," *British Journal of Haematology*, vol. 110, no. 1, pp. 98–103, 2000.

[64] H. S. Ballard, "Hematological complications of alcoholism," *Alcoholism: Clinical and Experimental Research*, vol. 13, no. 5, pp. 706–720, 1989.

[65] H. E. Gerritsen, P. L. Turecek, H. P. Schwarz, B. Lämmle, and M. Furlan, "Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF. A tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP)," *Thrombosis and Haemostasis*, vol. 82, no. 5, pp. 1386–1389, 1999.

[66] A. E. Schorer, C. F. Moldow, and M. E. Rick, "Interleukin 1 or endotoxin increases the release of von Willebrand factor from human endothelial cells," *British Journal of Haematology*, vol. 67, no. 2, pp. 193–197, 1987.

[67] I. Tornai, J. Hársfalvi, Z. Boda, M. Udvardy, G. Pfliegler, and K. Rak, "Endothelium releases more von Willebrand factor and tissue-type plasminogen activator upon venous occlusion in patients with liver cirrhosis than in normals," *Haemostasis*, vol. 23, no. 1, pp. 58–64, 1993.

[68] A. Bernardo, C. Ball, L. Nolasco, J. F. Moake, and J. F. Dong, "Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand-factor multimers under flow," *Blood*, vol. 104, no. 1, pp. 100–106, 2004.

[69] W. J. Cao, M. Niiya, X. W. Zheng, D. Z. Shang, and X. L. Zheng, "Inflammatory cytokines inhibit ADAMTS13 synthesis in hepatic stellate cells and endothelial cells," *Journal of Thrombosis and Haemostasis*, vol. 6, no. 7, pp. 1233–1235, 2008.

[70] Y. Kume, H. Ikeda, M. Inoue et al., "Hepatic stellate cell damage may lead to decreased plasma ADAMTS13 activity in rats," *FEBS Letters*, vol. 581, no. 8, pp. 1631–1634, 2007.

[71] M. Niiya, M. Uemura, X. W. Zheng et al., "Increased ADAMTS-13 proteolytic activity in rat hepatic stellate cells upon activation in vitro and in vivo," *Journal of Thrombosis and Haemostasis*, vol. 4, no. 5, pp. 1063–1070, 2006.

[72] Y. Fujimura and M. Matsumoto, "Registry of 919 patients with thrombotic microangiopathies across Japan: database of Nara Medical University during 1998–2008," *Internal Medicine*, vol. 49, no. 1, pp. 7–15, 2010.

[73] H. Fukui, B. Brauner, J. C. Bode, and C. Bode, "Plasma endotoxin concentrations in patients with alcoholic and non-alcoholic liver disease: reevaluation with an improved chromogenic assay," *Journal of Hepatology*, vol. 12, no. 2, pp. 162–169, 1991.

[74] P. Tandon and G. Garcia-Tsao, "Bacterial infections, sepsis, and multiorgan failure in cirrhosis," *Seminars in Liver Disease*, vol. 28, no. 1, pp. 26–42, 2008.

[75] M. Sala, A. Forner, M. Varela, and J. Bruix, "Prognostic prediction in patients with hepatocellular carcinoma," *Seminars in Liver Disease*, vol. 25, no. 2, pp. 171–180, 2005.

[76] R. A. Claus, C. L. Bockmeyer, M. Sossdorf, and W. Lösche, "The balance between von-Willebrand factor and its cleaving protease ADAMTS13: biomarker in systemic inflammation and development of organ failure?" *Current Molecular Medicine*, vol. 10, no. 2, pp. 236–248, 2010.

[77] R. A. Reiter, K. Varadi, P. L. Turecek, B. Jilma, and P. Knöbl, "Changes in ADAMTS13 (von-Willebrand-factor-cleaving protease) activity after induced release of von Willebrand factor during acute systemic inflammation," *Thrombosis and Haemostasis*, vol. 93, no. 3, pp. 554–558, 2005.

[78] C. L. Bockmeyer, R. A. Claus, U. Budde et al., "Inflammation-associated ADAMTS13 deficiency promotes formation of ultra-large von Willebrand factor," *Haematologica*, vol. 93, no. 1, pp. 137–140, 2008.

[79] K. Iyoda, M. Kato, T. Nakagawa et al., "Thrombotic thrombocytopenic purpura developed suddenly during interferon treatment for chronic hepatitis C," *Journal of Gastroenterology*, vol. 33, no. 4, pp. 588–592, 1998.

[80] A. Hori, M. Kami, T. Hamaki et al., "Thrombotic thrombocytopenic purpura without any evidence of thrombotic lesions at autopsy," *Annals of Hematology*, vol. 80, no. 8, pp. 496–498, 2001.

[81] A. Shibuya, A. Satomichi, S. Nagaba, H. Endoh, S. Kuwao, and K. Saigenji, "Thrombotic thrombocytopenic purpura in autoimmune hepatitis," *Journal of Gastroenterology*, vol. 36, no. 8, pp. 569–573, 2001.

[82] J. V. Nally and E. N. Metz, "Acute thrombotic thrombocytopenic purpura. Another cause for hemolytic anemia and thrombocytopenia in cirrhosis," *Archives of Internal Medicine*, vol. 139, no. 6, pp. 711–712, 1979.

[83] S. Killick, S. Jeffery, M. Otter, C. Rist, and D. Bevan, "Thrombotic thrombocytopenic purpura in a patient with genetic haemochromatosis, liver cirrhosis and an iron-free focus," *British Journal of Haematology*, vol. 99, no. 4, pp. 839–841, 1997.

[84] K. Kitano, Y. Gibo, A. Kamijo et al., "Thrombotic thrombocytopenic purpura associated with pegylated-interferon alpha-2a by an ADAMTS13 inhibitor in a patient with chronic hepatitis C," *Haematologica*, vol. 91, article ECR34, supplement 8, 2006.

[85] F. Lambot, B. Hanson, and B. Sztern, "Thrombotic thrombocytopenic purpura mediated by an ADAMTS13-inhibitor related to a treatment with pegylated-interferon alpha-2a and ribavirine in a patient with chronic hepatitis C," *Presse Medicale*, vol. 39, pp. 1207–1210, 2010.

[86] M. Deutsch, E. K. Manesis, E. Hadziyannis, D. Vassilopoulos, and A. J. Archimandritis, "Thrombotic thrombocytopenic purpura with fatal outcome in a patient with chronic hepatitis C treated with pegylated interferon-a/2b," *Scandinavian Journal of Gastroenterology*, vol. 42, no. 3, pp. 408–409, 2007.

[87] M. Sallée, E. Crétel, R. Jean et al., "Thrombotic thrombocytopenic purpura complicating interferon therapy in chronic C hepatitis," *Gastroenterologie Clinique et Biologique*, vol. 32, no. 2, pp. 145–146, 2008.

[88] M. Shimizu, S. Nomura, K. Ishii et al., "The significance of ADAMTSI3 in a patient with thrombotic thrombocytopenic purpura complicated autoimmune hepatitis," *Thrombosis and Haemostasis*, vol. 101, no. 3, pp. 599–600, 2009.

[89] K. Sonomoto, T. Miyamura, H. Watanabe et al., "A case of systemic lupus erythematosus complicated with autoimmune hepatitis and thrombotic thrombocytic purpura," *Japanese Journal of Clinical Immunology*, vol. 32, no. 2, pp. 110–115, 2009.

[90] Y. Usta, F. Gurakan, Z. Akcoren, and S. Ozen, "An overlap syndrome involving autoimmune hepatitis and systemic lupus erythematosus in childhood," *World Journal of Gastroenterology*, vol. 13, no. 19, pp. 2764–2767, 2007.

[91] M. Lenzi, S. Bellentani, G. Saccoccio et al., "Prevalence of non-organ-specific autoantibodies and chronic liver disease in the general population: a nested case-control study of the Dionysos cohort," *Gut*, vol. 45, no. 3, pp. 435–441, 1999.

[92] M. Y. Hsieh, C. Y. Dai, L. P. Lee et al., "Antinuclear antibody is associated with a more advanced fibrosis and lower RNA levels of hepatitis C virus in patients with chronic hepatitis C," *Journal of Clinical Pathology*, vol. 61, no. 3, pp. 333–337, 2008.

[93] S. Tamura, Y. Sugawara, Y. Matsui et al., "Thrombotic microangiopathy in living-donor liver transplantation," *Transplantation*, vol. 80, no. 2, pp. 169–175, 2005.

[94] B. D. Clifford, D. Donahue, L. Smith et al., "High prevalence of serological markers of autoimmunity in patients with chronic hepatitis C," *Hepatology*, vol. 21, no. 3, pp. 613–619, 1995.

[95] A. Iorio, M. Basileo, E. Marchesini et al., "The good use of plasma. A critical analysis of five international guidelines," *Blood Transfusion*, vol. 6, no. 1, pp. 18–24, 2008.

[96] W. I. Youssef, F. Salazar, S. Dasarathy, T. Beddow, and K. D. Mullen, "Role of fresh frozen plasma infusion in correction of coagulopathy of chronic liver disease: a dual phase study," *American Journal of Gastroenterology*, vol. 98, no. 6, pp. 1391–1394, 2003.

[97] H. Yagi, M. Konno, S. Kinoshita et al., "Plasma of patients with Upshaw-Schulman syndrome, a congenital deficiency of von Willebrand factor-cleaving protease activity, enhances the aggregation of normal platelets under high shear stress," *British Journal of Haematology*, vol. 115, no. 4, pp. 991–997, 2001.

Pflugers Arch - Eur J Physiol (2011) 461:623–633
DOI 10.1007/s00424-011-0958-x

CARDIOVASCULAR PHYSIOLOGY

In vivo imaging analysis of the interaction between unusually large von Willebrand factor multimers and platelets on the surface of vascular wall

Miroslaw Rybaltowski · Yuko Suzuki · Hideo Mogami · Iwona Chlebinska · Tomasz Brzoska · Aki Tanaka · Fumiaki Banno · Toshiyuki Miyata · Tetsumei Urano

Received: 15 November 2010 / Revised: 13 February 2011 / Accepted: 9 March 2011 / Published online: 15 April 2011

Abstract To elucidate how unusually large von Willebrand factor (UL-VWF) multimers facilitate thrombus formation, their behavior was analyzed together with that of platelets in living mice deficient in the gene encoding the protease that cleaves UL-VWF, a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13 (ADAMTS13−/−). By crossing ADAMTS13−/− mice with green fluorescent protein-expressing transgenic mice (GFP mice), GFP-ADAMTS13−/− mice were obtained. The dynamics of GFP-expressing platelets were monitored employing intravital confocal fluorescent microscopy. Administration of a vasopressin derivative, DDAVP, a secretagogue of VWF increased the number of platelets adhered to vascular endothelial cells (VECs) on mesentery at sites recognized by an anti-VWF antibody. Some of these platelets were interconnected and aligned as beads on a string. They reached their maximum length at 5 min and were longer in GFP-ADAMTS13−/− mice than in GFP mice (5.3±4.3, N=6 vs 2.9±2.1 μm, N=4) (mean±SE). Focal injury of VECs by topical application of $FeCl_3$ developed longer (25, 3–50 vs 10, 2–25 μm, $P<0.01$) (mean, 10th–90th percentile) and more stable (1.3, 0.3–6.3 vs 0.3, 0.2–1.3 s, $P<0.01$) connected platelets in GFP-ADAMTS13−/− mice than in GFP mice. This study revealed that ADAMTS13 cleaves platelet-bound UL-VWF multimers, both during their secretion from VECs and after their adherence to injured vascular walls in veins. UL-VWF multimers either being secreted from VECs or circulating in plasma of ADAMTS13−/− mice appeared to facilitate the accumulation of longer and more stable VWF strings with more associated platelets on injured vascular walls.

Keywords Von Willebrand factor (VWF) · A disintegrin-like and metalloprotease with thrombospondin type 1 motif 13 (ADAMTS13) · Thrombotic thrombocytopenic purpura (TTP) · Intravital confocal fluorescent microscopy

Introduction

Von Willebrand factor (VWF) is a macromolecular, multidomain glycoprotein that is synthesized both in vascular endothelial cells (VECs) and in megakaryocytes (see reviews [15, 37]). VWF forms dimers by C-terminal disulphide bonding in the endoplasmic reticulum, and it subsequently forms multimers by N-terminal disulphide bridging in the Golgi apparatus. VWF multimers are either constitutively secreted from VECs or are stored in secretory granules in VECs (Weibel-Palade bodies) [44] as "unusually large" VWF (UL-VWF) multimers [41], having a wide range of molecular masses from 500 to 20,000 kDa [18]. The stored VWF can be secreted from

Electronic supplementary material The online version of this article (doi:10.1007/s00424-011-0958-x) contains supplementary material, which is available to authorized users.

M. Rybaltowski · Y. Suzuki · H. Mogami · I. Chlebinska · T. Brzoska · A. Tanaka · T. Urano (✉)
Department of Physiology,
Hamamatsu University School of Medicine,
1-20-1, Handa-yama, Higashi-ku,
Hamamatsu 431–3192, Japan
e-mail: uranot@hama-med.ac.jp

F. Banno · T. Miyata
Department of Molecular Pathogenesis,
National Cerebral and Cardiovascular Center, Research Institute,
5-7-1 Fujishiro-dai, Suita,
Osaka 565-8565, Japan

Weibel-Palade bodies by a variety of secretagogues, such as the vasopressin derivative desmopressin acetate (DDAVP) [25]. VWF plays an essential role in hemostasis by mediating the initial adhesion of platelets at sites of endothelial denudation, through binding of its A3 domain with collagen and of its A1 domain with glycoprotein Ib (GPIb) on platelets, especially under high shear stress conditions [1, 35]. VWF also plays a crucial role in aggregation of fluid-phase platelets by interacting with the platelet glycoprotein IIb/IIIa (GPIIb/IIIa) [11, 12]. Thus, deficiencies in either the quality or quantity of plasma VWF result in the severe bleeding tendency known as von Willebrand disease [18].

The length of VWF multimers determines their capacity to bind platelets [30], and this is likely due to the existence of multiple interactive sites on VWF for platelets and for vessel wall components [18]. UL-VWF multimers, once released into the circulation, are rapidly cleaved into smaller, less thrombogenic VWF multimers by a metalloprotease called a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13 (ADAMTS13) [24, 40, 48]. ADAMTS13 is a 195-kDa glycoprotein that is primarily synthesized in hepatic stellate cells [43, 49]. It cleaves a peptide bond between Tyr1605 and Met1606 in the A2 domain of VWF, producing VWF subunit fragments of 176 and 140 kDa [13]. This cleavage proceeds more efficiently when the VWF A2 domain is unfolded by mechanical force [46, 47]. A deficiency of ADAMTS13 increases the plasma levels of UL-VWF multimers [29] and is clinically linked to the development of thrombotic thrombocytopenic purpura (TTP) [19, 24, 42]. Pathological findings in TTP include the accumulation of platelet-rich multiple thrombi, which are distinct from those in disseminated intravascular coagulation. These thrombi are abundant in VWF antigen but have little or no fibrin, and they occur in multiple organs including heart, brain, kidneys, adrenals, spleen, and liver [3].

It is still not clear, however, how and where ADAMTS13 cleaves UL-VWF in the vasculature, and how UL-VWF multimers facilitate thrombus formation. We have recently established a method having advantages in the analysis of dynamics of platelets on VECs in vivo, in which intravital confocal microscopy [17] is applied to green fluorescent protein-expressing transgenic mice (GFP mice) [20]. Specifically, ADAMTS13 knockout mice [6] were crossed with GFP mice [32] to generate GFP-expressing ADAMTS13 knockout mice (GFP-ADAMTS13−/− mice), in which all platelets can be visualized without labeling. This made it possible to analyze the behavior of intact GFP-expressing platelets and to quantify their exact numbers bound to either DDAVP-stimulated VECs or to the injured vascular wall through UL-VWF multimers, in living animals lacking ADAMTS13.

Materials and methods

Animals

Green fluorescent protein-expressing transgenic mice, based on the C57BL/6J strain (GFP mice), were supplied by Dr. Okabe (Osaka University, Osaka, Japan) [32]. ADAMTS13 knock-out mice (ADAMTS13−/− mice: C57BL/6 strain background) [4, 6], which lack its specific activity to cleave recombinant substrate, GST-mVWF73-H, as well as a fluorogenic substrate, FRETS-VWF73 [4, 6], was crossed with GFP mice to obtain hybrid mice lacking the ADAMTS13 gene and expressing GFP (GFP-ADAMTS13−/− mice). Genomic DNA was analyzed to confirm the lack of the ADAMTS13 gene [6]. The experimental protocol was reviewed and approved by the Animal Experiments Committee of the Hamamatsu University School of Medicine.

Materials

Desmopressin ([deamino-Cys^1, D-Arg^8]-vasopressin acetate hydrate, abbreviated DDAVP) was obtained from Sigma (St. Louis, MO, USA). Polyclonal rabbit anti-human VWF antibody was purchased from DakoCytomation (Glostrup, Denmark) and was labeled with Alexa Fluor 568 obtained from Molecular Probes (Eugene, OR, USA).

Hematologic analysis

Blood samples were collected from the inferior vena cava with a syringe containing 0.1 volume of 3.8% trisodium citrate. Blood cell counts and hematocrits were determined using a whole-blood cell counter (Celltac, Nihon Kohden, Tokyo, Japan). Plasma VWF antigen levels were determined by an enzyme-linked immunosorbent assay as previously reported [34], with a slight modification. The polyclonal rabbit anti-human VWF antibody (DakoCytomation) was used as both a capturing and a tagging antibody. When it was used as a tagging antibody, it was biotinylated using the ECL™ Protein Biotinylation Module (Amersham Biosciences, Buckinghamshire, England). Human plasma was collected from five normal volunteers, pooled, and then employed to obtain a standard curve for VWF levels after sequential dilution.

In vivo imaging analyses using intravital fluorescence confocal microscopy

For intravital fluorescence microscopy of the microcirculation of a living mouse, we used a Yokogawa Real Time 3D Workstation (Yokogawa Electric Co., Tokyo, Japan), composed of a Nikon TE 600 microscope (×40, NA 0.8

water immersion objective), a Yokogawa CSU-21 confocal scanner unit, either EB-CCD or EM-CCD (C7190 or C9100-12, Hamamatsu Photonics, Hamamatsu, Japan), and a piezo electric driver (P-721.17, Physik Instrumente GmbH & Co. KG, Germany). Using this system, a focal plane image (one optical section) can be taken at 33 ms along the *z*-axis. GFP and Alexa Fluor 568 were simultaneously excited by 488- and 568-nm lasers (Krypton Argon-ion lasers; 643-YB-A01, Melles Griot Laser Group, CA, USA). For the simultaneous monitoring of both fluorescent wavelengths, an emission beam splitter, a W-view Optics (A 4313–11, Hamamatsu Photonics), was installed between the scanner unit and the CCD. The W-view consists of a dichroic mirror of 550 nm, two emission filters, a 510/23-nm band pass filter for GFP and a 590 nm long pass filter for Alexa Fluor 568 excited by light at 568 nm, so that two separate images of GFP and Alexa Fluor 568 fluorescence can be produced at the same time.

Mouse preparation for in vivo imaging

Mice were anaesthetized at least 20 min before the surgical procedure with an intraperitoneal injection of Nembutal (Dainippon Sumitomo Pharma, Osaka, Japan) at a dose of 0.06 mg/g of body weight in an atmosphere of diethyl ether. The mesenteric vascular bed was exposed after making a midline incision in the ventral abdominal wall followed by careful exteriorization. Special precautions were taken not to stretch the mesentery or touch it with metallic instruments. Only one to three different vessels per mouse, 200 to 250 μm in diameter with a shear rate ~100 s^{-1} (2.5 dyn/cm^2) were studied [10]. Segments of exteriorized mesenteric veins 142×107 μm in size were evaluated. During the procedure, the mesentery and viscera were moistened by dropwise addition of preheated saline at 37°C to prevent them from drying. Mice were placed in a supine position on the stage of the intravital fluorescence microscope. Digital video recordings were coded for subsequent blinded analysis. Upon completion of the invasive study on the mesentery vessels, mice were euthanized by anaesthetic overdose.

Intravenous DDAVP administration and VWF secretion

DDAVP, a known stimulant of VWF exocytosis, was administered, although its effect on mice is limited [7]. A dose of 0.3 μg/kg body weight [25] was administered intravenously to GFP-ADAMTS13−/− mice (n=6) and GFP mice (n=4). Using an intravital fluorescence confocal microscopy system, and IPLab software (BD Biosciences Bioimaging, MD, USA), the in vivo kinetics of GFP-expressing platelet adhesion on VECs was evaluated. When more than two platelets were connected to each other and adhered on VECs, these connected platelets were considered as one "platelet string". The numbers of adhered single platelets and of "platelet strings" were counted for each venular segment, and the sum of them was indicated as the "total number". The diameter of each single platelet as well as the length of each "platelet string" were also measured for each venular segment, and their sum was indicated as the "total length". The average lengths were also calculated by dividing "total length" by the "total number". During this experiment, only one vein per mouse was evaluated. The results represent data for an average venular segment, with five segments counted and recorded over the same period of time (100 frames, 30 frames per sec). Recordings were made just before and just after DDAVP administration, in 5-min intervals for up to 1 h. For visualizing VWF multimer strings, Alexa Fluor 568-labeled anti-VWF antibody (0.1 ml of approximately 1 mg/ml solution) was administered intravenously together with DDAVP through the tail vein into GFP-ADAMTS13−/− mice, and the adhesion of GFP-expressing platelets on the strings was confirmed by another set of experiments.

VECs injury by 2.5% ferric chloride ($FeCl_3$)

Mesenteric venules were exteriorized and injured by topical use of $FeCl_3$ solution. Lavage of mesentery by dropwise addition of saline, which was continued after its exteriorization, was stopped for 1 min and then 30 μl of 2.5% $FeCl_3$ was topically applied to the surface of the targeted vessel. After 15 s of exposure of the vein to $FeCl_3$, the mesentery was exposed to saline again and the applied $FeCl_3$ was washed out. Precisely 1 min after application of $FeCl_3$, changes in fluorescent intensity in a standard venular segment were recorded for about 23 s (700 frames taken with a video rate of 30 frames per second). Recorded sequences were evaluated at each frame using IPLab software, and the length of connected platelets aligned as beads on a string ("platelet string") was measured. The duration of each string's binding to a vascular surface was also calculated by multiplying the numbers of sequential frames in which the strings were observed by the frame interval of 0.033 s. One segment per vein, and one to three veins per mouse were assessed in GFP-ADAMTS13−/− mice (n=9) and in GFP mice (n=8).

Laser-induced vessel wall injury and image analysis of microthrombi

The mesenteric venule was exteriorized and the endothelium was focally injured by a 514-nm argon ion laser (543-GS-A03; Melles Griot Laser Group, CA). The laser beam was aimed at the endothelium through the microscope objective lens. The injured area of endothelium, the

diameter of which was approximately 10 μm [20], was kept constant by changing the intensity and duration of laser illumination (typically 140 mW, 5 s). Changes in the fluorescence intensity of GFP-expressing platelets were measured with an EB-CCD camera to monitor thrombus formation.

A z-stack of 25 optical sections (2 μm optical slice thickness), at up to 30 frames per second from the vessel wall to the luminal surface of a thrombus, was captured exactly 15, 30, 45, 120, and 300 s after injury and analyzed using a Yokogawa Real Time 3D Workstation and IPLab software in both GFP-ADAMTS13−/− mice (n=6) and GFP mice (n=6). A freehand-defined region of interest was traced along the outline of GFP–platelet thrombus areas. After blinding, scans were analyzed for fluorescence intensity of GFP using Adobe Photoshop CS2 and Scion Image to measure the area of the thrombus. The size of the thrombus was then calculated based on the fact that the thickness of each plane is 2 μm, and its volume was expressed in μm^3. In another set of experiments, Alexa Fluor 568-labeled anti-VWF antibody was injected intravenously through the tail vein into GFP-ADAMTS13−/− mice (n=4) and GFP mice (n=4) before laser irradiation. The scans were taken at exactly 15, 30, 45, 60, 120, and 300 s after laser injury, and the fluorescent intensity of Alexa Fluor 568 was measured to analyze the localization of VWF in the thrombus.

Statistical analysis

To evaluate the significance of differences between two groups, the parametric Student's T test was used unless otherwise indicated. The non-parametric Mann–Whitney U test was also used when the population was not normally distributed. Differences were considered significant at $P<0.05$.

Results

Characteristics of GFP-ADAMTS13−/− mice

GFP-ADAMTS13−/− mice were viable and fertile, and their blood cell counts were normal (Table 1). Plasma VWF levels in GFP-ADAMTS13−/− mice (0.372±0.069 U/ml, mean±SD, n=12) were approximately 1.4 times higher than those in GFP mice (0.259±0.004 U/ml, mean value±SD, n=10). As noted previously for ADAMTS13−/− mice [5, 6, 31], they did not show any sign of developing spontaneous TTP.

In vivo analyses of platelet adhesion to VECs after DDAVP administration: role of ADAMTS13 in proteolysis of secreted UL-VWF multimers

In both GFP-ADAMTS13−/− mice and GFP mice, some platelets adhered to normal VECs even before DDAVP stimuli. Most of these events were observed as a single platelet adhesion but doublet or triplet platelets, which were connected like beads on strings, were also observed. There was no difference in the number of platelets adhered to VECs between the two animal groups before DDAVP stimuli.

Although the ability of DDAVP to stimulate VWF secretion in mice is controversial, we observed enhanced VWF secretion from VECs in the present study, as was reported before [7]. After DDAVP administration, the number of platelets adhered to VECs increased in both GFP-ADAMTS13−/− mice and in GFP mice, and some of them were attached to each other. A simultaneous injection of Alexa568-labeled anti-VWF antibody revealed that these platelets appeared to attach close to the spots labeled by the anti-VWF antibody (Fig. 1). Several platelets connected and aligned on VWF strings were also observed (Supplement Fig. 1). The numbers of both single platelets and "platelet strings" attached to VECs (total number) in a vascular segment were counted. There was no significant difference in these numbers for the first 20 min, whereas a significant difference was observed thereafter between GFP-ADAMTS13−/− mice and GFP mice, which continued until the end of the observation, i.e., for up to 60 min (Fig. 2a). Administration of DDAVP also significantly increased the "total length" of the attached platelets to VECs, which was obtained as a sum of the diameters of attached single platelets, as well as of the length of each "platelet string" per venular segment in GFP-ADAMTS13−/− mice, while in GFP mice, we did not see a similar response (Fig. 2b). The average length of those in GFP-ADAMTS13−/− mice reached a maximum at 5 min, and these strings were significantly longer than those in the GFP mice (Fig. 2c). Plasma levels of VWF, however, did not significantly increase at 5, 15, 30, 60, or

Table 1 Blood cell counts

	WBC	RBC	Hb	Hct	Plt
GFP-ADAMTS13−/− mice	3650±940	796±23	10.5±0.2	37.1±1.2	46.8±13.0
GFP mice	4950±840	820±44	10.7±0.5	37.4±1.7	42.0±8.9

Mean±SD N=6, *WBC* white blood cells, *RBC* red blood cells, *Hb* hemoglobin, *Hct* hematocrit, *Plt* platelet