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Ticlopidine- and clopidogrel-associated thrombotic thrombocytopenic purpura (TTP): review of clinical, laboratory, epidemiological, and pharmacovigilance findings (1989–2008)

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Thrombotic thrombocytopenic purpura (TTP) is a fulminant disease characterized by platelet aggregates, thrombocytopenia, renal insufficiency, neurologic changes, and mechanical injury to erythrocytes. Most idiopathic cases of TTP are characterized by a deficiency of ADAMTS13 (a disintegrin and metalloprotease, with thrombospondin-1-like domains) metalloprotease activity. Ironically, use of anti-platelet agents, the thienopyridine derivates clopidogrel and ticlopidine, is associated with drug induced TTP. Data were abstracted from a systematic review of English-language literature for thienopyridine-associated TTP identified in MEDLINE, EMBASE, the public website of the Food and Drug Administration, and abstracts from national scientific conferences from 1991 to April 2008. Ticlopidine and clopidogrel are the two most common drugs associated with TTP in FDA safety databases. Epidemiological studies identify recent initiation of anti-platelet agents as the most common risk factor associated with risks of developing TTP. Laboratory studies indicate that most cases of thienopyridine-associated TTP involve an antibody to ADAMTS13 metalloprotease, present with severe thrombocytopenia, and respond to therapeutic plasma exchange (TPE); a minority of thienopyridine-associated TTP presents with severe renal insufficiency, involves direct endothelial cell damage, and is less responsive to TPE. The evaluation of this potentially fatal drug toxicity can serve as a template for future efforts to comprehensively characterize other severe adverse drug reactions.

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Thrombotic thrombocytopenic purpura (TTP) is a microvascular occlusive disorder characterized by systemic or intrarenal aggregation of platelets, leading to thrombocytopenia and mechanical injury to erythrocytes.[1] Conditions and factors associated with TTP include organ transplantation, infectious diseases, and drugs.[2] The most common drugs reported to the Food and Drug Administration (FDA) in association with TTP are the thienopyridine-derivative anti-platelet agents, ticlopidine and clopidogrel.[3,4] Before 1999, ticlopidine was widely used for prevention of cerebrovascular, cardiovascular, and peripheral vascular complications and following coronary artery stent procedures.[5] Since 2000, owing to concerns over ticlopidine-associated agranulocytosis, clinicians switched to clopidogrel in these settings.[5–9] Herein, we summarize the clinical, laboratory, and epidemiological information on thienopyridine-associated TTP.

PHARMACOLOGY

Ticlopidine and clopidogrel, thienopyridine derivatives that inhibit platelet aggregation,[5] differ structurally by a carboxymethyl side group. Animal studies and *in vitro* laboratory studies indicate that ticlopidine, but not clopidogrel, is associated with bone marrow toxicity.[5] As all clopidogrel metabolites contain the carboxymethyl side group, the two drugs have no common metabolites.[10] Ticlopidine and clopidogrel are administered orally, requiring hepatic breakdown to an active metabolite to achieve *in vivo* activity. The major therapeutic target of the thienopyridines is one of the adenosine diphosphate receptor types on human platelets, $P2Y_{12}$. Blockade of this receptor impairs adenosine diphosphate-induced platelet aggregation and decreases the propensity for arterial thrombosis.

EPIDEMIOLOGY

Epidemiological investigations identified a strong association of TTP with ticlopidine (Table 1). The first cases of ticlopidine-associated TTP were identified in 1991 at an apheresis center in Paris.[11] In 1998, a survey of apheresis centers supplemented by FDA adverse event reports identified 60 cases of ticlopidine-associated TTP; one-third had died from the TTP.[12] Most patients had received between 2–12 weeks of ticlopidine.[12,13] Subsequently, after the introduction of coronary artery stent procedures, additional ticlopidine-associated TTP cases were identified at interventional cardiology laboratories and therapeutic plasma exchange (TPE) centers.[13,14] Two surveys of interventional cardiology laboratories that had placed coronary artery stents in 8000 and 45,000 persons identified rates of TTP after ticlopidine administration of 1 in 1600 and 1 in 5000 patients, respectively.[15,16] These findings placed ticlopidine as the drug with the highest reported rate of TTP.

Recent investigations evaluated the association of clopidogrel with TTP. The first two cases were identified by the directors of apheresis centers in 1998, shortly after the drug received FDA approval.[17] In 2000, eleven cases of TTP after administration of clopidogrel were identified at apheresis centers in six cities.[17] By 2004, 37 cases of

Table 1 | Comparison of basic science, epidemiological, clinical, and pharmacovigilance findings for ticlopidine- versus clopidogrel-associated TTP

Category	Ticlopidine-associated TTP	Clopidogrel-associated TTP
Basic science[20]		
Probable underlying pathophysiology	Antibody to ADAMTS13 and microvascular endothelial cell damage	Microvascular endothelial cell damage
High molecular weight vWF identified during the acute TTP phase	Yes	Yes
ADAMTS13 deficiency during the acute TTP phase	Yes	No
Functional IgG inhibitors to ADAMTS13 identified during acute phase	Yes	No
Clinical[20]		
Usual time period for onset	Two to 12 weeks after drug initiation	Within 2 weeks of drug initiation
Renal insufficiency	Mild to none	Severe
Thrombocytopenia	Severe	Mild
Survival after plasma exchange	>90%, usually within days of initiation of plasma exchange	70%, often takes several weeks of plasma exchange
Survival without plasma exchange	30%	70%
Spontaneous relapse	Occasional	Infrequent
Likelihood of relapse occurring with exposure to the other thienopyridine	High	Low
Epidemiological		
Epidemiological studies identifying cases of TTP after thienopyridine administration	Surveys of directors of interventional cardiology laboratories as well as directors of therapeutic plasma exchange centers (n=33 cases)[26,29]	Surveys of directors of therapeutic plasma exchange centers (n=13 cases)[5,31]
Estimated incidence based on information included in the FDA-approved package insert	0.01-0.02%[37]	0.0001%[18] (threefold greater than estimated incidence of idiopathic TTP)
Population-based case-control studies	None	Recent initiation of anti-platelet agents (clopidogrel, aspirin, or dipyridamole) is associated with 19.8-fold increased risk of developing TTP (n=86 cases; 177 age- and gender-matched controls)[41]
Pharmacovigilance		
Number of thienopyridine-associated TTP cases identified in the first year of marketing of the relevant drug	4 (year=1991)[11]	2 (year=1999)[38,39]
Number of cases included in the largest case series	98 patients[14]	50 patients[3]
Year of FDA approval	1991 (current sales are $100,000)[9]	1998 (current sales are $7.3 billion)[9]
Time from FDA approval to identification of first cases	0 years (4 cases) (1991)	1 year (2 cases) (1999)
Time from FDA approval to reporting of first case series	7 years (1998)	1.5 years (2000)
Rank in FDA MedWatch database in association with drug-associated TTP reports (1998–2006)	First—overall (first in the years 1998 and 1999)	Second—overall (first since 2000)
Advisories from the FDA	Package insert warning (1995)[40] Black box warning (1998)[37]	Package insert warning (2000)[18]
'Dear Doctor' warnings describing drug-associated TTP mailed by the pharmaceutical supplier	1998	2000

ADAMTS13, a disintegrin and metalloprotease, with thrombospondin-1-like domains; FDA, Food and Drug Administration; TTP, thrombotic thrombocytopenic purpura.

clopidogrel-associated TTP had been reported to the FDA.[3] The pharmaceutical supplier reported an estimated incidence rate of 12 TTP cases per million clopidogrel-treated patients,[18] three times the background rate for TTP in the general population.[19]

CLINICAL FEATURES

Thienopyridine-associated TTP is characterized by two clinical syndromes (Figure 1a). Most cases of ticlopidine-associated TTP and a minority of clopidogrel-associated TTP cases present with severe thrombocytopenia, microangiopathic hemolytic anemia, markedly elevated serum levels of lactate dehydrogenase, and normal renal function; and they occur between 2 and 12 weeks after initiation of thienopyridine therapy. Most cases of clopidogrel-associated TTP and a minority of ticlopidine-associated TTP cases present with mild thrombocytopenia, microangiopathic hemolytic anemia, mildly elevated serum levels of lactate dehydrogenase, and marked renal insufficiency. Onset usually occurs within 2 weeks of thienopyridine initiation.[3,17,20] Both syndromes differ from those reported for other drug-associated TTP syndromes.[2] Thrombotic microangiopathy associated with the calcineurin inhibitors gemcitabine and mitomycin-C is dose dependent, occurs after several weeks or months of use, and is attributed to the cumulative toxic effects on vascular endothelium.[2] Renal dysfunction is generally present, with many patients requiring hemodialysis. TPE is not effective.[2] Thrombotic microangiopathy developing after organ transplantation is associated with calcineurin inhibitors. The most common treatment strategy is discontinuation of the drug.[2] Although quinine-associated TTP/HUS is antibody mediated, the antibodies are directed against granulocytes, lymphocytes, endothelial cells, or platelet glycoprotein Ib/IX or IIb/IIIa complexes.[21] The syndrome can occur after ingestion of a single tablet of quinine in previously exposed persons, and is characterized by neurologic complications, thrombocytopenia, and hemolysis. Renal failure is absent occasionally. Treatment includes discontinuation of quinine, TPE, and hemodialysis.

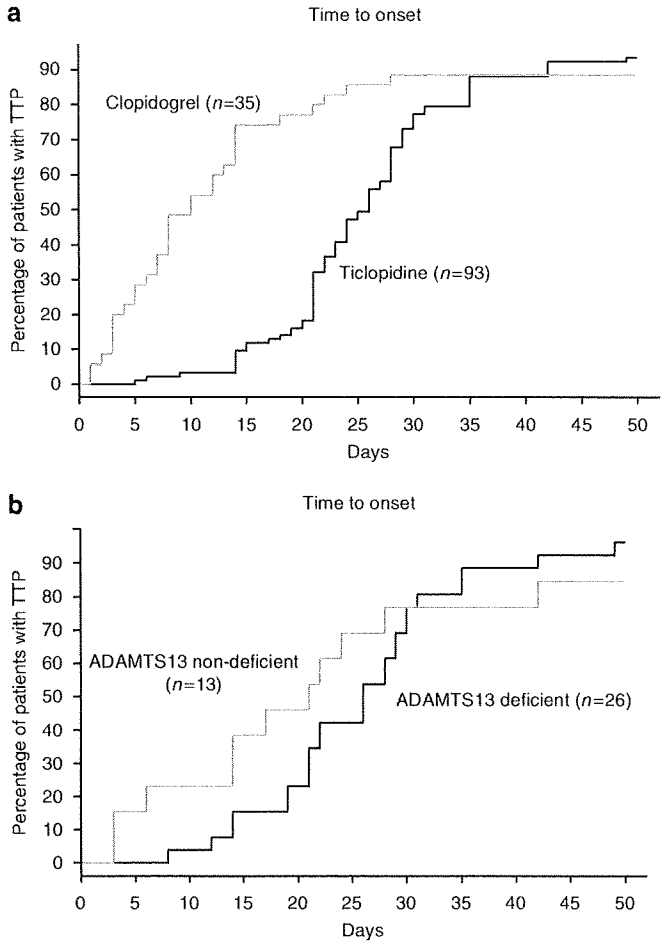

Figure 1 | Duration of thienopyridine exposure prior to TTP onset. (**a**) Thienopyridine-associated thrombotic thrombocytopenic purpura (TTP) onset: ticlopidine versus clopidogrel ($P = 0.0016$). (**b**) Thienopyridine-associated TTP onset: ADAMTS13 (a disintegrin and metalloprotease, with thrombospondin-1-like domains) deficient (<15%) versus near-normal levels (>15%) of ADAMTS13 activity ($P > 0.05$). This figure has previously been published in Bennett *et al.*[20]

PATHOPHYSIOLOGY

For most patients with ticlopidine-associated TTP and a minority of patients with clopidogrel-associated TTP, *in vitro* assessments of plasma ADAMTS13 activity show severely diminished activity at the time of TTP onset.[20] Onset of TTP occurs between 2 and 12 weeks after thienopyridine initiation (Figure 1b). Reduced *in vitro* ADAMTS13 activity correlates with deficient ADAMTS13 activity near the surface of stimulated endothelial cells that secrete ULVWF multimers. Plasma from six of seven patients with ticlopidine-associated TTP and from two of eleven patients with clopidogrel-associated TTP contained inhibitors to the ADAMTS13 metalloprotease.[17,22] Failure to process ULVWF multimers seems to lead to the binding of ULVWF to platelets, systemic platelet aggregation, and TTP.[23] After TPE and thienopyridine discontinuation, most patients with ADAMTS13 deficiency, anti-ADAMTS13 autoantibodies, and thienopyridine-associated TTP recover. Plasma exchange may lead to removal of ULVWF multimers, removal of autoantibodies to ADAMTS13, and replacement of the ADAMTS13 with that present in fresh frozen plasma. Clinical findings also require stimulation of endothelial cells to secrete ULVWF. Such a double-insult model is exemplified by the ADAMTS13 knockout mouse, which requires endothelial cell stimulation to evoke a TTP-like microvascular thrombosis.[24] In genetically predisposed individuals, thienopyridine may stimulate an autoimmune anti-ADAMTS13 antibody response and microvascular endothelial injury. Ticlopidine and clopidogrel are protein-bound in plasma and can function as haptens capable of eliciting IgE and IgG antibody formation.[25] However, they do not directly bind to ADAMTS13 and stimulate production of antibodies that inhibit ADAMTS13

enzyme activity. Anti-ADAMTS13 antibodies generated in a fraction of thienopyridine-treated patients do not require the presence of the drug (or metabolite).[17,22,26] Thienopyridine/anti-ADAMTS13 antibodies are analogous to warm autoantibodies against red blood cell antigens that emerge in a subset of patients treated with the antihypertensive agent, α-methyldopa.[27] Binding of thienopyridines to $P2Y_{12}$ molecules on different cell types may, in a fraction of exposed individuals, initiate anomalous intracellular signaling patterns or provoke antibody production against the haptenic thienopyridine–$P2Y_{12}$ protein complex on cell surfaces. Malfunction or injury to lymphocytes, CD34+ stem cells, or endothelial cells may result.

For most clopidogrel-associated and a minority of ticlopidine-associated TTP patients, the syndrome is characterized by mild thrombocytopenia, microangiopathic hemolytic anemia, and marked renal insufficiency.[20,28] Onset of TTP is generally within 2 weeks of thienopyridine initiation (Figure 1a). Most cases have ULVWF in their plasma and near-normal levels of plasma ADAMTS13 metalloprotease activity during the acute phase of the syndrome, suggesting endothelial cell injury or stimulation with release of ULVWF.[20] Thienopyridines may bind to $P2Y_{12}$ receptors (with or without anti-thienopyridine antibodies) on CD34+ stem cells, altering cell proliferation and differentiation. Interaction of thienopyridines with endothelial cells has been shown to result in nitric oxide and possibly prostacyclin (PGI_2) generation.[29,30] At least some thienopyridine binding to human endothelial cells is likely to be through the $P2Y_{12}$ receptors on these cells. In a few thienopyridine-treated patients, the endothelial cell response to the thienopyridine (with or without antibody) attachment may be a combination of cell injury, excessive secretion of ULVWF multimeric strings, or apoptosis.[23]

THERAPY

All patients who develop thienopyridine-associated TTP should have prompt plasma exchange.[1,3,12,17,31,32] Plasma exchange is continued until the goals of resolution of neurologic symptoms, improvement of LDH to near normal, and achieving and maintaining for 2–3 days a platelet count of 150,000/mm^3 are achieved.[33] After this, plasma exchange may be either discontinued or reduced in frequency.[32] Among thienopyridine-associated TTP patients who have antibody-mediated ADAMTS13 deficiency, a few days of plasma exchange are required. Although patients generally recover without permanent organ damage, a spontaneous relapse occurs occasionally. Among thienopyridine-associated TTP patients without autoantibodies against ADAMTS13, several weeks of plasma exchange are often required and spontaneous relapses are rare.[3,14,17,20] One report describes a patient with a drug-eluting coronary artery stent who developed TTP within days of clopidogrel initiation. After the TTP resolved with TPE and clopidogrel discontinuation, the patient was re-challenged with ticlopidine and did not experience a TTP relapse.[34]

CONCLUSIONS

Thienopyridine-associated TTP has served as the focus of intensive scientific investigation over the past two decades.[12,14,15,17,20,22,23,26,35,36] As with idiopathic TTP, most thienopyridine-associated TTP cases are associated with autoantibodies that inhibit the plasma metalloprotease, ADAMTS13. For these individuals, TTP onset usually occurs within 2–12 weeks after initiation of ticlopidine (rarely clopidogrel) and resolves rapidly with TPE. For a minority of cases of thienopyridine-associated TTP, autoantibodies directed against ADAMTS13 metalloprotease have not been implicated. For these persons, the syndrome occurs within 2 weeks of initiating clopidogrel (rarely ticlopidine) and is less responsive to TPE. Prominent warnings in FDA-approved labels for ticlopidine and clopidogrel describe clinical findings, and the importance of timely initiation of plasma exchange for patient who develop this toxicity. The clinical, laboratory, and epidemiological evaluation and related pharmaceutical safety interventions for thienopyridine-associated TTP can serve as a template for future efforts to investigate and protect patients against harm from other severe adverse drug reactions.

DISCLOSURE

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Pivotal role of ADAMTS13 function in liver diseases

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

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

1 Introduction

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

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

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

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

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

2 Hepatic microcirculation and hypercoagulability in liver diseases

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

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

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

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

3 ADAMTS13 assays

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

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

4 The physiological significance of ADAMTS13 in liver diseases

4.1 Liver cirrhosis

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

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

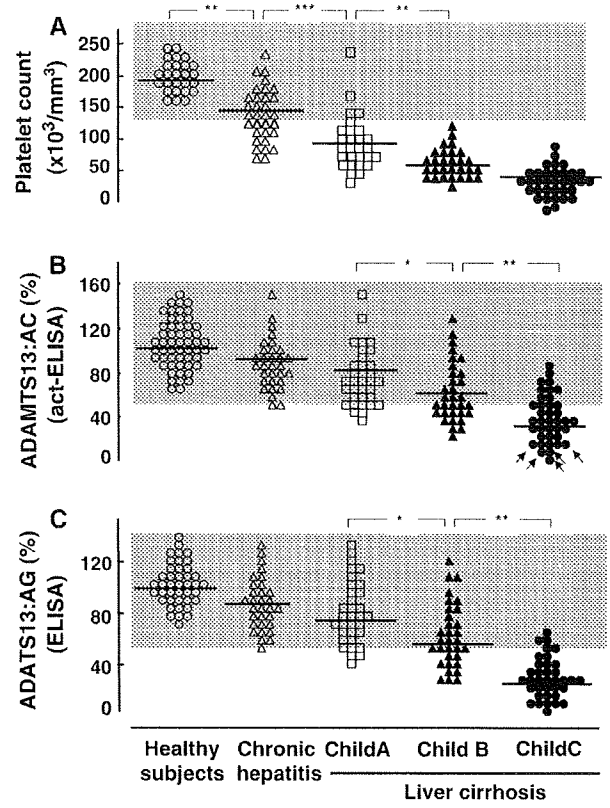

Fig. 1 Platelet counts and plasma levels of ADAMTS13:AC and ADAMTS13:AG in patients with chronic liver diseases. Platelet count decreased with the severity of chronic liver diseases, but no difference was found between Child B and C (**a**). Plasma ADAMTS13:AC determined by the ELISA progressively decreased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

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

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

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

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

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

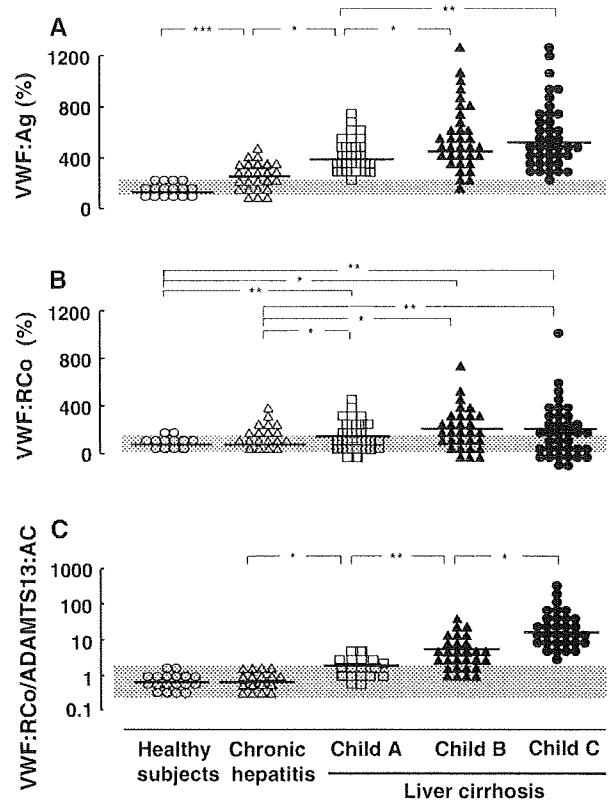

Fig. 2 Plasma levels of VWF:Ag, VWF:RCo, and VWF:RCo/ADAMTS13:AC ratio in patients with chronic liver disease. The VWF:Ag increased with the progression of chronic liver diseases, but the difference between Child B and C did not reach statistical significance (**a**). The VWF:RCo is higher in liver cirrhosis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

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

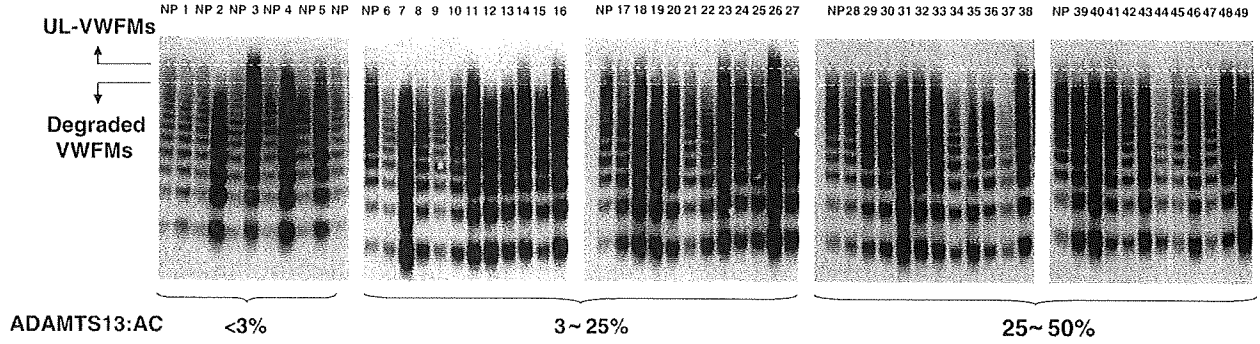

Fig. 3 Plasma VWF multimer in 49 liver cirrhosis patients with severe to mild deficiency of ADAMTS13:AC. The VWF multimer was analyzed by a vertical SDS–1.0% agarose gel electrophoresis system. Five patients (patients no. 1–5) were originally identified as a severe deficiency of plasma ADAMTS13:AC by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

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

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

4.2 Alcoholic hepatitis (AH)

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

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

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

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

4.3 Hepatic veno-occlusive disease (VOD)

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

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

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

4.4 Liver transplantation

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

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

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

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

5 Conclusion and future perspectives

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

conventional hemostatic factors. One could, then, notice that the origin of VWF, the substrate of ADAMS13, is indeed transformed hepatic sinusoidal and/or extrahepatic endothelial cells, but not hepatocytes. The procoagulant and anticoagulant proteins synthesized in hepatocytes decrease as liver disease progresses, whereas VWF markedly increases. Under such circumstances, ADAMTS13 deficiency may lead to microcirculatory disturbance not only in the liver, but also in the systemic circulation. The determination of ADAMTS13 and its related parameters will thus be quite useful for better understanding the pathophysiology and for providing appropriate treatments especially in severe liver disease patients. It will be necessary to measure ADAMTS13:AC when patients with unexplained thrombocytopenia in the course of liver disease are encountered. Further investigation will be necessary to clarify potential roles of ADAMTS13:AC in patients with liver disease.

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ORIGINAL ARTICLE

Cut-off values of D-dimer and soluble fibrin for prediction of deep vein thrombosis after orthopaedic surgery

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Abstract Soluble fibrin (SF) and D-dimer are considered to be useful for the diagnosis of thrombosis; however, the efficacy of the diagnosis of deep vein thrombosis (DVT) after orthopaedic surgery by SF and D-dimer is still not well established. The present study was designed to evaluate the efficacy of SF and D-dimer in the diagnosis of DVT after orthopaedic surgery. The plasma concentrations of SF and D-dimer were measured in 99 patients following orthopaedic surgery. The plasma concentrations of D-dimer and SF in patients undergoing orthopaedic surgery were markedly high in comparison to healthy volunteers, and these markers were increased after surgery. The plasma concentrations of D-dimer were significantly higher in patients with DVT than in those without DVT at days 4, 7, 10 and 14, and those of SF were significantly higher in patients with DVT than in those without DVT at days 1, 4 and 14. A receiver operating characteristic (ROC) analysis of SF and D-dimer for diagnosis of DVT after surgery generated an ROC curve that showed SF to be better than D-dimer at day 1, while D-dimer was better than SF at day 4. In addition, less than 7.2 μg/ml of D-dimer or 3.6 μg/ml of SF at day 1 after surgery, or less than 7.0 μg/ml of D-dimer at day 4 excluded DVT. These findings suggest that the D-dimer and SF are useful for the diagnosis and exclusion of DVT after orthopaedic surgery.

Keywords SF · D-dimer · Orthopaedic surgery · Total hip arthroplasty · Total knee arthroplasty

1 Introduction

Soluble fibrin (SF) and D-dimer are sensitive markers for thrombotic diseases [1, 2]. These markers are reported to be elevated in deep vein thrombosis (DVT)/pulmonary embolism (PE) [3–5], disseminated intravascular coagulation (DIC) [6–8], acute myocardial infarction (AMI) [9, 10] and thrombotic thrombocytopenic purpura (TTP) [11]. The International Society of Thrombosis and Haemostasis (ISTH) established the diagnostic criteria for overt-DIC using SF and D-dimer [12]. Since PE is a common, frequently undiagnosed and potentially fatal event, and the symptoms of PE are common, including dyspnoea and chest pain, the early recognition of DVT and PE by D-dimer and SF is clinically important [13, 14]. D-dimer is widely used to diagnose thrombosis such as DVT, but many of the commercially available D-dimer assay kits contain different monoclonal antibodies and standard substances, and are based on different assay systems. Since the issue of standardization of D-dimer assays remains to be

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resolved, several studies [15, 16] reported the basic data for the standardization of D-dimer. The *elevated* soluble fibrin (SF) [17] in plasma is an indicator of thrombin activation in the blood, as are the thrombin–antithrombin complex and prothrombin fragment F1+2.

Orthopaedic surgeries, such as total hip arthroplasty (THA) and total knee arthroplasty (TKA), are frequently associated with DVT/PE [18, 19]. Several clinical trials have been recently reported for the prevention of DVT [20, 21]. The diagnosis of DVT depends on venography or echography, but these methods are time consuming and expensive. The exclusion of DVT by D-dimer or SF might be helpful. But the usual cut-off value of D-dimer is not appopriate for patients after surgery, since all patients after surgery have high plasma concentrations of D-dimer and SF.

The present study was designed to evaluate the efficacy of the SF and D-dimer in the diagnosis of DVT after surgery. For this purpose, the changes of the plasma concentration of D-dimer and SF were examined in the clinical course after surgery of 99 patients who underwent orthopaedic surgery.

2 Materials and methods

2.1 Subjects

From January 1, 2006 to May 31, 2007, 99 patients (median age 66 years of age, 25–75% percentile, range 58–73 years of age; sex, 86 females and 13 males) had orthopaedic surgery (71 THA and 28 TKA) in the Mie University Graduate School of Medicine. The plasma concentrations of SF and D-dimer were examined in these patients before the operation, and 1, 4, 7, 10, 14, 21, 24 and 32 days after surgery. At 4 and 10 days after orthopaedic surgery, echography was carried out for DVT. The study protocol was approved by the Human Ethics Review Committees of the Mie University Graduate School of Medicine and a signed consent form was obtained from each subject. Among these patients, 84 patients (66 years of age, 57–73 years of age, 73 females and 11 males) had no thrombosis, and 15 patients had a DVT (68 years of age, 60–73 years of age, 13 females and 2 males). Confirmation of DVT was diagnosed with echography or venography. Several mechanical prophylaxis were carried out in all patients until 4 days after surgery and 10 patients with high risk (evaluated by a physician) or superficial vein thrombosis were treated with unfractionated heparin (UFH) or low-dose warfarin 4 days after orthopaedic surgery. Fifteen patients, who were diagnosed to have DVT, were treated with UFH.

Citrated blood samples were obtained from the peripheral veins of healthy subjects (see below) and patients under fasting conditions and then centrifuged for 20 min at 3,000 rpm. The supernatants (plasma) were analysed within 4 h. The same parameters were also measured in 99 healthy subjects (mean age 22 years, range 21–30 years; 41 females and 58 males), who were free of any diseases including thrombotic disease or hyperlipidaemia, as confirmed by an annual medical checkup.

2.2 Measurement of plasma concentrations of D-dimer and soluble fibrin

Plasma D-dimer levels were measured with LPIA-ACE D-dimer (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) using JIF23 monoclonal antibody. The JIF23 monoclonal antibody, which recognizes plasmin-digested N-terminus of the γ chain on the D region, was used for latex agglutination [22]. SF was also determined by the latex agglutination method using IATRO SF (Mitsubishi Kagaku Iatron Inc.) containing the monoclonal antibody IF-43, which recognizes a segment of the fibrin Aα chain [(Aα-17–78) residue segment] exposed in the E region of fibrin monomer (FM) when the FM molecule binds the D region of another FM or fibrinogen. The antibody is coated for the SF assay [23].

2.3 Statistical analysis

The data are expressed as the median (25–75% percentile). The differences between the groups were examined for statistical significance using the Mann–Whitney U test. A P value of less than 0.05 was considered to be significant. The usefulness of D-dimer and SF levels in the diagnosis of thrombosis and VTE was examined by a receiver operating characteristic (ROC) analysis [24]. The cut-off values were determined by the ROC analysis. All statistical analyses were performed using the SPSS II software package (SPSS Japan, Tokyo).

3 Results

The plasma concentrations of D-dimer in patients without DVT were significantly increased on day 1 (median 13.4 μg/ml, 25–75 percentile, 8.4–24.1 μg/ml, $P < 0.01$) and day 7 (9.4 μg/ml, 7.6–12.6 μg/ml, $P < 0.05$) after surgery in comparison to day 0 (7.5 μg/ml, 5.9–9.3 μg/ml). While in patients with DVT, the plasma concentrations of D-dimer were significantly increased on day 1 (16.1 μg/ml, 9.9–26.3 μg/ml, $P < 0.01$), day 4 (19.0 μg/ml, 10.6–28.7 μg/ml, $P < 0.01$), day 7 (12.3 μg/ml, 8.9–25.4 μg/ml, $P < 0.05$) and day 14 (16.0 μg/ml, 9.6–23.0 μg/ml, $P < 0.01$) after surgery in

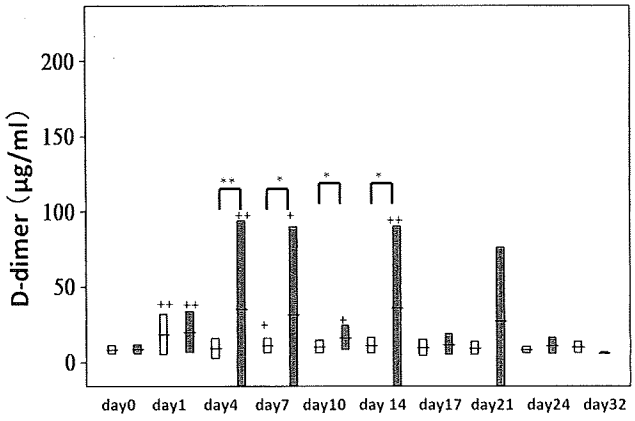

Fig. 1 Plasma concentrations of D-dimer in patients after orthopaedic surgery. *Open bar* patients without DVT, *closed bar* patients with DVT. $^{*}P < 0.05$, $^{**}P < 0.01$ difference between patients without DVT and those with DVT. $^{+}P < 0.05$, $^{++}P < 0.01$ in comparison to day 0

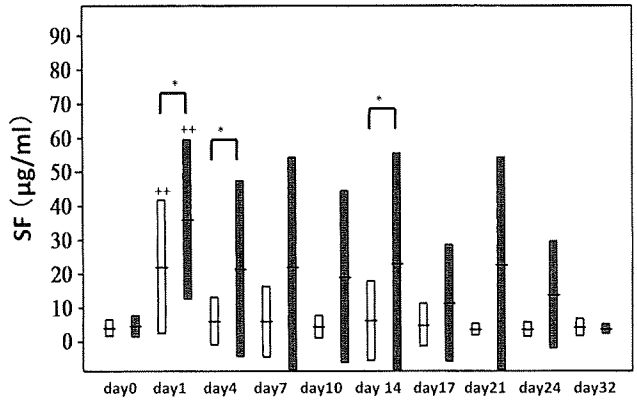

Fig. 2 Plasma concentrations of SF in patients after orthopaedic surgery. *Open bar* patients without DVT, *filled bar* patients with DVT. $^{*}P < 0.05$, $^{**}P < 0.01$ difference between patients without DVT and those with DVT. $^{+}P < 0.05$, $^{++}P < 0.01$ in comparison to day 0

comparison to day 0 (7.6 μg/ml, 6.1–9.5 μg/ml). The plasma concentrations of D-dimer were significantly higher in patients with DVT than in those without DVT on day 4 ($P < 0.01$), 7 ($P < 0.05$), 10 ($P < 0.05$) and 14 ($P < 0.05$; Fig. 1). The plasma concentration of D-dimer was markedly high in each of the days after surgery in comparison to healthy volunteers (0.4 μg/ml, 0.2–0.5 μg/ml).

The plasma concentrations of SF in patients without DVT were significantly increased on day 1 (18.2 μg/ml, 6.0–33.1 μg/ml, $P < 0.01$) after surgery in comparison to day 0 (3.7 μg/ml, 2.5–8.3 μg/ml). While in patients with DVT, the plasma concentrations of SF were significantly increased on day 1 (32.0 μg/ml, 16.7–48.0 μg/ml, $P < 0.01$) after surgery in comparison to day 0 (3.5 μg/ml, 2.8–10.1 μg/ml). The plasma concentrations of SF were significantly higher in patients with DVT than in those without DVT on days 1, 4 and

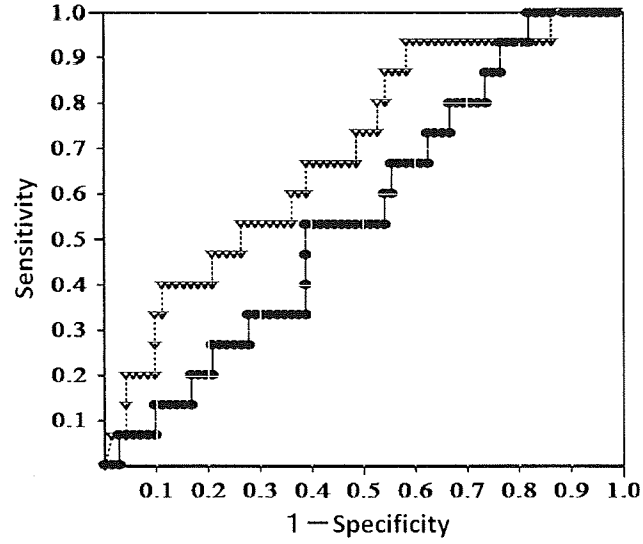

Fig. 3 ROC analysis for diagnosis of DVT 1 day after orthopaedic surgery. *Filled circle* D-dimer, *open triangle* SF. AUC: D-dimer 0.557, SF 0.692

14, respectively ($P < 0.05$) (Fig. 2). The plasma concentration of SF was markedly high in each of the days after surgery in comparison to healthy volunteers (0 μg/ml, 0–0.6 μg/ml).

In an ROC analysis of SF and D-dimer for diagnosis of DVT at day 1 after surgery (Fig. 3), the ROC curve showed that SF was better than D-dimer on day 1. The area under the curve (AUC) was 0.692 in SF and 0.557 in D-dimer. The 100% negative predictive value (NPV) of SF and D-dimer was 3.6 and 7.2 μg/ml, respectively. In the highest odds ratio, D-dimer was 8.0 μg/ml and SF was 11.9 μg/ml (Table 1). The ROC analysis of SF and D-dimer for diagnosis of DVT at day 4 after surgery (Fig. 4) showed that D-dimer was better than SF on day 4. The AUC was 0.737 in SF and 0.862 in D-dimer. The 100% of NPV for SF was not detected, but that of D-dimer was 7.0 μg/ml. In the highest odds ratio, D-dimer was 17.7 μg/ml and SF was 17.8 μg/ml (Table 2).

4 Discussion

In the present study, the plasma concentrations of D-dimer and SF in patients after orthopaedic surgery were markedly high in comparison to those in healthy volunteer. These findings are consistent with previous reports [1, 2, 14]. Under these conditions, the cut-off value in previous reports might not be useful for the diagnosis of thrombosis. Therefore, a new cut-off value of SF or D-dimer for thrombosis should be determined in the patients after orthopaedic surgery. Patients with DVT had high levels of D-dimer on day 0 in comparison to healthy volunteers. This reason for this may be due to the presence of some occult thrombosis in these patients.

Table 1 Cut-off values of SF and D-dimer on day 1 after surgery

Cut-off value (μg/ml)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Odds ratio
SF					
3.6	100	12.5	19.5	100	–
11.9	93.3	41.7	25.0	96.8	10.0
D-dimer					
7.2	100	18.1	20.3	100	–
8.0	93.3	23.7	20.3	94.4	4.33

PPV positive predictive value, *NPV* negative predictive value

Table 2 Cut-off values of SF and D-dimer on day 4 after surgery

Cut-off value (μg/ml)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Odds ratio
SF					
–	100			100	–
17.8	36.4	95.6	66.7	86.0	12.3
D-dimer					
7.0	100	55.6	35.5	100	–
17.7	54.5	97.8	85.7	90.0	52.8

PPV positive predictive value, *NPV* negative predictive value

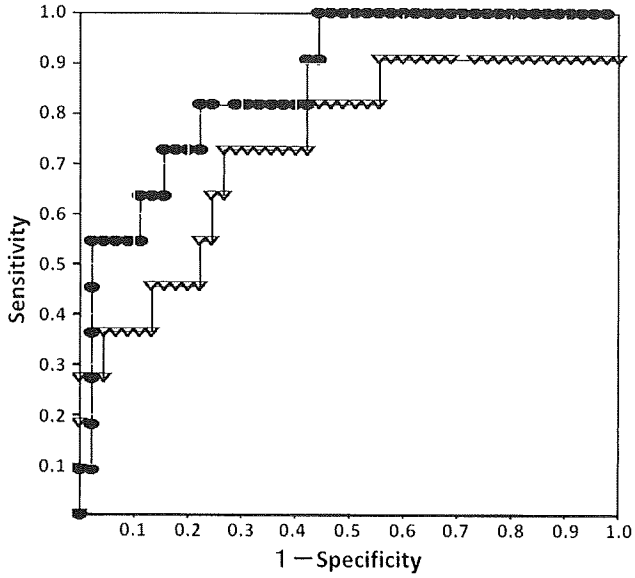

Fig. 4 ROC analysis for diagnosis of DVT 4 days after orthopaedic surgery. *Filled circle* D-dimer, *open triangle* SF. AUC: D-dimer 0.862 SF 0.737

From the significant difference in the plasma concentrations of D-dimer between patients with DVT and those without DVT, D-dimer may be useful for the diagnosis of DVT in patients on day 4, 7, 10 and 14 after orthopaedic surgery. However, no significant difference of plasma D-dimer concentration was observed between the two groups on day 1 after surgery, suggesting that D-dimer was not useful for the diagnosis of DVT at that time. The ROC analysis also showed that D-dimer was not useful for the diagnosis of DVT on day 1 after surgery. As the half life of the plasma SF levels is short, the elevation of SF due to the operation decreased on day 1. The onset of DVT is thus considered to begin on day 1. Therefore, an elevation of SF on day 1 after operation is a useful marker for the prediction of DVT. In contrast, the half life of the plasma D-dimer levels is long, and therefore the elevation of D-dimer due to the operation continues for several days, thus making it difficult to detect the onset of DVT on day 1.

From the significant difference of plasma concentrations of SF between patients with DVT and those without DVT, SF may be useful for the diagnosis of DVT on day 1, 4 and 14 after orthopaedic surgery. The ROC analysis also showed that SF was useful for the diagnosis of DVT on day 1 after surgery. As the patients with high risk and with superficial vein thrombosis were treated with UFH or low-dose warfarin from day 4 after surgery, the value of D-dimer and SF at only 1 and 4 days after orthopaedic surgery were not affected by the use of anticoagulant drugs in this study.

In previous reports [2, 14, 25], the high concentrations of SF and D-dimer could be considered to be markers of thrombosis including venous thromboembolism (VTE). An appropriate cut-off value for the diagnosis of VTE in patients without operation was reported to be 5.9 μg/ml in SF and 4.8 μg/ml in D-dimer with different assays (data from SF and D-dimer by different assays were similar; unpublished data) [25]. In this study, more than 11.9 μg/ml of SF on day 1 or more than 17.7 μg/ml of D-dimer on day 4 was suggested to be associated with DVT in the patients after surgery.

In Europe and North America, D-dimer concentrations of less than 0.5 μg/ml are considered to exclude DVT/PE in patients without surgery [26], but some D-dimer kits that are frequently used in Japan have a different cut-off value (1.2 μg/ml) for the exclusion of DVT/PE [14]. In this study, a new cut-off value for exclusion of DVT in patients after orthopaedic surgery could be determined: for D-dimer 7.2 μg/ml on day 1 and 7.0 μg/ml on day 4, and for SF 3.6 μg/ml on day 1. This discrepancy in the cut-off values between outpatients and postoperative patients may be due to thrombin formation as a result of the operation.

Fondaparinux and enoxaparin were recently approved as prophylaxis drugs for orthopaedic surgery by the Japanese Ministry Health, Labour and Welfare, but these sometimes cause severe bleeding [27]. The current results could allow recommending treatment with fondaparinux or enoxaparin in patients with more than 11.9 μg/ml of SF on day 1 or more than 17.7 μg/ml of D-dimer on day 4, but not in those with less than 7.2 μg/ml of D-dimer or less than 3.6 μg/ml of SF on day 1. For the prevention of DVT, it is therefore