Increased production of ADAMTS13 in hepatic stellate cells contributes to enhanced plasma ADAMTS13 activity in rat models of cholestasis and steatohepatitis

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Summary
Although hepatic stellate cells, endothelial cells, glomerular podocytes and platelets were reported to be a source of ADAMTS13, it is not clarified which source is involved in the regulation of plasma ADAMTS13 activity. It was demonstrated previously that selective hepatic stellate cell damage in rats caused decreased plasma ADAMTS13 activity. To further elucidate the potential contribution of hepatic stellate cells to the regulation of plasma ADAMTS13 activity, this study examined plasma ADAMTS13 activity when hepatic stellate cells proliferate during the process of liver fibrosis by employing rat models of liver fibrosis due to cholestasis, bile duct ligation, and steatohepatitis, a choline-deficient L-amino acid-defined-diet. ADAMTS13 expression was increased with co-localisation with smooth muscle α-actin, a marker of hepatic stellate cells, in bile duct-ligated livers up to four weeks, in which a close correlation between ADAMTS13 and smooth muscle α-actin mRNA expressions was determined. Plasma ADAMTS13 activity, measured by a sandwich ELISA involving a specific substrate to ADAMTS13, was increased in bile duct-ligated rats with a significant correlation with ADAMTS13 mRNA expression levels in the liver. Furthermore, ADAMTS13 mRNA expression was increased with enhanced mRNA expression in smooth muscle α-actin in the livers of rats fed a choline-deficient L-amino acid-defined-diet for 16 weeks, in which increased plasma ADAMTS13 activity was determined. Thus, increased plasma ADAMTS13 activity in cholestasis and steatohepatitis in rats may be due, at least in part, to enhanced ADAMTS13 production in the liver, suggesting a significant role of hepatic stellate cells in the regulation of plasma ADAMTS13 activity.

Keywords
ADAMTS13, hepatic stellate cell, smooth muscle α-actin, bile duct ligation, steatohepatitis

Introduction
A disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) has gained attention, because it has been revealed that a defect in ADAMTS13 activity explains the pathogenesis of thrombotic thrombocytopenic purpura (1). ADAMTS13 is an enzyme in normal plasma, which is required for proteolysis of von Willebrand factor (VWF) following its secretion from endothelial cells; the absence of ADAMTS13 in the circulation leads to abnormally large multimers of VWF, which may spontaneously bind to circulating platelets and result in microvascular thrombosis. Thus, a deficiency of ADAMTS13 activity creates a prothrombotic state (2, 3), while bleeding may occur due to the resultant thrombocytopenia.

ADAMTS13 activity in plasma has been revealed to be regulated by the balance of ADAMTS13 production and degradation or inactivation. Deficient plasma ADAMTS13 activity in Upshaw-Schulman syndrome, a congenital bleeding disorder characterised by repeated episodes of thrombocytopenia and microangiopathic haemolytic anaemia, is caused by inability of ADAMTS13 production by genetic mutations (4–6). On the other hand, reduced plasma ADAMTS13 activity in thrombotic...
thrombocytopenic purpura is explained by an inhibitory auto-
antibody to ADAMTS13 (7–11) as well as congenital deficiency of
ADAMTS13. Furthermore, a deficiency of plasma ADAMTS13 in sepsis-induced disseminated intravascular co-
agulation was reportedly caused by its cleavage by protease, such as thrombin, plasmin or granulocyte elastase (12).

Regarding a site of ADAMTS13 production, ADAMTS13 mRNA expression was first shown exclusively in the liver in hu-
mans (13–15), and subsequently ADAMTS13 was shown to be primarily produced in hepatic stellate cells among the liver cells based on evidence of co-localisation with smooth muscle α-actin in the liver in humans (16) and rats (17) and direct cloning of
ADAMTS13 cDNA from hepatic stellate cells in mice (18). Fur-
thermore, recent evidence showed that cultured human endothelial cells (19) and glomerular podocytes in the kidney in humans (20) express ADAMTS13. ADAMTS13 was also detected in human platelets and found to be secreted in response to intense stimulation by thrombin receptor-activating peptide (21). Thus, it is not known which site(s) as a source of ADAMTS13 may play a role in the regulation of plasma ADAMTS13 activity. To ad-
dress this issue, we previously determined that selective hepatic stellate cell damage in rats leads to decreased plasma
ADAMTS13 activity (22), suggesting that hepatic stellate cells may be involved in the regulation of plasma ADAMTS13 activity. We then wondered whether plasma ADAMTS13 activity could be up-regulated when hepatic stellate cells proliferate during
the process of liver fibrosis (23). To determine this, we em-
ployed two distinct rat models of liver fibrosis due to cholestasis caused by bile duct ligation and steatohepatitis induced by a choline-deficient L-amino acid-defined (CDAA) diet.

Materials and methods

Animals

Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were fed a standard pelleted diet and water ad libitum, and used in all experiments. They were housed in groups of three or four per cage under normal laboratory con-
ditions. All animals received humane care, and the experimental protocol was approved by the Animal Research Committee of the University of Tokyo and followed the NIH guidelines for the care and use of laboratory animals.

Bile duct ligation

The common bile duct was interrupted by double ligation and subsequent scission in rats as described previously (24). At one, two and four weeks after the operation, the rats were sacrificed. Each experimental group was comprised of seven rats; ADAMTS13 activity were measured in all the seven rats, histo-
logical analyses of the liver in four rats and RNA isolation from
the liver in three rats.

CDAA diet

The CDAA and choline-supplemented L-amino acid-defined (CSAA) diets were obtained in powdered form (Dyets Inc., Be-
thlehem, PA, USA; product numbers 518753 and 518754) (25). Five rats were fed with CDAA diet and six rats with the control, CSAA diet for 16 weeks.

Blood collection and separation of plasma

Blood was collected from rats through the inferior vena cava with a 21G needle and plastic syringe. Whole blood (1.8 ml) was mixed with 0.2 ml of 3.2% sodium citrate (12, 26), and was cen-trifuged at 3,000 g at 4°C for 15 minutes (min) (27) to separate the plasma for the measurement of ADAMTS13 activity.

Histological analysis

Excised liver specimens were fixed immediately in 10% forma-
lin and embedded in paraffin. Serial 4-μm-thick liver tissue sec-
tions were deparaffinised and analysed by hematoxylin-eosin and Masson’s trichrome staining (28).

Immunohistochemical analysis

Immunohistochemical staining for smooth muscle α-actin or ADAMTS13 was performed in liver tissue fixed in 10% forma-
lin and embedded in paraffin using a Vector immunodetection kit in accordance with the protocol specified by the manufacturer (Vector Laboratories, Burlingame, CA, USA), with rabbit poly-
clonal antibody against smooth muscle α-actin (ab5694, Abcam plc., Cambridge, MA, USA) or mouse monoclonal antibody against ADAMTS13 (A10) (16). To simultaneously detect smooth muscle α-actin and ADAMTS13 immunoreactivity, imm-
unofluorescence labelling was performed; liver tissue was in-
cubated with anti-smooth muscle α-actin antibody and anti-
ADAMTS13 antibody; and subsequently with Alexa 546-con-
jugated anti rabbit-IgG (Invitrogen, Carlsbad, CA, USA) and Alexa 488-conjugated anti mouse-IgG (Invitrogen). Samples were examined under a Nikon E-600 fluorescence microscope (Nikon, Tokyo, Japan). Images were captured by a Spot charged
coupled device camera (ORCA-ER; Hamamatsu Photonics, Ham-
amatsu, Japan), digitised by AQUA-Lite (ver 1.3; Hamamatsu Photonics), and then edited using the Adobe Photoshop CS soft-
ware program (Adobe Systems Inc., Mountain View, CA, USA).

Quantitative real-time PCR

Total RNA of the liver, abdominal aorta, inferior vena cava, and right kidney was obtained from rats using TRIZOL reagent (In-
vitrogen) in accordance with the manufacturer’s protocol. One microgram of purified total RNA was transcribed using a Super-
ScriptTM First-Strand Synthesis System for RT-PCR (Invit-
rogen). A real-time PCR was performed with the same sets of rat
ADAMTS-13 primers (5’-cggaggctccagctaatgat-3’ and 5’-cag-
catccagagggtgaag-3’), rat-specific smooth muscle α-actin-
primers (5’-gctctctctctctctctctcagag-3’ and 5’-gatggtggagaa-
gaggtc-3’), rat-specific 18S ribosomal RNA primers (5’-cagaggtggtggtggtcata-3’ and 5’-gatcaggacccatcactac-3’), and GAPDH primers (5’-cggaggccacactcaacta-3’ and 5’-gaggccgcatgctgtc-3’). (17). The PCR reactions were per-
formed in a total volume of 20 μl in the presence of 0.5 μM of
primers and LightCycler FastStart Reaction Mix SYBR Green I (Roche Molecular Diagnostics, Mannheim, Germany). The samples were incubated initially for 10 min at 95°C, followed by 35 cycles of 95°C for 5 seconds (s), 63°C for 10 s, and 72°C for 10 s. The fluorescence signal from PCR reactions was monitored in real time using a Light Cycler 2.0 instrument (Roche Mole-
cular Diagnostics). The relative amount of ADAMTS-13 or smooth muscle α-actin was determined from a standard curve generated.
Measurement of ADAMTS13 activity

ADAMTS13 enzymatic activity was measured using a novel commercial chromogenic ELISA kit, ADAMTS13-act-ELISA (Kainos Inc., Tokyo, Japan / Technoclone GmbH, Vienna, Austria), which captures products cleaved by ADAMTS13 using a sandwich method. Because ADAMTS13 specifically cleaves the Y1605-M1606 bond of the VWF-A2 domain, Escherichia coli-expressed recombinant VWF-A2 polypeptides tagged with glutathione S-transferase (GST)-histidine (GST-VWF73-His) are used in this method. First, the anti-GST antibody against GST-VWF73-His is immobilised onto a microplate. The sample was then applied to the microplate, in which ADAMTS13 cleaves GST-VWF73-His. By applying the horseradish peroxidase-conjugated antibody against the cleaved product, the cleaved product is sandwiched between the two antibodies. Then, ADAMTS13 enzymatic activity can be determined colorimetrically by quantitation of the cleaved substrate of ADAMTS13. Thus, this ELISA system can measure ADAMTS13 activity, irrespective of the species of the sample. The very high correlation of the values measured by classical VWF multimer assay and this novel chromogenic ADAMTS13-act-ELISA was reported previously (29).

Statistical analyses

Quantitative data are presented as means ± standard deviation (SD). Comparisons between groups were made using Student t-test. Correlations were determined using Spearman’s rank correlation coefficient. Statistical significance was set at p<0.05.
Figure 2: ADAMTS13 expression in the liver, the aorta, the inferior venous cava, and the kidney and plasma ADAMTS13 activity in rats with bile duct ligation. Liver tissue sections from untreated control rats and bile duct-ligated rats at 4 weeks were submitted for ADAMTS13 immunolabelling (A). Results are representative of four animals at each time point. Bar = 100 µm. The sections of bile duct-ligated livers at 4 weeks were subjected to immunofluorescent labelling with anti-ADAMTS13 and anti-smooth muscle α-actin antibodies (B). Original magnification is ×600. Green fluorescence indicates ADAMTS13 immunoreactivity and red fluorescence smooth muscle α-actin. Co-localisation of ADAMTS13 and smooth muscle α-actin yielded yellow in the merged figure. Total RNA was obtained from the livers of untreated control rats and bile duct-ligated rats for 1, 2 and 4 weeks (n = 3), and ADAMTS13 mRNA expression was quantified by real-time PCR (C). A relation of ADAMTS13 and smooth muscle α-actin mRNA expressions in the liver was investigated in each rat with the untreated control and bile duct ligation for 1, 2 or 4 weeks (n = 12) (D). Plasma ADAMTS13 activity was determined in untreated control rats and bile duct-ligated rats at 1, 2 and 4 weeks (n = 7). The relationship of plasma ADAMTS13 activity and ADAMTS13 mRNA expression level in the liver was investigated in untreated control and bile duct-ligated rats at 1, 2 or 4 weeks (n = 12) (E). The relationship of plasma ADAMTS13 activity and ADAMTS13 mRNA expression was quantified by real-time PCR (G-I).
Results

Plasma ADAMTS13 activity was increased with enhanced ADAMTS13 production in the liver in bile duct-ligated rats

Bile duct ligation in rats is well known as a model of cholestatic liver injury, which results in liver fibrosis (30). Liver fibrosis forming fine lobular septa with massive periductal deposits was confirmed at four weeks after bile duct ligation, as shown in Figure 1A. Morphometric analysis of liver fibrosis showed a marked progression of liver fibrosis up to four weeks (Fig. 1B). Then, immunohistochemical staining of smooth muscle α-actin was performed in bile duct-ligated livers. In keeping with ongoing progression of fibrosis, smooth muscle α-actin immuno-reactivity was increased along with fibrous septa as well as ductular structures at four weeks after the ligation (Fig. 1C). As shown in Figure 1D, smooth muscle α-actin mRNA expression determined by quantitative real-time PCR was increased in bile duct-ligated livers at four weeks after the ligation up to 2.4 ± 0.3 fold of untreated control, as liver fibrosis became apparent. These results indicate that the number of smooth muscle α-actin-expressing cells was increased in bile duct-ligated livers in accordance with the development of liver fibrosis.

Next, ADAMTS13 expression was examined by immunohistochemical staining in bile duct-ligated livers. As demonstrated in Figure 2A, ADAMTS13 immunoreactivity was enhanced in bile duct-ligated livers for four weeks compared to untreated control livers. Co-localisation of ADAMTS13 and smooth muscle α-actin was previously demonstrated in liver specimen from rats with carbon tetrachloride-induced liver fibrosis (17) and a patient with hepatitis C-related chronic hepatitis (16), and it was confirmed that ADAMTS13 is localised in smooth muscle α-actin expressing cells similarly in bile duct-ligated rat livers for four weeks by immunofluorescence labeling (Fig. 2B). Quantitative analysis of mRNA expression of ADAMTS13 in bile duct-ligated livers showed that ADAMTS13 mRNA expression was increased after the bile duct ligation up to 1.7 ± 0.3 fold of untreated control at four weeks (Fig. 2C). Of note, a close direct linear correlation between ADAMTS13 and smooth muscle α-actin mRNA expressions was determined in untreated and bile duct-ligated livers at one, two and four weeks after the operation (Fig. 2D) (Spearman’s rank correlation coefficient; ρ = 0.8391, p<0.001, n = 12), which is in line with co-localisation of ADAMTS13 and smooth muscle α-actin in those livers determined by immunofluorescence labelling. Finally, this study examined plasma ADAMTS13 activity in bile duct-ligated rats. As shown in Figure 2E, plasma ADAMTS13 activity was increased in bile duct-ligated rats up to 2.0 ± 0.4 fold of untreated control at four weeks. The relationship of ADAMTS13 mRNA expression in the liver and plasma ADAMTS13 activity was then investigated in each rat with untreated control and bile duct ligation for one, two and four weeks. As depicted in Figure 2F, a significant, direct linear correlation between ADAMTS13 mRNA expression in the liver and plasma ADAMTS13 activity existed in untreated control and bile duct-ligated rats (Spearman’s rank correlation coefficient; ρ = 0.7762, p<0.01, n = 12).

Because ADAMTS13 production has been reported in endothelial cells (19) and glomerular podocytes in the kidney (20), ADAMTS13 mRNA expression in the abdominal aorta, inferior vena cava or kidney was examined in untreated rats and bile duct-ligated rats at four weeks. In line with previous reports demonstrating scarce ADAMTS13 mRNA expression in the aorta (15), kidney (13, 14) or heart (13, 14), ADAMTS13 mRNA expression of some samples of the aorta, inferior vena cava or kidney was in the undetectable range of our real-time PCR system. Nonetheless, the significant increase of ADAMTS13 mRNA expression was not determined in the aorta, inferior vena cava nor kidney of bile duct-ligated rats at four weeks compared to that of untreated control rats, as demonstrated in Figure 2G-I.

Plasma ADAMTS13 activity was increased with enhanced ADAMTS13 production in the liver in the CDDA-diet fed rats

The CDDA-diet has been shown to cause steatohepatitis and liver fibrosis (25). Massive steatosis and fibrosis with extensive accumulation of extracellular matrices was determined in the liver of the CDDA-diet fed rats for 16 weeks compared to the control CSAA-diet fed rats for 16 weeks, as shown in Figure 3A. Morphometric analysis of fibrosis area in the liver confirmed a marked progression of liver fibrosis (Fig. 3B). As shown in Figure 3C, smooth muscle α-actin mRNA expression determined by quantitative real-time PCR was increased in the livers of rats fed with the CDDA-diet for 16 weeks up to 4.8 ± 0.1 fold of those fed the control CSAA-diet. These results indicate that smooth muscle α-actin expression was increased in the livers of rats fed the CDDA-diet in accordance with the development of liver fibrosis.

Then, ADAMTS13 expression was determined in the livers of rats fed with the CDDA-diet. As demonstrated in Figure 4A, quantitative analysis of mRNA expression of ADAMTS13 in the livers of rats fed the CDDA-diet for 16 weeks showed the increase of ADAMTS13 mRNA expression, up to 1.9 ± 0.1 fold of those fed the CSAA-diet. As shown in Figure 4B, plasma ADAMTS13 activity was increased in rats fed the CDDA-diet for 16 weeks up to 2.3 ± 0.5 fold of those fed the control CSAA-diet. Thus, enhanced ADAMTS13 production in the liver led to increased plasma ADAMTS13 activity in rats with liver fibrosis due to steatohepatitis.
Discussion

The current study revealed the strong relation between the increased production of ADAMTS13 in the liver and the enhanced plasma ADAMTS13 activity in rats in the process of liver fibrosis, where hepatic stellate cells are known to proliferate (23), due to bile duct ligation, a model of cholestatic liver injury (30), and CDAA-diet feeding, a model of steatohepatitis (25). It was previously shown that hepatic stellate cells express both ADAMTS13 and smooth muscle α-actin in humans (16) and rats (17). In line with these findings, ADAMTS13 production in the liver was increased in relation with enhanced smooth muscle α-actin expression in the liver in rats with bile duct ligation and in rats fed the CDAA diet. In bile duct-ligated livers, co-localisation and correlation of the expression levels of ADAMTS13 and smooth muscle α-actin were determined. Thus, increased plasma ADAMTS13 activity in cholestasis and steatohepatitis in rats may be due, at least in part, to enhanced ADAMTS13 production in the liver, suggesting a significant role of hepatic stellate cells in the regulation of plasma ADAMTS13 activity.

This study contrasts with the previous report, which showed that increased number of hepatic stellate cells resulted in increased ADAMTS13 activity in carbon tetrachloride-induced fibrotic liver tissue (17), which is compatible with our current findings. However, increased plasma ADAMTS13 activity was found with increased ADAMTS13 production in the liver, suggesting a significant role of hepatic stellate cells in the regulation of plasma ADAMTS13 activity.

What is known about this topic?

− Hepatic stellate cells, endothelial cells, glomerular podocytes and platelets are reportedly capable of producing or releasing ADAMTS13.
− Hepatic stellate cell damage leads to reduced plasma ADAMTS13 activity.

What does this paper add?

− Plasma ADAMTS13 activity is enhanced correlatively with increased ADAMTS13 production by hepatic stellate cells in rat model of cholestasis.
− Enhanced plasma ADAMTS13 activity is correlated to increased ADAMTS13 production in the liver in rat model of steatohepatitis, suggesting that hepatic stellate cells in the liver play a significant role in the regulation of plasma ADAMTS13 activity.
production in the liver in bile duct ligation- and the CDAA diet-induced liver fibrosis, but not in carbon tetrachloride-induced liver fibrosis. The authors suggested that other sources of plasma ADAMTS13 (19–21) may be at work and that the contribution of hepatic stellate cells in the regulation of plasma ADAMTS13 activity may be minimal (17). However, our findings that not only the decrease (22) but also the increase of ADAMTS13 production by hepatic stellate cells caused the concordant change in plasma ADAMTS13 activity suggest that such minimal contribution of hepatic stellate cells in the regulation of plasma ADAMTS13 activity may be less likely. Although we do not exclude the possibility that other sources such as endothelial cells or glomerular podocytes might alter the production of ADAMTS13 in accordance with that in hepatic stellate cells, our current evidence that significant increase of ADAMTS13 mRNA expression was not found at least in the aorta, inferior vena cava or kidney in bile duct-ligated rats suggests that endothelial cells or glomerular podocytes may not play a major role in the regulation of plasma ADAMTS13 activity in bile duct-ligated rats. Of note, VWF antigen level was shown to be increased in patients with inflammatory diseases, indicative for endothelial activation (26), but no specific rise of plasma ADAMTS13 activity was determined despite the presence of ADAMTS13 in endothelial cells (19). Because plasma ADAMTS13 activity was rather reduced in those patients, it was suggested that ADAMTS13 is not released from endothelial cells or that it is rapidly cleared or consumed upon release (31).

As to the discrepancy between the previous evidence dealing with carbon tetrachloride-intoxicated rats and the current one, it might be explained by the inhibitory mechanism of ADAMTS13 activity in carbon tetrachloride-intoxicated rats. Alternatively, the methodology to measure ADAMTS13 activity might explain the discrepancy. It has been recently shown that bilirubin interferes with fluorescence evolution in the FRETS-VWF73 assay (32) by acting as a quencher (33), which was used in the previous study (17), whereas bilirubin does not interfere with chromogenic ADAMTS13 assay employed in this study. It is known that the plasma bilirubin levels are increased in carbon tetrachloride-intoxicated rats.

In liver diseases, the alteration of plasma ADAMTS13 activity has been reported (26, 27, 31, 34–36), which may be compatible with the current evidence suggesting that the liver plays a significant role in the regulation of plasma ADAMTS13 activity. Plasma ADAMTS13 levels were reduced in paediatric patients with acute hepatitis, but not in those with chronic hepatitis (34). Reduced plasma ADAMTS13 activity was also reported in patients with alcoholic hepatitis (35). Thus, the damage to hepatic stellate cells may cause decreased ADAMTS13 production in acute hepatitis and alcoholic hepatitis. In patients with liver cirrhosis, plasma ADAMTS13 activity was shown to be decreased (26) in relation to the severity of cirrhosis (27), although the wide range of values were detected compared to normal controls (31). In contrast, there was a report showing that plasma ADAMTS13 activity in patients with liver cirrhosis was highly variable and not significantly different from that in normal controls (36). Because it is well known that the number of hepatic stellate cells is increased in cirrhotic liver of humans as well as rats, the regulatory mechanism ADAMTS13 production by hepatic stellate cells in advanced cirrhosis in human should be further clarified. Furthermore, potential degradation or inactivation of ADAMTS13 in liver cirrhosis in human should be evaluated, which could explain also the variable data of plasma ADAMTS13 activity in those patients.

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References


