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Complete deficiency in ADAMTS13 is prothrombotic, but it alone is not sufficient to cause thrombotic thrombocytopenic purpura

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ADAMTS13 is a plasma metalloproteinase that regulates platelet adhesion and aggregation through cleavage of von Willebrand factor (VWF) multimers. In humans, genetic or acquired deficiency in ADAMTS13 causes thrombotic thrombocytopenic purpura (TTP), a condition characterized by thrombocytopenia and hemolytic anemia with microvascular platelet thrombi. In this study, we report characterization of mice bearing a targeted disruption of the Adamts13 gene. ADAMTS13-deficient mice were born in the expected mendelian distribution; homozygous mice were viable and fertile. Hematologic and histologic analyses failed to detect any evidence of thrombocytopenia, hemolytic anemia, or microvascular thrombosis. However, unusually large VWF multimers were observed in plasma of homozygotes. Thrombus formation on immobilized collagen under flow was significantly elevated in homozygotes in comparison with wild-type mice. Thrombocytopenia was more severely induced in homozygotes than in wild-type mice after intravenous injection of a mixture of collagen and epinephrine. Thus, a complete lack of ADAMTS13 in mice was a prothrombotic state, but it alone was not sufficient to cause TTP-like symptoms. The phenotypic differences of ADAMTS13 deficiencies between humans and mice may reflect differences in hemostatic system functioning in these species. Alternatively, factors in addition to ADAMTS13 deficiency may be necessary for development of TTP. (Blood. 2006;107:3161-3166)

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening systemic disease, characterized by anemia, thrombocytopenia, and microvascular thrombosis.[1-4] Hemolysis, the cause of the anemia, generates pointed red cell fragments, schistocytes. Thrombocytopenia is caused by the consumption of platelets in thrombi, which cause renal and neurologic dysfunction. Without treatment, the mortality rate of affected patients exceeds 90%, but plasma exchange reduces the death rate to approximately 20%.[5]

Our understanding of TTP pathophysiology increased considerably with the identification of ADAMTS13, which specifically cleaves the Tyr^{1605}-Met^{1606} peptidyl bond of von Willebrand factor (VWF).[6-10] VWF is a large glycoprotein that mediates platelet adhesion to vascular lesions. It is mainly synthesized in endothelial cells and secreted into the blood as "unusually large" VWF (UL-VWF) multimers, the highly active forms for platelet adhesion and aggregation.[11,12] ADAMTS13 cleaves UL-VWF multimers into smaller forms under flow, limiting platelet thrombus formation under normal conditions. Severe deficiency in ADAMTS13 activity is observed in most patients with TTP, allowing UL-VWF multimers to persist in the circulation.[1-4] UL-VWF multimers mediate enhanced platelet clumping under shear stress, which is thought to cause the clinical symptoms of TTP. Congenital TTP is associated with mutations in the ADAMTS13 gene, whereas acquired TTP results from the production of autoantibodies against ADAMTS13. A number of causative mutations for congenital TTP have been identified within the ADAMTS13 gene.[3,4] In vitro expression studies have confirmed the deleterious effects of mutant ADAMTS13 on proteolytic activity or secretion.[13-15]

Here, we generated a mouse model of ADAMTS13 deficiency by a gene-targeting approach, to further understand the pathophysiologic process of TTP. We found that the complete deficiency in ADAMTS13 is not sufficient to produce in mice the typical TTP phenotype. Other triggers may be needed to provoke the disease.

Materials and methods

Generation of ADAMTS13-deficient mice

The isolation of • phage genomic clones containing Adamts13 has been previously described.[16] The targeting vector was constructed from a 12.3-kb fragment including exons 3-12, in which the 3.6-kb SalI-EcoRI region containing exons 3-6 was replaced by a neomycin resistance cassette. A

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F.B. designed research, performed research, analyzed data, and wrote the paper; K.K. designed research, performed research, and wrote the paper; T.O. contributed vital analytical tools and interpreted the data; S.H. contributed vital analytical tools and interpreted the data; S.M. contributed vital analytical tools and interpreted the data; H.K. performed research, contributed vital analytical tools, and interpreted the data; Y.T. contributed vital analytical tools and interpreted the data; and T.M. designed research and wrote the paper.

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diphtheria toxin A fragment expression cassette was inserted into downstream of the 3•-homologous region. The vector was introduced into 129/Sv-derived R-CMTI-1A embryonic stem cells by electroporation. Cells were selected in medium containing G418 (Invitrogen, Carlsbad, CA) and screened by polymerase chain reaction (PCR) and Southern blot analyses. Targeted cells were microinjected into C57BL/6 blastocysts to generate chimeric mice. The resulting male chimeras were bred to wild-type 129/Sv females to produce heterozygous F1 offspring on the 129/Sv genetic background. Heterozygotes were interbred to obtain homozygous mice. Male mice aged 8 to 12 weeks were used for phenotypic analyses. Pregnant female mice aged 8 months were used for renal histology analysis. Female mice aged 15 to 20 weeks (20-30 g) were used for in vivo thrombosis experiments. All animal procedures were performed in accordance with institutional guidelines and were approved by the Animal Care and Use Committee of the National Cardiovascular Center Research Institute.

Genotypic analysis

gDNA, isolated from ear or kidney, was used for genotyping by PCR or Southern blot analyses. For PCR analysis, DNA amplification was performed using a mixture of 3 primers: an intron 2–specific forward primer (5•-ACCCTATCTCTGGCCTGTATTCCT-3•), an intron 3–specific reverse primer (5•-TACTGACTTGTGACCACAAGCCCT-3•), and a neo cassette-specific reverse primer (5•-ATCGAGTCTAGCTTGGCTGGACGT-3•). For Southern blot analysis, a 580-bp fragment upstream of the 5•-homologous region was generated by PCR with primers 5•-TGTCTGCAAGTGCAGTGAGAGGCA-3• and 5•-AATGAAGATGGCACCAGTGAGGAT-3• and used for the synthesis of a fluorescein-labeled probe. The probe was hybridized to HindIII-digested gDNA and detected using a CDP-Star detection module (Amersham, Piscataway, NJ).

RT-PCR analysis

Total RNA was prepared from liver using ISOGEN reagent (Nippon Gene, Tokyo, Japan) and subjected to 1-step reverse transcription-PCR (RT-PCR; Qiagen, Hilden, Germany). An exon 21/22-specific sense primer (5•-TTGTGGGAGAGGTCTGAAGGAACT-3•) and an exon 24/25-specific antisense primer (5•-ACAGGAGACAGAGCACTCTGTCCA-3•) were used to amplify ADAMTS13 mRNA.

In situ hybridization

In situ hybridization was performed as described.[17] A 435-bp mouse Adamts13 cDNA fragment (nucleotides: 679-1113) was used to synthesize digoxigenin-labeled sense and antisense RNA probes by in vitro transcription with a DIG RNA labeling mix (Roche, Basel, Switzerland). The probe was hybridized to liver sections and detected using an anti-DIG AP conjugate (Roche) and NBT/BCIP solution (Roche). Sections were counterstained with Kernechtrot solution.

Measurement of plasma ADAMTS13 activity

With the mice under ether anesthesia, blood was collected from the retro-orbital plexus into tubes containing a 0.1 volume of 3.8% sodium citrate. Plasma was prepared from blood by centrifugation at 800g for 15 minutes at room temperature. ADAMTS13 activity was measured using a recombinant substrate, GST-mVWF73-H, as described.[16,18] Activity was also measured using a fluorogenic substrate, FRETS-VWF73 (Peptide Institute, Minoh, Japan).[19]

VWF multimer analysis

Plasma samples, diluted in sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris-HCl, 2% SDS, 2 mM EDTA, 0.02% bromphenol blue, and 43.5% glycerol, pH 6.8) were electrophoresed on a 1% agarose gel (Agarose IEF; Amersham) at a constant current of 15 mA at 4°C. After transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA) by capillary blotting, the membrane was incubated in peroxidase-conjugated rabbit anti–human VWF (1:500, Dako, Glostrup, Denmark) in 5% skim milk to detect VWF multimers. Bound antibody was detected with Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer, Boston, MA) on an image analyzer (Fujifilm, Tokyo, Japan). The chemiluminescent intensities of each lane were scanned using Image Gauge software (Fujifilm); the relative intensity profiles were shown.

Hematologic analysis

Blood cell counts and hematocrit were determined using an automatic cell counter (KX-21NV; Sysmex, Kobe, Japan). Peripheral blood smears were stained with May-Grünwald-Giemsa and examined under light microscopy. Plasma haptoglobin levels were analyzed using a mouse haptoglobin enzyme-linked immunosorbent assay (ELISA) test kit (Life Diagnostics, West Chester, PA).

Plasma VWF antigen was measured by ELISA using antibodies against human VWF. Plasma samples in 1% BSA were applied to rabbit anti–human VWF-coated (Dako) ELISA plates for 2 hours at room temperature. Bound VWF was detected by incubation with peroxidase-conjugated rabbit anti–human VWF (1:4000, Dako) in 1% BSA for 1 hour. Bound antibody was detected using a SureBlue Reserve TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD); the absorbance at 450 nm was measured. A standard curve was constructed from the pooled plasma of 129/Sv mice.

Plasma fibrinogen levels were also measured by ELISA using rabbit anti–human fibrinogen (Dako) and peroxidase-conjugated goat anti–mouse fibrinogen (Nordic Immunological Laboratories, Tilburg, The Netherlands) antibodies. Plasma factor VIII (FVIII) activity was measured using a Testzym FVIII Kit (Daiichi Pure Chemicals, Tokyo, Japan). To assess the ELISA and FVIII activity data, the levels measured in wild-type mice were arbitrarily defined as 100%.

Histologic analysis

The kidneys of pregnant female mice were fixed in phosphate-buffered 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin or periodic acid-Schiff reagent. VWF antigen was detected using an ENVISION• system (Dako) with rabbit anti–human VWF (Dako).

Coagulation tests and bleeding assay

The prothrombin time (PT) and activated partial thromboplastin time (APTT) of plasma samples were determined using Thrombocheck PT (Sysmex) and Thrombocheck APTT (Sysmex) reagents, respectively. Bleeding analysis was performed on mice anesthetized with sodium pentobarbital (50 • g/g). Tails were amputated 3 mm from the tip and immersed in 1 mL PBS at 37°C for 15 minutes. Blood loss was estimated from the comparison of the absorbance of the PBS at 562 nm with that of PBS containing known volumes of mouse blood.

Platelet aggregation analysis

Platelet aggregation was measured using an aggregometer (MC Medical, Tokyo, Japan) as described.[20] Platelet counts in platelet-rich plasma (PRP) were adjusted to 3.0 • 10^5/• L by adding platelet-poor plasma (PPP). Aggregation was initiated by addition of acid-insoluble type I collagen (MC Medical) or botrocetin to PRP. PPP was used as a standard indicating 100% aggregation.

Perfusion assay with a parallel plate flow chamber

Platelet thrombus formation in flowing blood on immobilized collagen was analyzed using a parallel plate flow chamber as described.[21,22] Acid-insoluble type I collagen-coated (Sigma, St Louis, MO) glass coverslips were placed in a flow chamber. The chamber was mounted on a fluorescence microscope (Axiovert S100; Carl Zeiss, Oberkochen, Germany) equipped with a 40 • /0.75 numeric aperture objective lens (Carl Zeiss) and a CCD camera system (DXC-390; Sony, Tokyo, Japan). Blood was collected into tubes containing argatroban (240 • M; Mitsubishi Chemical Corporation, Tokyo, Japan). The fluorescent dye mepacrine (10 • M; Sigma) was added to the blood. Whole blood samples were aspirated through the chamber and across the collagen-coated coverslip by a syringe

pump (Harvard Apparatus, South Natic, MA) at a constant flow rate producing a wall shear rate of 750 s^{-1} The shear rate was calculated from the assumption that the viscosity of mouse blood is equal to that of human blood. To analyze the cumulative thrombus volume, image sets at 1.0-• m z-axis intervals within a defined area (156.4 • 119.6 • m) was captured using MetaMorph software (version 6.1.4; Universal Imaging, West Chester, PA). After blind deconvolution of image sets processed by AutoDeblur software package (version 8.0.2; AutoQuant Imaging, Troy, NY), 3-dimensional volumetric measurements of thrombi were accomplished using VoxBlast software (version 3.0; Vartek, Fairfield, IA).

In vivo thrombosis model

A mixture of 600 ng/g collagen (Nycomed, Roskilde, Denmark) and 60 ng/g epinephrine (Sigma) was injected into tail vein of mice.[23] Blood was collected 15 minutes after the injection and platelet counts were determined.

Statistical analysis

Statistical significance was assessed by the Student t test or the •^{2} test. Differences were considered to be significant at P below .05.

Results

Generation of ADAMTS13-deficient mice

We previously reported 2 strain-specific forms of the mouse Adamts13 gene.[16] In the 129/Sv strain, the Adamts13 gene contains 29 exons, as in human ADAMTS13, encoding a protein with a similar domain organization as human ADAMTS13. Several strains of mice, including the C57BL/6 strain, harbor a retrotransposon insertion, encoding a variant form of ADAMTS13 that lacks the C-terminal domains. Therefore, we generated and analyzed ADAMTS13-deficient mice on a 129/Sv genetic background.

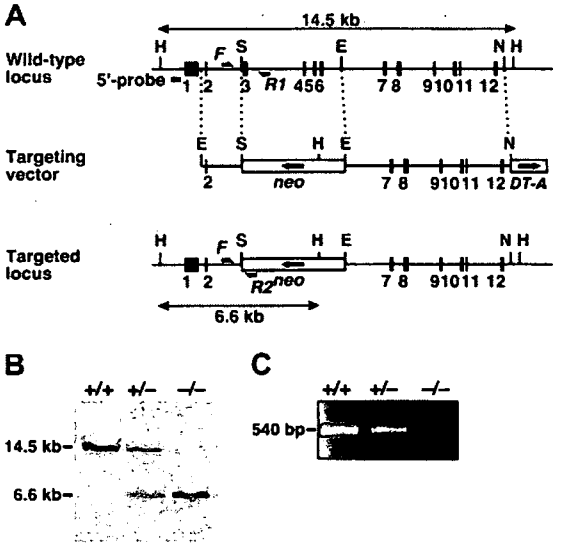

Figure 1. Targeted disruption of the mouse Adamts13 gene. (A) Structure of the targeted locus in the mouse Adamts13 gene. Exons are represented by filled boxes. A neomycin-resistance cassette (neo), in the opposite transcriptional orientation, and a forward-oriented diphtheria toxin A fragment expression cassette (DT-A) are indicated. Homologous fragments are indicated by dotted lines; the HindIII fragments detected by Southern analysis of the wild type and targeted alleles are indicated by double-headed arrows. The sites of primers used for the genotyping PCR (F, R1, and R2) are indicated by arrows. H indicates HindIII; S, SalI; E, EcoRI; N, NcoI. (B) Southern blot analysis. gDNA from offspring obtained from heterozygous intercrosses was digested with HindIII and detected with the 5•-specific probe (wild type: 14.5 kb; targeted allele: 6.6 kb). (C) RT-PCR analysis. Total RNA isolated from mouse liver was reverse-transcribed and amplified using the Adamts13-specific primer set to generate a 540-bp fragment.

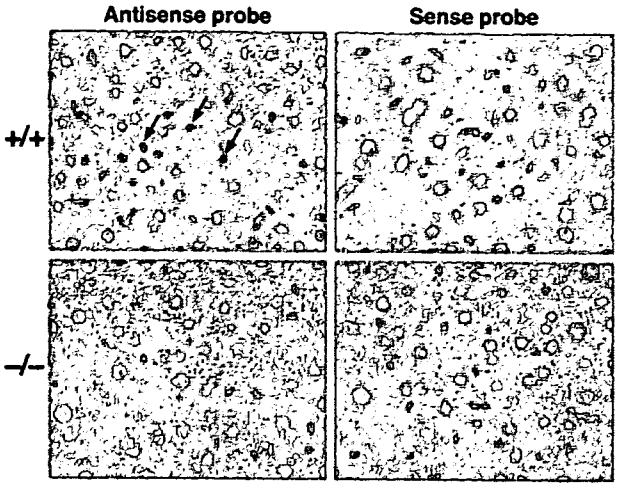

Figure 2. In situ hybridization analysis of ADAMTS13 mRNA. Liver sections from Adamts13$^{+/+}$ (top panels) and Adamts13$^{-/-}$ (bottom panels) mice were hybridized to the antisense (left panels) or sense (right panels) Adamts13 RNA probes. The hybridized sections were counterstained with Kernechtrot solution. Typical positive signals are indicated by arrows.

The Adamts13 gene was disrupted using a targeting vector that eliminated exons 3-6, encoding the catalytic domain (Figure 1A). The expected structure of the targeted locus was confirmed by PCR (data not shown) and Southern blotting (Figure 1B). Elimination of ADAMTS13 mRNA in Adamts13$^{+/+}$ mice was verified by RT-PCR of total RNA from liver (Figure 1C), the primary site of synthesis.[16] In situ hybridization analysis also confirmed the loss of ADAMTS13 mRNA in Adamts13$^{-/-}$ mice (Figure 2). Because ADAMTS13 is expressed in hepatic stellate cells,[24,25] we detected hybridization with an antisense probe in the nonparenchymal liver cells of Adamts13$^{-/-}$ mice. According to their morphology, these cells were hepatic stellate cells. Specific hybridization was not detected in sections from Adamts13$^{+/+}$ mice.

No ADAMTS13 enzymatic activity could be detected in plasma samples of Adamts13$^{-/-}$ mice by either qualitative (Figure 3A) or quantitative (Figure 3B) methods using GST-mVWF73-H and FRETS-VWF73, respectively, as substrates. Enzymatic activity in Adamts13$^{+/-}$ mice was reduced to approximately 35% that seen in Adamts13$^{+/+}$ mice (Figure 3B).

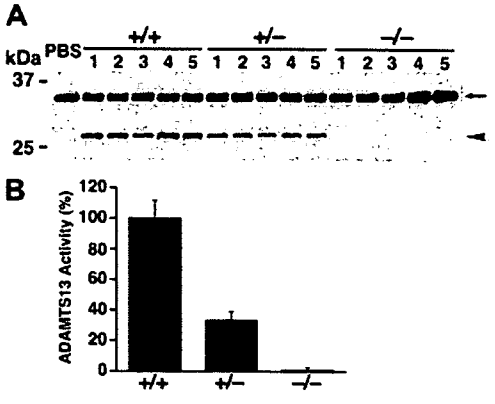

Figure 3. ADAMTS13 activity in plasma. (A) Qualitative assay using a recombinant substrate, GST-mVWF73-H. The substrate and product bands are indicated by arrows and arrowheads, respectively. (B) Quantitative assay using a fluorogenic substrate, FRETS-VWF73. Data are mean • SD from 4 mice for each genotype. The average activity measured in wild-type mice was defined as 100%.

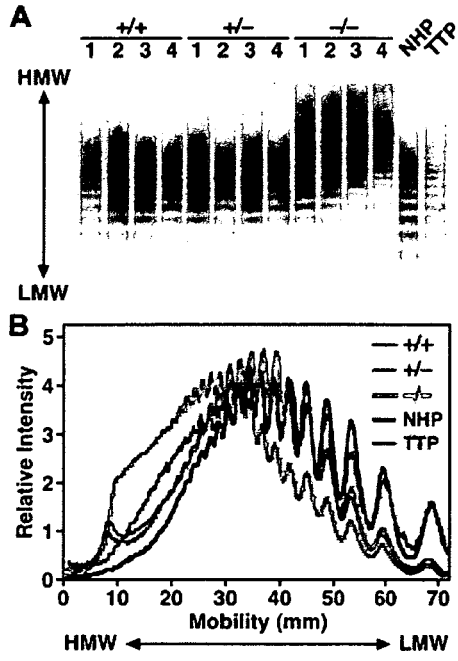

Figure 4. Analysis of plasma VWF multimers. (A) VWF multimer patterns. Plasma samples (1 • L/lane) from Adamts13$^{+/+}$, Adamts13$^{+/-}$, and Adamts13$^{-/-}$ mice were electrophoresed on SDS-agarose gels and transferred to nitrocellulose membranes. VWF multimers were detected with anti-VWF antibodies. Normal human plasma (NHP) and ADAMTS13-deficient TTP patient plasma (TTP) were analyzed in parallel (0.2 • L/lane). (B) Relative intensities of plasma VWF multimers. The chemiluminescent intensities of the VWF multimer patterns (A) were scanned using image analysis software. HMW indicates high molecular weight; LMW, low molecular weight.

Accumulation of UL-VWF multimers in plasma

In humans, genetic defects in ADAMTS13 lead to the accumulation of UL-VWF multimers in plasma. Analysis of VWF-multimer patterns in plasma detected UL-VWF multimers in Adamts13$^{-/-}$ mice (Figure 4), suggesting ADAMTS13 deficiency supports the accumulation of plasma UL-VWF multimers. Because the laddering patterns of VWF multimers in Adamts13$^{+/-}$ and Adamts13$^{-/-}$ mice were similar, less than half of the normal plasma ADAMTS13 activity (Figure 3B) was sufficient to regulate VWF multimer size. VWF multimers in mice were larger than those in humans (Figure 4B); the multimer sizes seen in Adamts13$^{-/-}$ mice were similar to those observed in patients with TTP.

No TTP symptoms in ADAMTS13-deficient mice

Genotyping of 195 offspring of Adamts13$^{+/-}$ intercrosses showed the expected 1:2:1 mendelian distribution of Adamts13$^{+/+}$ (52 of 195), Adamts13$^{+/-}$ (91 of 195), and Adamts13$^{-/-}$ (52 of 195). Thus, ADAMTS13 deficiency did not cause embryonic lethality. Adamts13$^{-/-}$ mice were viable and fertile. To date, 4 Adamts13$^{-/-}$ mice exhibited lateral flexion of upper body; one of them had a cloudy eye. Further study is required to uncover whether this rare phenotype is caused by ADAMTS13 deficiency. Although pregnancy is a triggering event for TTP,[26] deficient females survived pregnancy, delivering viable offspring in normal-sized litters. No significant difference in blood cell counts (Table 1) or plasma haptoglobin levels (Table 2) was observed between Adamts13$^{+/+}$ and Adamts13$^{+/-}$ mice. Peripheral blood smears from Adamts13$^{+/-}$ mice did not show erythrocyte fragmentation (data not shown), demonstrating a lack of spontaneous thrombocytopenia and hemolytic anemia in Adamts13$^{+/-}$ mice. The renal histology of Adamts13$^{+/-}$ mice during pregnancy did not exhibit microvascular thrombi deposition or excessive accumulation of VWF antigen (data not shown). Thus, Adamts13 disruption in mice did not cause TTP-like symptoms.

Table 1. Blood cell counts

	Adamts13$^{+/+}$	Adamts13$^{-/-}$
Red blood cell count, • 10^{12}/L	8.19 • 0.41	7.97 • 0.25
Hemoglobin level, g/L	129 • 5	126 • 4
Hematocrit concentration	.426 • .021	.422 • .008
Platelet count, • 10^9/L	512 • 42	532 • 62

Values are mean • SD of 7 mice in each genotype.

Increased thrombogenesis in ADAMTS13-deficient mice

Plasma VWF antigen levels in Adamts13$^{-/-}$ mice were elevated in comparison with those from Adamts13$^{+/+}$ mice (Table 2). The activity of plasma FVIII, which correlates with VWF levels, was also significantly increased in Adamts13$^{-/-}$ mice (Table 2). The plasma fibrinogen levels, however, were comparable between Adamts13$^{+/+}$ and Adamts13$^{+/-}$ mice (Table 2). PT and APTT suggested the coagulant state in Adamts13$^{-/-}$ mice was normal (Table 2). To investigate the effects of ADAMTS13 deficiency on hemostasis in vivo, we measured blood loss after tail transection. There were no significant differences in blood loss between Adamts13$^{+/+}$ and Adamts13$^{+/-}$ mice (Table 2), suggesting UL-VWF multimers did not impair hemostasis.

To uncover a latent prothrombotic state caused by the presence of UL-VWF multimers in Adamts13$^{-/-}$ mice, we investigated platelet aggregation under static or flow conditions. We examined agonist-induced platelet aggregation under static conditions. Aggregation responses to botrocetin and collagen in Adamts13$^{-/-}$ mice were indistinguishable from those seen in Adamts13$^{+/+}$ mice (Figure 5). Thus, an UL-VWF–mediated prothrombotic state could not be detected in Adamts13$^{-/-}$ mice under static conditions.

Focusing on thrombus formation under flow, whole blood was perfused over a collagen-coated surface in a parallel plate flow chamber. Even though mice have smaller platelets than humans, thrombus formation was more prominent in mice than in humans, under our flow chamber system. The maximum shear rate to follow up thrombus formation in mouse blood was 750 s^{-1} and we selected this rate for comparing thrombogenesis between the groups. Cumulative thrombus volume was recorded every 0.5 minute after beginning perfusion (Figure 6). Until 3.5 minutes of perfusion, thrombus formation progressed slowly; the thrombus volume did not differ between the Adamts13$^{+/+}$ and Adamts13$^{+/-}$ groups. After 3.5 minutes, the thrombus grew rapidly in Adamts13$^{-/-}$ mice; the thrombus volume at 5.5 minutes was significantly higher in Adamts13$^{-/-}$ mice than in Adamts13$^{+/-}$ mice. Thus, ADAMTS13 deficiency in mice does not affect the

Table 2. Hematologic and coagulation parameters

	Adamts13$^{+/+}$	Adamts13$^{-/-}$
Haptoglobin, %	100 • 67	103 • 69
VWF antigen, %	100 • 23	129 • 31*
FVIII activity, %	100 • 10	146 • 22†
Fibrinogen, %	100 • 5	98 • 7
PT, s	16.1 • 0.8	16.0 • 1.0
APTT, s	44.2 • 3.7	43.3 • 2.5
Blood loss, • L	12.5 • 8.4	9.5 • 3.1

Values are mean • SD of 12 mice in each genotype except for the blood loss, where it is mean • SD of 18 mice.

*P • .05 when compared with Adamts13$^{+/+}$ mice

†P • .001 when compared with Adamts 13$^{+/+}$ mice.

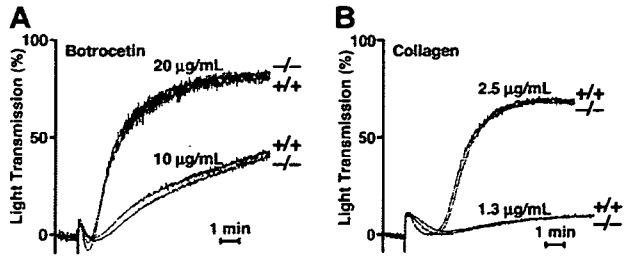

Figure 5. Platelet aggregation under static condition. (A) Botrocetin-induced aggregation. Pooled PRP samples from Adamts13$^{+/+}$ or Adamts13$^{-/-}$ mice were treated with botrocetin at a final concentration of 10 or 20 • g/mL. Aggregation was measured using an aggregometer at 37°C with constant stirring. (B) Collagen-induced aggregation. Pooled PRP samples were treated with acid-insoluble type I collagen at a final concentration of 1.3 or 2.5 • g/mL. Bars indicate 1 minute. The results of 3 typical experiments are shown.

initial adhesion of platelets to collagen, but enhances thrombus growth under shear stress.

To evaluate in vivo consequence of a lack of ADAMTS13, we examined a model of collagen-induced thrombosis. Under the conditions we examined, the mortality was not different between Adamts13$^{+/+}$ and Adamts13$^{-/-}$ mice (1 of 12 and 1 of 15 died, respectively, P • .87 by •^{2} test). However, platelet counts of treated mice were significantly lower in Adamts13$^{-/-}$ mice than in Adamts13$^{+/+}$ mice (Figure 7), whereas platelet counts of untreated mice were not different between groups. These results indicate that ADAMTS13 deficiency generates prothrombotic state in vivo as well as in vitro.

Discussion

This study suggests 2 perspectives on the etiology of TTP. First, deficiency in ADAMTS13 alone is sufficient to generate UL-VWF multimers in plasma, leading to a prothrombotic state. Second, ADAMTS13 deficiency is insufficient to produce the typical symptoms of TTP in mice. ADAMTS13 deficiency may induce TTP only when combined with other triggering factors.

Under static conditions, platelet aggregation responses to collagen and botrocetin were indistinguishable in ADAMTS13-deficient mice from those seen in wild-type mice, although the plasma VWF multimer size was larger in ADAMTS13-deficient mice. This result is consistent with the previous report that botrocetin is active on rodent platelets, reacting to a broad

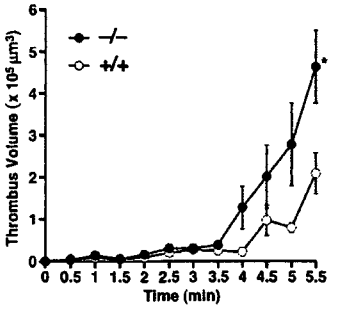

Figure 6. Thrombogenesis on collagen surface under flow. Whole blood from Adamts13$^{+/+}$ or Adamts13$^{-/-}$ mice containing mepacrine-labeled platelets was perfused over an acid-insoluble type I collagen-coated surface at a wall shear rate of 750 s^{-1}. The cumulative thrombus volume, analyzed using a multidimensional imaging system, was measured every 0.5 minute until 5.5 minutes. Data are the mean • SEM of 5 mice for each genotype. *Significant differences at P • .05 in comparison with Adamts13$^{+/+}$ mice.

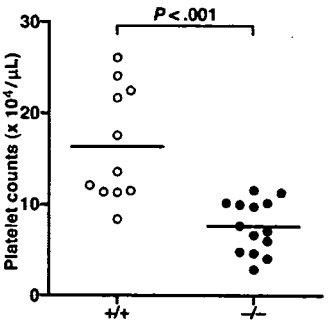

Figure 7. Platelet counts following collagen plus epinephrine challenge. Mice were given injections of 600 ng/g collagen plus 60 ng/g epinephrine via tail vein and platelet counts were measured 15 minutes after injection. Symbols represent platelet counts of a single mouse. Bars represent the mean values of groups. Platelet counts after the challenge were significantly lower in Adamts13$^{-/-}$ mice (n • 14) than Adamts13$^{+/+}$ mice (n • 11) at 7.7 • 2.9 • 10^4/• L and 16.4 • 6.2 • 10^4/• L, respectively (mean • SD; P • .001), whereas platelet counts without challenge were not different between groups (Adamts13$^{-/-}$, 86.2 • 13.2 • 10^4/• L; Adamts13$^{+/+}$, 83.7 • 3.3 • 10^4/• L; mean • SD of 4 mice).

spectrum of high to low molecular weight VWF multimers.[27] Under flow conditions, however, thrombus formation on a collagen surface was enhanced in ADAMTS13-deficient mice. Although initial platelet adhesion to immobilized collagen was not affected, the growth rate of thrombus was significantly faster in ADAMTS13-deficient mice. In an in vivo thrombosis model, ADAMTS13-deficient mice were more sensitive to collagen-induced thrombocytopenia than wild-type mice, confirming in vitro observation in the flow chamber study. Thus, it was concluded that ADAMTS13 deficiency produces the prothrombotic state. Further study will be necessary to elucidate whether this prothrombotic state is ascribable to hyperreactivity of UL-VWF multimers in ADAMTS13-deficient mice.

Although prolonged coagulation time was not observed, plasma levels of VWF antigen and FVIII activity were elevated in ADAMTS13-deficient mice, potentially reflecting endothelial damage induced by undetectable platelet aggregates. Alternatively, the plasma clearance rate of VWF multimers without cleavage by ADAMTS13 might be slower than cleaved VWF multimers. High levels of VWF antigen are also seen in the plasma of patients with low ADAMTS13 activity.[28]

ADAMTS13 deficiency in mice did not cause a major defect in hemostasis that would lead spontaneously to typical TTP symptoms. ADAMTS13 deficiency may cause a milder prothrombotic state in mice than in humans. The plasma VWF multimer sizes in wild-type mice were larger than those seen in humans, comparable to those in human TTP patients (Figure 4B). Mice lacking VWF exhibit milder tendencies to bleed than patients with type 3 von Willebrand disease.[29] Thus, the dependence of platelet aggregation on VWF might differ in laboratory mice from humans.

Alternatively, ADAMTS13 deficiency may not be sufficient for the development of TTP, even in humans. There is a large variation in the phenotypes of TTP patients with ADAMTS13 deficiency. Most TTP patients with congenital ADAMTS13 deficiency had their first acute episode in the newborn period or early infancy. Only a number of exceptional cases remain asymptomatic until adulthood.[30] Patients with identical ADAMTS13 genotypes, but different symptoms, have also been described,[31,32] suggesting that the etiology of TTP cannot be explained by a single defect in ADAMTS13. Secondary triggering factors may promote the pathogenic platelet thrombus formation that results in TTP. Indeed,

Motto et al[32] independently reported generation of ADAMTS13-deficient mice and revealed that the injection of shigatoxin, a substance toxic to endothelium, provoked TTP-like symptoms in the ADAMTS13-deficient mice. In the present study, we observed enhanced thrombus formation on collagen surface under flow and promoted thrombocytopenia induced by the injection of a mixture of collagen and epinephrine in ADAMTS13-deficient mice. Genetic defects or environmental factors may stimulate endothelial activation or damage via TTP triggers, such as oxidative stress,[33] infection,[34] antiendothelial cell antibodies,[35] or complement dysfunction.[36,37] ADAMTS13-deficient mice may be useful to identify TTP triggers.

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To the editor:

Protein S–K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients

Deep vein thrombosis (DVT) is a multifactorial disease caused by interactions between acquired risk factors and coagulation abnormalities.[1] In whites, the factor V–Leiden and the prothrombin-20210G>A are widely recognized as genetic risk factors for DVT. However, these 2 mutations are not present in Japanese populations, and little is known about the genetic risk factors for DVT in these populations. In this study, we evaluated the genetic contributions of 5 polymorphisms in Japanese DVT patients. The plasminogen-A620T mutation, formerly referred to as plasminogen-Tochigi, and the protein S–K196E mutation, formerly referred to as protein S–Tokushima, exhibited decreased activities of plasminogen and protein S despite normal antigen levels.[2-4] The ADAMTS13-P475S mutation exhibited low von Willebrand factor–cleaving activity in vitro.[5] The factor XII–4C>T substitution in the 5′-untranslated region, formerly referred to as 46C>T, showed decreased plasma levels of both antigen and activity.[6] The plasminogen activator inhibitor-1 (PAI-1) 4G/5G polymorphism is related to in vitro differences in transcription activity.[7] We genotyped subjects for these 5 polymorphisms and compared their genotypic frequencies between 161 DVT patients and 3655 population-based controls. The protocol for this study was approved by the ethical review committee, and only those subjects who provided written informed consent for genetic analyses were included in this study. All participants of this study were Japanese. The controls were from a general population randomly selected from the residents of Suita City located in the second largest urban area in Japan (the Suita Study).[8] One hundred sixty-one DVT patients, 78 men and 83 women, were registered by the Study Group of Research on Measures for Intractable Diseases, working under the auspices of the Ministry of Health, Labor, and Welfare of Japan. Six centers (Tochigi, Tokyo, Nagoya, Kyoto, and 2 in Osaka) participated in this study. The patients' mean age was 49.5 years (range, 12-87 years) and their mean body mass index was 23.6 ± 3.3. Thirteen percent of patients had a family history of thrombosis, and 16% of the patients had recurrent thrombosis.

Of all the polymorphisms tested, only the frequency of protein S–K196E was statistically different between the 2 groups ($\chi^2 = 38.3$, $P < .001$) (Table 1). No other frequency differences were statistically significant. Two DVT patients were homozygous for the protein S–196E allele; however, no homozygotes were identified in the control group. One patient with the 196EE genotype first developed DVT following surgery at age 47, while the other patient developed DVT during pregnancy at age 32.

The mutant protein S with the E allele has already been intensively studied as protein S–Tokushima.[11] The protein S mutant showed the reduced activated protein C cofactor activity compared with wild-type protein S, suggesting a direct link between the protein S–K196E mutation and the development of DVT. By the genotyping of the general population, the protein S–196E allele frequency was estimated as 0.009. Thus, a substantial portion of the Japanese population harbors this mutant allele and is at higher risk for DVT.

Table 1. Numbers and genotypic frequencies of protein S–K196E mutation in the DVT and control groups

Genotypes	General population, no. (%)	DVT group, no. (%)
Additive model*		
KK	3585 (98.2)	146 (90.7)
KE	66 (1.8)	13 (8.1)
EE	0 (0.0)	2 (1.2)
Total	3651 (100.0)	161 (100.0)
Dominant model†		
KK	3585 (98.2)	146 (90.7)
KE + EE	66 (1.8)	15 (9.3)
Total	3651 (100.0)	161 (100.0)

DNA genotyping was performed by the TaqMan allele discrimination method.[9] We have adopted the numbering standards of the Nomenclature Working Group, wherein the A of the ATG of the initiator Met codon is denoted as nucleotide + 1, and the initial Met residue is denoted as amino acid + 1, resulting in the renaming of several mutant alleles.[10] Comparisons between the DVT cases and the controls were analyzed using a χ^2 test with the genotypes as independent variables (indicated by P and OR) or using multiple logistic analyses with the genotypes as independent variables and age and sex as covariates (indicated by P' and OR').

*For comparison of general population to DVT group, P was not determined.

†For comparison of general population to DVT group, $P < .001$; OR = 5.58 (3.11-10.01); $P' < .001$; OR' = 4.72 (2.39-9.31).

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SHORT REPORT

Plasma ADAMTS13 Activity May Predict Early Adverse Events in Living Donor Liver Transplantation: Observations in 3 Cases

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A disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) is a metalloproteinase that specifically cleaves the multimeric von Willebrand factor (VWF). Deficiency of ADAMTS13 increases the unusually large VWF multimers (UL-VWFM), which leads to platelet clumping and/or thrombus formation, resulting in microcirculatory disturbance. We serially determined the activity of plasma ADAMTS13, together with VWF antigen (VWF:Ag) and UL-VWFM, in association with the development of early graft dysfunction in 3 liver transplant recipients and 4 patients with major hepatectomy as controls. In case 1, ADAMTS13 activity decreased markedly from 108% to less than 3% with concomitant thrombocytopenia on posttransplantation day 7, when acute rejection occurred. Simultaneously, UL-VWFM were detected. During the second episode of rejection, VWF:Ag increased to 368% with the appearance of UL-VWFM, while ADAMTS13 activity was as low as 18%, indicating an imbalance between a large amount of UL-VWFM and low activity of ADAMTS13. Administration of fresh frozen plasma (FFP) together with treatment for acute rejection resulted in an improvement of ADAMTS13 activity and disappearance of the UL-VWFM. In case 2, ADAMTS13 activity promptly decreased to 9% with thrombocytopenia on day 1, when ischemia-reperfusion injury occurred. Subsequently, the ADAMTS13 activity increased steadily without appearance of UL-VWFM, and the patient recovered uneventfully. ADAMTS13 activity decreased to 15% immediately after transplantation in case 3 as well. In contrast, ADAMTS13 activity never decreased below 20% in 4 patients with major hepatectomy as controls. In conclusion, these results indicate that the kinetics of ADAMTS13 and UL-VWFM could be good indicators of adverse events after liver transplantation. Our findings not only suggest a novel mechanism for thrombocytopenia, but also provide a useful tool for diagnosis of graft dysfunction in the early stage after transplantation. ***Liver Transpl 12:859-869, 2006.***

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Thrombotic microangiopathies (TMAs) are defined as life-threatening generalized disorders, characterized by microangiopathic hemolytic anemia, destructive thrombocytopenia, and organ dysfunction caused by microvascular platelet thrombi.[1,2] Because of these features, TMAs are usually expressed heterogeneously, and can include thrombotic thrombocytopenic purpura (TTP) with neurotropic signs prevalent in adults, but not exclusively, and hemolytic-uremic syndrome with predominant nephrotropic signs.[1,2] TMA is also recognized as a critical complication after solid organ transplantation.[3-9] However, most cases of transplantation-associated TMA are not clearly distinguishable as either TTP or hemolytic-uremic syndrome.

Abbreviations: ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13; VWF, von Willebrand factor; UL-VWFM, unusually large VWF multimers; FFP, fresh frozen plasma; VWF:Ag, VWF antigen; TTP, thrombotic thrombocytopenic purpura; TMA; thrombotic microangiopathy; ALT, alanine aminotransferase.

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The discovery of a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) has provided a breakthrough in our understanding of TMA pathogenesis. Recent studies indicate that ADAMTS13 is produced mainly in the liver, exclusively the stellate cells (formerly called Ito cells), and then thought to be released into the circulation via the microsinusoidal system,[10] where ADAMTS13 specifically cleaves multimeric von Willebrand factor (VWF) between Tyr1605 and Met1606 in the A2 domain.[11-14] VWF is synthesized in vascular endothelial cells, and released into the plasma as unusually large VWF multimers (UL-VWFM), which have potent biological activities.[2,15] Under physiological conditions, UL-VWFM are rapidly degraded into smaller VWF multimers by ADAMTS13.[2,15] Deficiency of the protease increases the level of UL-VWFM in plasma and leads to platelet aggregation and/or thrombus formation, finally resulting in TTP.[16,17] In fact, the activity of ADAMTS13 is significantly decreased in most patients with TTP, whereas it is relatively preserved in the majority of patients with hemolytic-uremic syndrome.[18]

On the other hand, thrombocytopenia is commonly observed during the first week after liver transplantation, with or without apparent TMA.[19-21] Some clinical studies have demonstrated a significantly poorer prognosis in recipients with severe thrombocytopenia than in those without,[22,23] suggesting a close relationship of thrombocytopenia to allograft dysfunction including ischemia-reperfusion injury and acute rejection, which are common adverse events in the early period after transplantation. The primary target for these adverse events is vascular endothelial cells, and injury to these cells in the graft liver results in a large amount of VWF production.[24-27] It is known that circulating VWF levels are markedly high in recipients with poor early graft function.[27] Platelet adhesion to the sinusoidal endothelium with a concomitant increase of VWF expression in the reperfused liver is one of the main deleterious effects of cold preservation of liver allografts.[26]

A few reports have described that ADAMTS13 activity can be used as a marker to diagnose TMA in recipients of liver transplants and renal allografts.[3,4] However, there is little information about the relationship between ADAMTS13 and allograft dysfunction and thrombocytopenia after liver transplantation. In the present study of 3 living-donor liver transplant recipients, we measured the plasma activity of ADAMTS13 together with VWF and UL-VWFM, and thereby attempted to clarify a potential role of the protease activity in adverse events including ischemia-reperfusion injury and/or acute rejection. As controls, 4 patients with major hepatectomy were also analyzed.

METHODS

Plasma levels of ADAMTS13 activity, VWF antigen (VWF:Ag), and UL-VWFM were sequentially evaluated before and after liver transplantation in 3 recipients. Inhibitor activity against ADAMTS13 was assayed on day 7 after transplantation in case 1, and on day 1 after transplantation in case 2. Also, as controls, plasma ADAMTS13 activity and VWF:Ag were measured in 4 patients with normal livers who underwent major hepatectomy in our hospital during July to November 2005. Blood was taken in plastic tubes containing a 1/10th volume of 3.8% sodium citrate, and platelet-poor plasma prepared by centrifugation at 3,000*g* at 4°C for 15 minutes was stored in aliquots at −80°C until analysis. The activity of plasma ADAMTS13 was assayed by the following 2 methods: 1) VWF-multimer assay using intact VWFM as a substrate according to Furlan et al.,[28] with slight modification.[18] The detection limit of the activity with this method was 3%, and the level obtained for 60 normal subjects was 102 ± 23% (mean ± standard deviation).[18] 2) Novel enzyme-linked immunosorbent assay using a murine monoclonal antibody specifically recognizing Tyr1605 residue of VWF-A2 domain,[29] generated by ADAMTS13 cleavage, and a recombinant GST-VWF73-His polypeptide[30] as a substrate. The detection limit of the activity with this enzyme-linked immunosorbent assay was 0.5%, and the normal level obtained for 55 healthy individuals was 99.1 ± 21.5% (mean ± standard deviation).[29] In the Case Reports and Results section and the Discussion section below, the values determined by VWF-multimer assay are described. Plasma UL-VWFM was evaluated by vertical agarose gel electrophoresis according to the method of Warren et al.,[31] with modifications. The activity of inhibitor against ADAMTS13 was evaluated using heat-inactivated plasma at 56°C for 30 minutes.[16,17] Plasma VWF:Ag was measured by a sandwich enzyme immunoassay using a rabbit anti-human VWF polyclonal antibody. The value obtained for healthy subjects in our laboratory (n = 54; 30 males, 24 females, 20-39 yr of age) was 100 ± 53% (mean ± standard deviation).

CASE REPORTS AND RESULTS

Case 1

A 27-yr-old man with a diagnosis of Budd-Chiari syndrome was referred to our department for liver transplantation on October 5, 2004. Radiological imaging showed complete occlusion of both the middle and left hepatic veins, together with stenosis of the orifice of the right hepatic vein. The liver had rapidly swollen because of severe liver congestion, and massive ascites was noted before transplantation. Preoperative blood examination revealed a serum total bilirubin level of 4.8 mg/dL, hemoglobin of 10.9 gm/dL, platelet count of 83,000/μL, creatinine of 0.7 mg/dL, and alanine aminotransferase (ALT) activity of 23 IU/L (Fig. 1). His prothrombin time was 18 seconds (international normalized ratio: 1.49) and bleeding time was 5 minute. Anticoagulation factors protein C (54%), protein S (56%), and antithrombin III (75%) were not severely decreased. He had no history of hematological disorders, thrombotic events or relevant family diseases. On November 22, the patient underwent living donor liver transplantation, receiving a cross-match-negative and blood group type-identical right liver graft from his brother, who was healthy and had no history of previ-

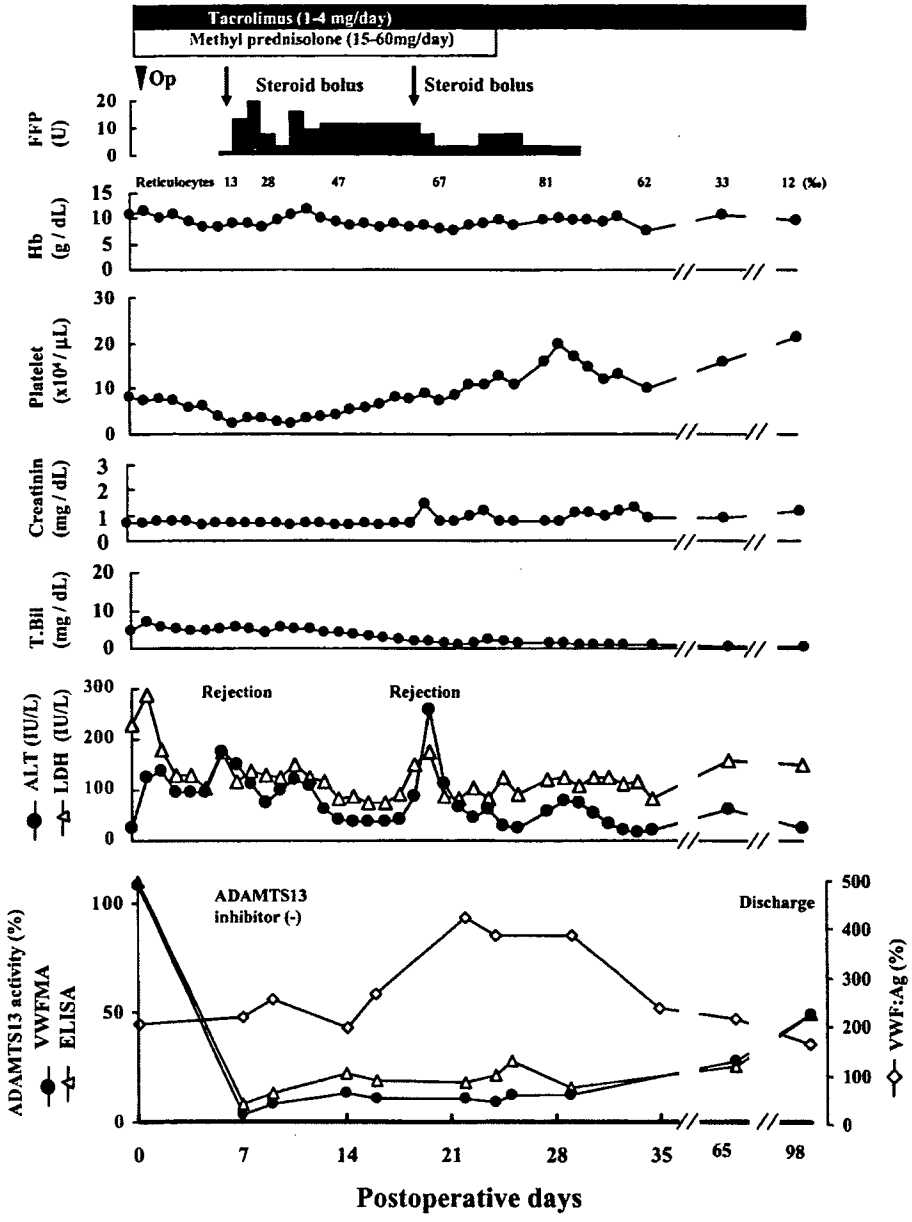

Figure 1. Clinical course and serial changes in plasma ADAMTS13 activity and VWF:Ag level after liver transplantation in case 1. Serum ALT level was mildly increased on days 1 and 2 because of ischemia-reperfusion injury, decreased thereafter, but rapidly increased again on day 7 due to acute rejection. The platelet count decreased gradually and reached a nadir on day 7, when ADAMTS13 activity decreased markedly to less than 3% from 108% before surgery. No inhibitor against the protease was detected. After the administration of FFP and bolus injection of methylprednisolone to treat acute rejection, ALT level decreased, and the platelet count gradually increased. The activity of ADAMTS13 increased to 22% on day 14. After the first episode of acute rejection, VWF:Ag increased further and reached 368% on day 21, when ALT again increased due to a second episode of acute rejection. Bolus injection of methylprednisolone led to a rapid decrease of ALT and a gradual increase in the platelet count. VWF:Ag decreased gradually, and ADAMTS13 activity finally recovered to 50%, corresponding to the lower limit of the normal range, on day 98.

ous hematological disorders or relevant family diseases. The graft weight and graft-to-recipient weight ratio were 588 gm and 0.93%, respectively. Operative blood loss was 8,410 mL and required 12 units of packed red blood cells and 10 units of fresh frozen plasma (FFP). Platelet concentrate was not required. Posttransplantation immunosuppressive treatment consisted of tacrolimus and methylprednisolone. The dose of tacrolimus was adjusted to maintain whole-blood trough levels of 10-15 ng/mL.

Before transplantation, the activity of ADAMTS13 was 108% of the normal control activity (Figs. 1 and 2), VWF:Ag was 175% (Fig. 1), and UL-VWFM was not detected (Fig. 3). During an uneventful course in the early stage after transplantation, the platelet count decreased gradually to 62,000/μL on postoperative day 5, and reached a nadir (25,000/μL) on day 7, when ADAMTS13 activity decreased markedly to less than 3% (Fig. 2), although no inhibitor against the protease was detected. Simultaneously, the serum ALT level increased to 124 IU/L on day 1 because of ischemia-reperfusion injury, decreased thereafter to 97 IU/l on day 5, and again increased rapidly to 175 IU/L on day 6 due to acute rejection, which was clinically diagnosed (Fig. 1). VWF:Ag was mildly increased to 188% on day 7. UL-VWFM was detectable even on day 1, diminished gradually during days 2 to 4, and again became evident on day 7 (Fig. 3). Based on the activity of ADAMTS13, we considered that TMA was the cause of thrombocytopenia, but the recipient never showed any apparent

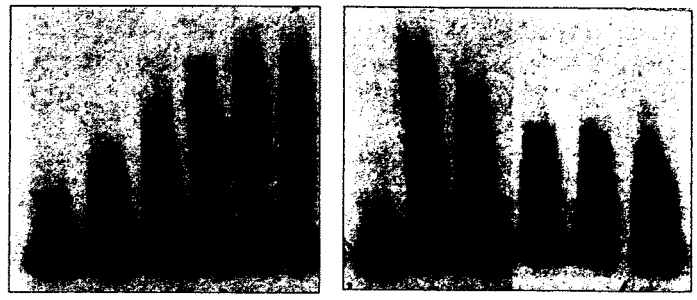

Figure 2. Serial determination of plasma ADAMTS13 activity in case 1. The polymeric size of degraded VWF multimers was analyzed by sodium dodecyl sulfate-1.4% agarose gel electrophoresis, followed by immunoblotting. The standard curve and representative values are shown for plasma samples obtained on preoperative day 4, and on postoperative days 7, 9, 22, 29, and 35. The activity of ADAMTS13 was as extremely low as 3% on day 7, in contrast to the preoperative value of 108%.

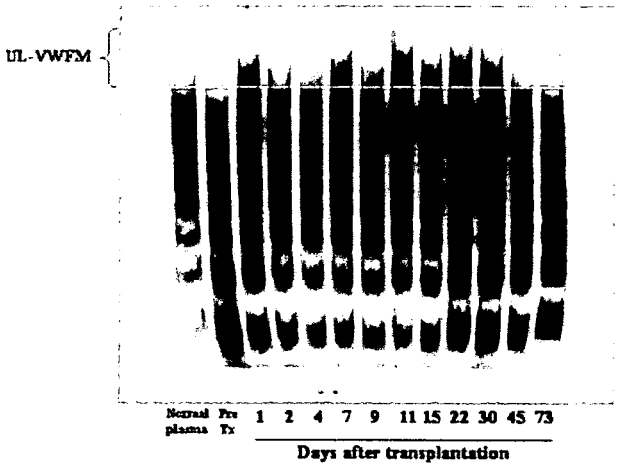

Figure 3. Serial determination of plasma UL-VWFM in case 1 using 0.9% sodium dodecyl sulfate-agarose gel electrophoresis. UL-VWFM was detectable on day 1 at the time of ischemia-reperfusion injury, thereafter diminishing gradually during days 2 to 4, and again becoming evident on day 7 when acute rejection developed. The UL-VWFM disappeared transiently on day 9, but reappeared on day 11, coinciding with a mild increase in transaminase. UL-VWFM tended to diminish on day 15, but again became prominent on day 22 during the second episode of acute rejection.

clinical features including renal dysfunction, neuropsychological symptoms or hemolytic anemia. We therefore administered a large amount of FFP (4 to 20 units daily) from day 7 to day 30 to restore the ADAMTS13 activity. Plasmapheresis was not performed, because no inhibitor against ADAMTS13 was detected. Bolus injection of methylprednisolone (500 mg daily) was added to treat the acute rejection from day 7 to 9, without conversion of tacrolimus to another drug. Thereafter, the platelet count increased gradually to 90,000/μL on day 20 without administration of platelet concentrate. The activity of ADAMTS13 also increased to 12% on day 14 (Fig. 2), and this was maintained until day 65. After the first episode of acute rejection around day 7, VWF:Ag increased further and reached 368% on day 21, when ALT level again increased to 259 IU/L due to a second episode of acute rejection. The amount of UL-VWFM diminished transiently on day 9 during remission of the first acute rejection episode, but increased again on day 11, coinciding with a mild elevation of transaminase. After the amount of UL-VWFM diminished on day 15, it became prominent again on day 22 during the second episode of acute rejection (clinically diagnosed). Bolus injection of methylprednisolone quickly reduced the level of ALT, and the platelet count gradually increased and reached 199,000/μL on day 30 (Fig. 1). ADAMTS13 increased gradually to 50%, corresponding to the lower limit of the normal range, and VWF:Ag decreased gradually and returned to the normal range at the time of discharge on day 98 (Fig. 1). UL-VWFM became undetectable until day 45 (Fig. 3). After liver transplantation, the patient lost a large amount of lymphatic fluid via the peritoneal drain, but this gradually decreased and had disappeared completely by about day 65.

Case 2

A 53-yr-old man with a diagnosis of hypercitrullinemia type II was referred to our department for liver transplantation on January 4, 2005. The activity of argininosuccinate synthetase was markedly low (0.36 U/gm liver; normal range 2.59 ± 1.13 U/gm liver), while the quantity of the enzyme was preserved (0.0052 U/mg protein; normal range 0.0033 ± 0.0012 U/mg protein). The serum ammonia level was sometimes higher than 600 μg/dL, and analysis of plasma amino acid showed a markedly high concentration of citrulline (338 nmol/mol, normal range 17-43 nmol/mol). The patient had several episodes of deep hepatic coma. On February 14, 2005, he underwent auxiliary partial orthotopic liver transplantation using a left liver graft from his wife, because his own liver function was normal except for amino acid metabolism. The day 0 biopsy of the donor liver revealed macrovesicular steatosis. The graft weight and graft-to-recipient weight ratio were 304 gm and 0.56%, respectively. Operative blood loss was 2,900 mL and required 8 units of packed red blood cells and 4 units of FFP. Platelet concentrate was not required. Posttransplantation immunosuppressive treatment consisted of tacrolimus and methylprednisolone, as used in case 1.

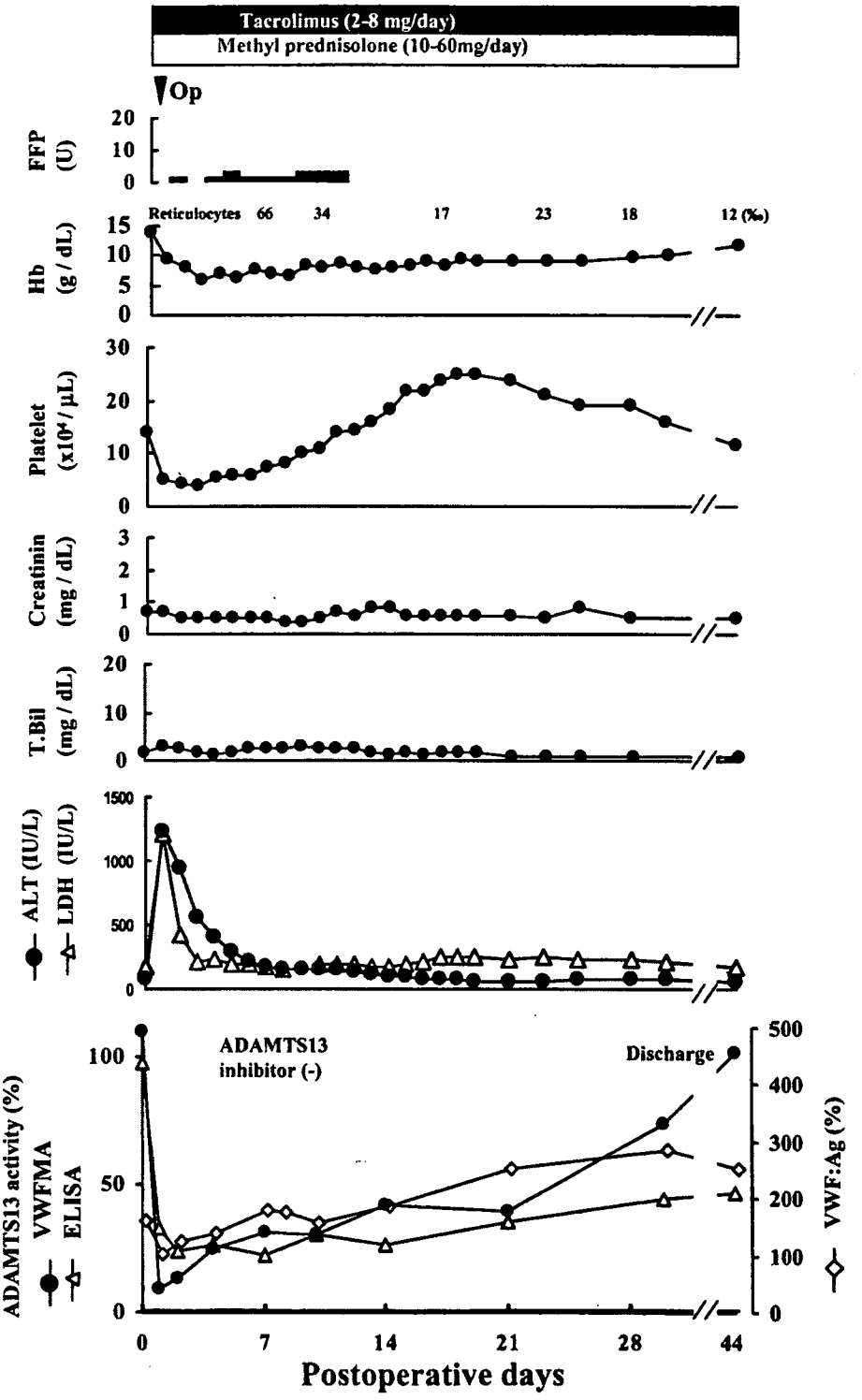

Figure 4. Clinical course and serial changes in plasma ADAMTS13 activity and VWF:Ag level after liver transplantation in case 2. On postoperative day 1, ALT level was markedly increased because of ischemia-reperfusion injury. The platelet count decreased rapidly on day 1 and reached a nadir on day 3. Simultaneously, ADAMTS13 activity decreased markedly and quickly to 9% on day 1 from the value of 110% before surgery. No inhibitor against the protease was detected. Subsequently the patient's recovery was uneventful. The activity of ADAMTS13 increased to 31% on day 7, 42% on day 14, and reached 102% on day 44. VWF:Ag did not increase significantly.

Preoperative blood examination revealed a serum total bilirubin level of 1.9 mg/dL, hemoglobin 13.8 gm/dL, platelet count 142,000/μL, creatinin 0.7 mg/dL, and ALT 85 IU/L (Fig. 4). The coagulation parameters were normal before transplantation, and had no history of hematological disorders or relevant family diseases. Pre-transplant ADAMTS13 activity was 110% of the normal control activity, VWF:Ag was 142%, and UL-VWFM was undetectable (Fig. 5). On day 1 after transplantation, ALT increased markedly to 1226 IU/L prob-

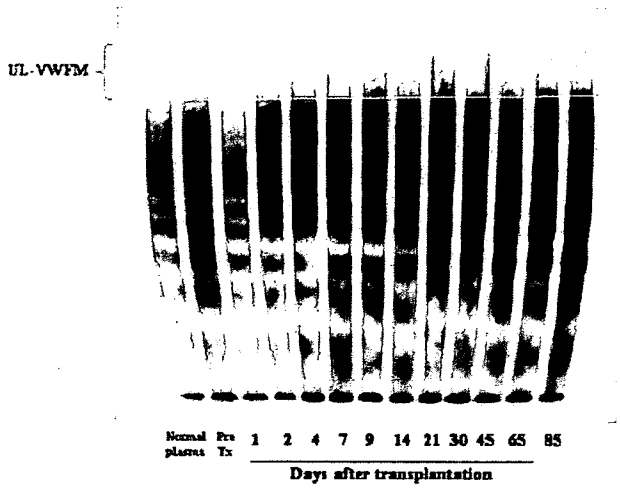

Figure 5. Serial determination of plasma UL-VWFM in case 2 using 0.9% SDS-agarose gel electrophoresis. UL-VWFM was not detectable during the observation period.

ably because of ischemia-reperfusion injury (Fig. 4). The platelet count decreased to 51,000/μL on postoperative day 1 and reached a nadir of 38,000/μL on day 3. The activity of ADAMTS13 decreased markedly to 9% on day 1, but no inhibitor against ADAMTS13 was detected. VWF:Ag decreased slightly to 89%. UL-VWFM was not detected (Fig. 5). Although the patient had no signs of TMA including renal dysfunction, neuropsychological symptoms, or hemolytic anemia, a small dose of FFP was administered from days 1 to 11 after transplantation to restore the activity of ADAMTS13. Thereafter, ALT promptly decreased, and the platelet count increased gradually to 182,000/μL on day 14. The levels of blood ammonia and citrulline were normalized within 2 weeks after transplantation, suggesting that the auxiliary partial liver graft was functioning very well. The activity of ADAMTS13 increased gradually to 31% on day 7, 42% on day 14, and reached 102% on day 44. VWF:Ag did not increase markedly, and UL-VWFM was never detected during hospitalization (Fig. 5).

Case 3

A 57-year-old man with a diagnosis of hepatitis B virus-related cirrhosis was referred to our department for liver transplantation on June 6, 2005. He had massive uncontrollable ascites and marked jaundice. Preoperative blood examination revealed a serum total bilirubin level of 22.0 mg/dL, hemoglobin of 11.4 g/dL, platelet count of 30,000/μL, creatinine of 0.8 mg/dL, and ALT of 32 IU/L (Fig. 1). His prothrombin time was 22.6 seconds (international normalized ratio: 1.97) and his Model for End-Stage Liver Disease score was 25. He had no history of hematological disorders or relevant family diseases. On July 6, the patient underwent living donor liver transplantation, receiving a cross-match-negative and blood group type-compatible (from B donor to AB recipient) right liver graft from his son, who was healthy and had no history of previous hematological disorders or relevant family diseases. The graft weight and graft-to-recipient weight ratio were 936 gm and 1.43%, respectively. Operative blood loss was 24,750 mL and required 77 units of packed red blood cells, 70 units of FFP and 5 units of platelet concentrate. The initial post-transplantation immunosuppressive treatment consisted of tacrolimus and methylprednisolone. The dose of tacrolimus was adjusted to maintain whole-blood trough levels of 10-15 ng/mL. Tacrolimus was converted to cyclosporine on day 12 due to tacrolimus-induced leukoencephalopathy, which was diagnosed by magnetic resonance imaging, and recovered completely after conversion to cyclosporine. The dose of cyclosporine was adjusted to maintain whole-blood trough levels of 150-200 ng/mL. The patient was positive for the YMDD-mutant of hepatitis B virus before transplantation and treated with lamivudine and adefovir before and after transplantation. Prophylactic infusion of human hepatitis B immunoglobulin was administered intravenously to prevent hepatitis B recurrence according to the reported protocol.[32] Hepatitis B did not recur and the hepatitis B virus-deoxyribonucleic acid levels were below the detection limit after transplantation during observation.

Before transplantation, the activity of ADAMTS13 showed a low level (32%) probably because of severely impaired hepatic functional reserve (Fig. 6). VWF:Ag was markedly increased to more than 500% (Fig. 6), but UL-VWFM was not detected before transplantation (Fig. 7). While initial elevation of ALT due to ischemia-reperfusion was minimal, ADAMTS13 activity decreased markedly to 14% on day 2 after transplantation. His platelet count decreased to 13,000/ μL during the first week, but platelet concentrate was never administered. VWF:Ag level significantly decreased on day 1 after transplantation, and increased gradually until day 6. ADAMTS13 activity begun to increase from day 3, but again markedly decreased to 8% on day 13 when hemolysis due to B cell-mediated graft-vs.-host disease occurred. This hemolysis was considered to be caused by donor-derived antibody against anti-blood type A antigen, because anti-blood type A antigen appeared on day 13 in the peripheral blood. Rapid decrease of hemoglobin associated with increase of total bilirubin and lactate dehydrogenase was observed, but ALT remained normal. Serum creatinine level was not elevated significantly. During this episode, increase of VWF:Ag to 335% was seen, but UL-VWFM was not up-regulated (Fig. 7). This hemolytic reaction was successfully treated with bolus injection of steroid. ADAMTS13 increased to 25% until day 21. Decrease of VWF:Ag was also seen after this treatment. ALT increased mildly around day 25, but we could not determine the specific reason. This increase of ALT recovered without treatment. ADAMTS13 did not increase during this episode, and reached 48% on day 90.

Patients With Major Hepatectomy

Four patients who underwent major hepatectomy were analyzed for ADAMTS13 activity and for VWF:Ag before

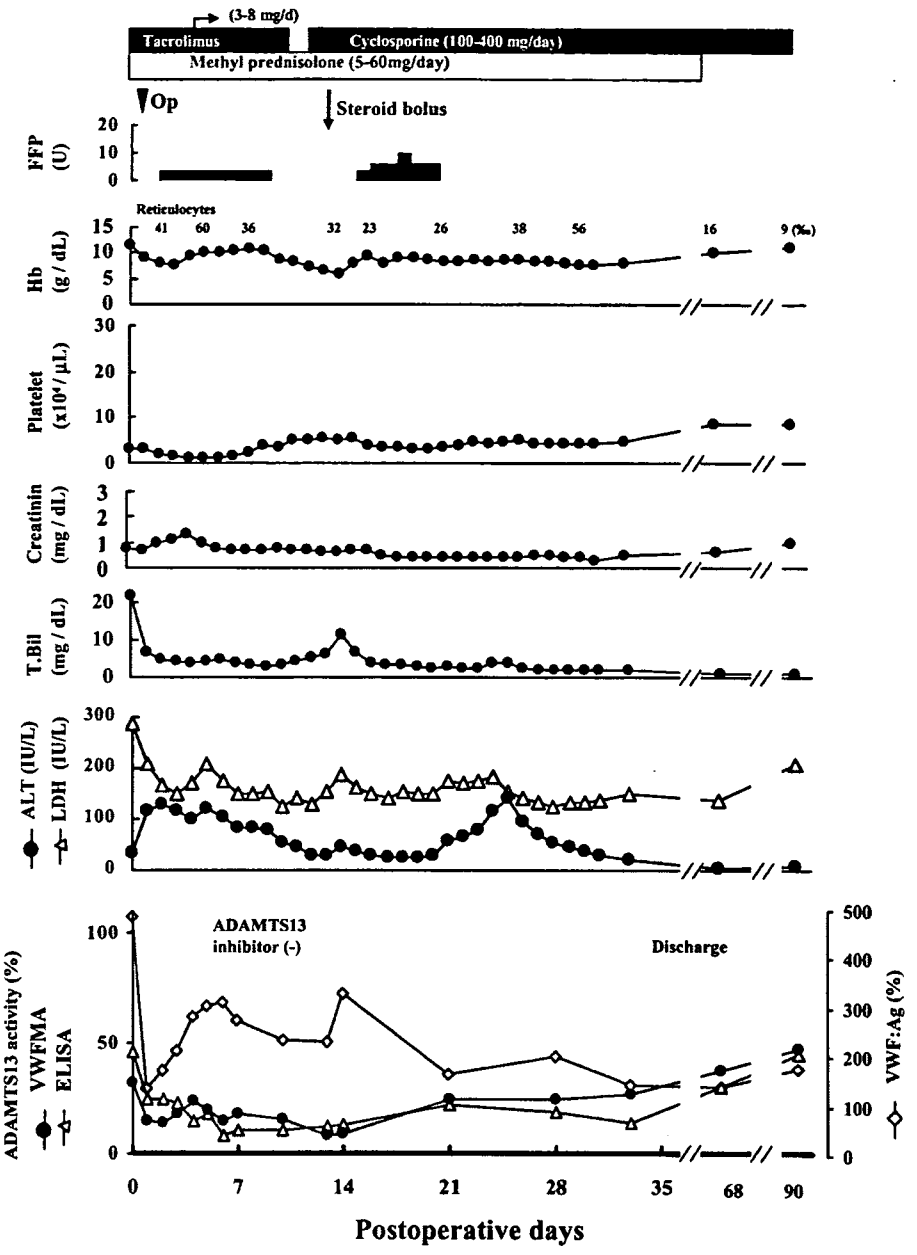

Figure 6. Clinical course and serial changes in plasma ADAMTS13 activity and VWF:Ag level after liver transplantation in case 3. The platelet count was only 30,000/μL before operation because of severe liver cirrhosis, and further decreased during the first postoperative week. Serum ALT level was mildly increased on days 1 because of ischemia-reperfusion injury, and decreased slowly. The ADAMTS13 activity decreased markedly to 15% on day 1, and recovered to 24% on day 4. The activity of ADAMTS13 decreased again to 8% on day 13. At that time, sever hemolytic attach developed with significant drop of hemoglobin levels and increase of total bilirubin and lactate dehydrogenase, but ALT did not increased. This was caused by B-cell mediated graft-vs.-host disease with transient increase of antibody against blood type A antigen of the recipient. The ADAMTS13 activity increased after remission of B cell graft-vs.-host disease by bolus injection of steroid. VWF:Ag increased transiently from day 13 to day 14 during B cell graft-vs.-host disease. ADAMTS13 activity finally recovered to 48% on day 90, which was higher than the preoperative level.

and after their operations, for comparison to the liver transplant patients. These 4 patients had normal hepatic parenchyma without cirrhotic change. While VWF:Ag levels were rather higher in these patients than those in cases 1-3 with liver transplantation, ADAMTS13 never decreased below 20% even in the very early phase after operation (Fig. 8).

DISCUSSION

In the present study, we serially determined the plasma values of ADAMTS13 activity, VWF:Ag, and UL-VWFM, and demonstrated their relationship to early adverse events including ischemia-reperfusion injury and/or acute graft rejection after liver transplantation. In case 1, the activity of plasma ADAMTS13 decreased markedly to less than 3% with concomitant thrombocytopenia on day 7 after transplantation, when acute rejection occurred. UL-VWFM was detected at the time of ischemia-reperfusion injury and also during 2 episodes of acute rejection. In case 2, the activity of the protease decreased markedly to 9% with concomitant thrombocytopenia on day 1, when ischemia-reperfusion injury was observed. The immediate marked decrease of ADAMTS13 was observed also in case 3. On the other hand, changes of ADAMTS13 levels were milder in patients with major hepatectomy than in liver transplant patients. These results indicate that decreased AD-

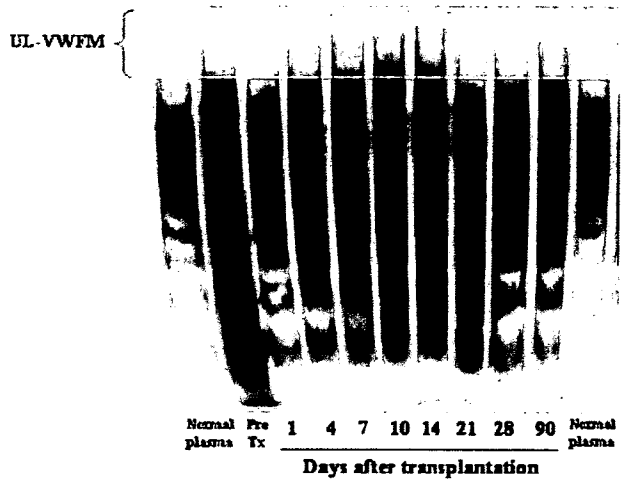

Figure 7. Serial determination of plasma UL-VWFM in case 3 using 0.9% sodium dodecyl sulfate-agarose gel electrophoresis. UL-VWFM was slightly detectable on day 10, but not significant. The dense band of VWF (not UL-VWFM) might represent very high plasma level of VWF:Ag before transplantation in this patient.

AMTS13 activity and the appearance of UL-VWFM are closely related to the development of early posttransplantation allograft dysfunction accompanied by thrombocytopenia.

We analyzed 3 liver transplant recipients, and all of these patients showed a significant decrease of ADAMTS13 with or without upregulation of UL-VWFM during adverse events after transplantation. However, no patient showed the typical clinical signs of TMA including neurological disorder or renal dysfunction during the significant drop in ADAMTS13, while various degrees of thrombocytopenia were associated with the drop in ADAMTS13. These results demonstrate that decrease of ADAMTS13 often occurs after liver transplantation without apparent clinical signs of TMA, and this phenomenon may have a functional relevance to the deterioration of the liver graft presumably due to the mechanism of local TMA within the graft site.

Posttransplantation thrombocytopenia is more often associated with early acute rejection in liver transplant recipients.[22] As to the mechanism of the thrombocytopenia, several factors involving sequestration of platelets in the reperfused liver graft, immunologic reactions, increased platelet consumption, reduced platelet production, impaired production of thrombopoietin, medication, or a combination of these have been suspected,[19-22,33,34] but our findings appear to suggest a novel mechanism of not only early posttransplantation thrombocytopenia, but also early graft dysfunction, which exerts a great influence on the prognosis of recipients.[22-25,27]

In case 1, we were unable to evaluate the activity of ADAMTS13 during the first few days after surgery, but it could have been low at the time of ischemia-reperfusion injury because of the apparent presence of UL-VWFM on day 1. In case 2, the marked decrease in ADAMTS13 activity on day 1 may have been due to consumption of the protease because of the weaker VWFM and lower plasma level of VWF compared with the situation before surgery. During the very early stage after transplantation (on day 1), it was noticeable that UL-VWFM was increased in case 1, but decreased in case 2. The decrease of UL-VWFM in case 2 may be explained by the consumption of UL-VWFM in the process of platelet aggregation, probably due to more severe ischemia-reperfusion injury than that in case 1.[28] Similarly, the decrease of UL-VWF in plasma was observed in patients with TTP during relapse.[35] Another possible explanation may have been the difference in the amount of UL-VWFM released from the damaged sinusoidal endothelium during the surgical procedures, including extirpation of the native diseased liver; case 1 with advanced cirrhosis may have produced a larger amount of UL-VWFM than case 2 with a normal liver, because sinusoidal endothelial damage with capillarization is further augmented as liver fibrosis progresses.[36,37] Regarding the mechanism responsible for the decrease in ADAMTS13 after liver transplantation, no antibody against ADAMTS13 was detected in either case. Therefore, we speculate that consumption of the protease due to a large amount of UL-VWFM, as seen in case 2, and unknown factors such as proinflammatory cytokines including interleukin-6, which might have inhibited the action of ADAMTS13, were involved.[38] In fact, the plasma concentration of interleukin-6 was significantly increased and reached a peak 2 hours after reperfusion of the liver graft.[39]

In case 3, plasma VWF:Ag was very high before the operation, probably because of sinusoidal endothelial injury due to severe liver cirrhosis.[36,37] The VWF:Ag rapidly decreased on day 1 after transplantation and UL-VWFM was not detected. We speculate that this significant and immediate decrease of VWF:Ag would be due to the washout effect of a large amount of transfusion during operation. That might be the reason why the VWF:Ag increased gradually during the first postoperative week in spite of the uneventful early recovery of the patient. Although transfusion of a large amount of FFP (70 units) during the operation in case 3 might have increased ADAMTS13 activity, the ADAMTS13 activity on day 1 decreased significantly to 15%. This result suggest that consumption of ADAMTS13 due to ischemia-reperfusion injury during liver transplantation would be so much as to wipe out the effect of 70 units of FFP.

The result of case 2 may be quite important in this study, because the native normal liver was preserved in this patient. The initial drop of ADAMTS13 activity immediately after transplantation was also significant in case 2 as well as case 3, although the production of ADAMTS13 might be preserved by the native right liver with auxiliary partial orthotopic liver transplantation in case 2. This result may suggest that consumption of the ADAMTS13 plays more important role in decrease of ADAMTS13 activity after transplantation during adverse events than decreased ADAMTS13 production due to impaired liver allograft function. Therefore, AD-

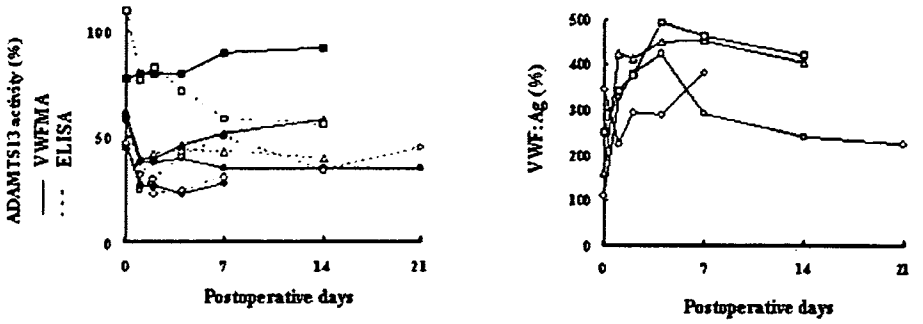

Figure 8. Plasma ADAMTS13 activity (left) and VWF:Ag level (right) after major hepatectomy. Four patients who underwent major hepatectomy were analyzed about ADAMTS13 activity and VWF:Ag for comparison to liver transplantation patients. Circles and triangles show the values of 2 patients who underwent right hepatectomy as living liver donors; Diamonds show a patient with hepatocellular carcinoma who underwent extended right hepatectomy; Squares show a patient with hepatocellular carcinoma who underwent left hepatectomy. Plasma ADAMTS13 activity never decreased below 20%. VWF:Ag levels were rather higher than those in cases 1 to 3 with liver transplantation.

AMTS13 may decrease in the initial phase of adverse events before impairment of graft function.

Interestingly, in case 1, VWF:Ag was markedly increased to 368% and UL-VWFM was detected during the second episode of rejection on day 21, while ADAMTS13 activity was as low as 18% and the platelet count was relatively well maintained at 75,000/μL. These findings indicate that the imbalance of a large amount of UL-VWFM relative to low ADAMTS13 activity could be a good indicator of allograft rejection, even in the absence of severe thrombocytopenia. Thus, our results may be able to explain the fact that liver transplant recipients with increased levels of circulating VWF, a reliable marker of endothelial damage,[40,41] show poor early graft function.[17]

In case 3, ADAMTS13 decreased during hemolysis by B cell-mediated graft-vs.-host disease. However, UL-VWFM was not upregulated during this episode. Differing from ischemia-reperfusion injury or acute rejection, this hemolytic reaction was a systemic reaction due to antibody against blood type A antigen. ADAMTS13, VWF:Ag, and UL-VWFM did not change during nonspecific elevation of ALT around day 25 in case 3. It may be important to analyze ADAMTS13 in combination with UL-VWFM to detect liver transplantation-specific adverse events.

The decrease of ADAMTS13 was milder in patients with major hepatectomy in comparison to liver transplant patients, while VWF:Ag increased higher. This result suggests that significant decrease of ADAMTS13 below 20% would be a liver transplantation-specific event, while the mechanism of difference in changes of ADAMTS13 activity between hepatectomy and liver transplantation is unknown.

The primary target of ischemia-reperfusion injury and allograft rejection is the sinusoidal endothelial cells of the liver graft.[24-26,42] Deposition of activated platelets on the sinusoidal endothelium with a concomitant increase of VWF expression have been found in the liver immediately after reperfusion or cold preservation.[24,25] In addition, upregulated VWF expression has been observed in liver allografts during acute rejection.[25] Furthermore, recipients with acute rejection show enhanced cytokinemia including tumor necrosis factor-α, which leads to endothelial activation and stimulates the release of UL-VWFM from endothelial cells.[38,42] VWF, the substrate of ADAMTS13, synthesized in vascular endothelial cells, mediates the initiation and progression of thrombus formation at sites of vascular injury.[14,40,41,43] VWF is released into plasma as UL-VWFM, which has high platelet aggregation activity. The deficiency of ADAMTS13, together with the excessive release of UL-VWFM from injured graft endothelial cells observed in our study, may cause sinusoidal microcirculatory disturbance and subsequent graft dysfunction.

Various degrees of thrombocytopenia were commonly observed after liver transplantation, especially during the first postoperative week, and many of these patients recover without specific treatment. However, it might be possible that thrombocytopenia is a sign of deterioration of the liver graft in some of the patients with thrombocytopenia, because clinical studies demonstrated that thrombocytopenia was associated with poor prognosis.[22-25,27] If thrombocytopenia is combined with significant decrease of ADMTS13, liver graft function may be deteriorated via the TTP like mechanism due to microcirculatory disturbance. Therefore, monitoring of ADAMTS13 would be quite important to judge the necessity of treatment for thrombocytopenia. In case 1, we administered a large amount of FFP from day 7 to prevent further deterioration of thrombocytopenia with TMA mechanism. In cases 2 and 3, a limited dose of FFP was administered as a prophylaxis of graft dysfunction due to TMA-like reaction, because the ADAMTS13 activity significantly decreased. However, it is to be elucidated whether such prophylactic use of FFP based on the ADAMTS13 activity would provide a beneficial effect.

The values of decreased plasma ADAMTS13 activity by VWF-multimer assay in the present study do not appear to be influenced by the elevated plasma UL-VWFM, because the comparable results are drawn by the novel enzyme-linked immunosorbent assay, which is totally insensitive to the presence of intact VWFM.[29]

This new enzyme-linked immunosorbent assay method would be very useful in clinical application of ADAMTS13 monitoring in liver transplantation, because the results can be obtained within several hours.

At present, FFP is the only available source of ADAMTS13 replacement.[18] Remarkably, in our patients, infusion of FFP, but not platelet concentrate, resulted in gradual improvement of severe thrombocytopenia together with an increase in ADAMTS13 activity. This is an extremely important issue in the treatment of thrombocytopenia associated with allograft dysfunction after liver transplantation, because administration of platelet concentrate under pathological conditions, including an imbalance between decreased ADAMTS13 activity and enhanced VWF production, would further exacerbate the formation of platelet aggregates mediated by uncleaved UL-VWFM, leading to multiorgan failure, as seen in TMA.[2] Platelet concentrate was never administered in case 3 even when the platelet count decreased to 13,000/μL on days 4 to 6, because the activity of ADAMTS13 was low. The mechanism of thrombocytopenia associated with early adverse events after liver transplantation is noteworthy. In the post-transplantation period, patients are especially susceptible to TMA because of administration of calcineurin inhibitors including tacrolimus and cyclosporine, which are well-documented to induce TMA.[44] In case 1, we successfully treated the thrombocytopenia by administering high-dose FFP without conversion of calcineurin inhibitors. Therefore, it would be particularly useful to determine the values of ADAMTS13 and its substrate, VWF:Ag, together with UL-VWFM in the early period after transplantation, not only for the diagnosis of TMA, but also for clarifying the mechanism of thrombocytopenia. Our experience, although based on only 3 liver transplantations and 4 major hepatectomy cases, may provide useful data that are relevant to the diagnosis and treatment of ischemia-reperfusion injury and allograft rejection, as well as for clarifying the pathogenesis of thrombocytopenia after liver transplantation.

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