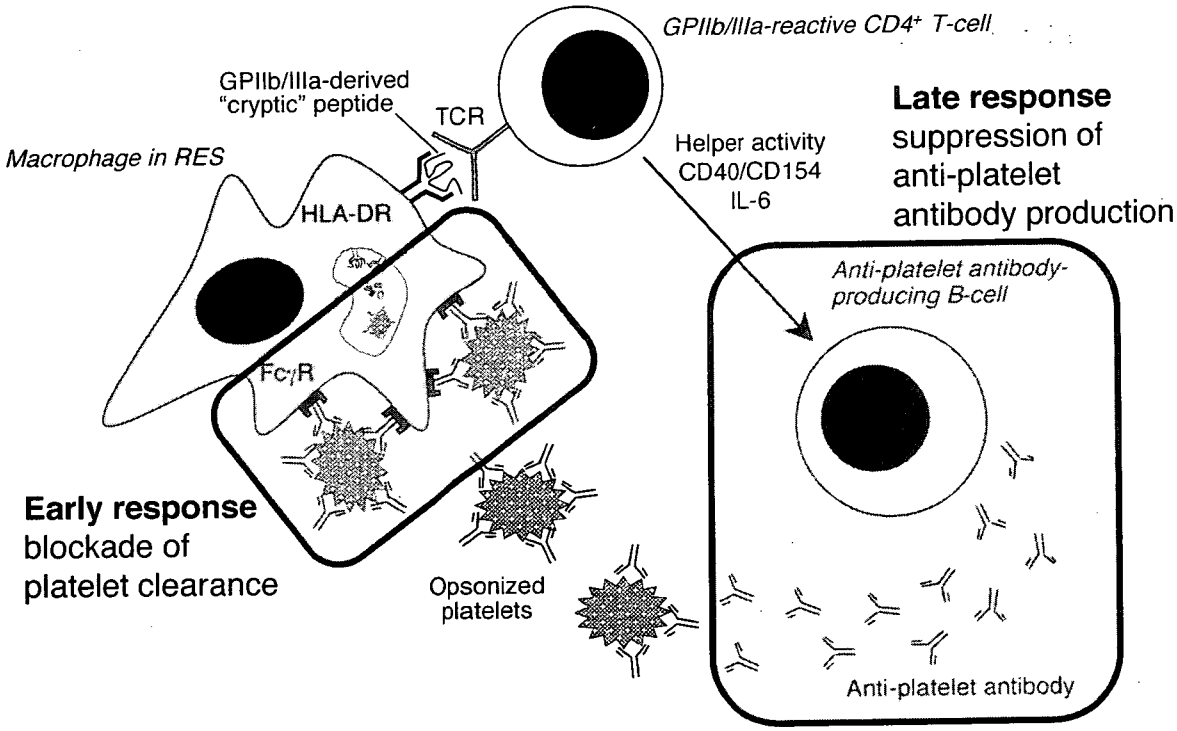

Figure 1. Schematic representation of mechanisms for early and late platelet responses following *H pylori* eradication in patients with ITP. A continuous pathogenic loop, consisting of macrophages in the reticuloendothelial system (RES), GPIIb/IIIa-reactive CD4+ T-cells, and anti-platelet antibody–producing B-cells, maintains anti-platelet antibody production in ITP patients. Early platelet response is likely to be mediated through blockade of platelet clearance by macrophages in RES, while late response results from suppression of anti-platelet autoantibody production. Modified with permission [3].

infection against the development of SLE [42]. Moreover, we failed to observe platelet recovery after eradication of *H pylori* in 5 *H pylori*–infected patients with liver cirrhosis and thrombocytopenia, whose pathogenic process also involves anti-GPIIb/IIIa autoantibody [43]. Thus, the effect of the *H pylori* eradication therapy on platelet count is likely to be restricted to *H pylori*–positive patients with idiopathic ITP, and the pathogenic process might be different between idiopathic and secondary ITP in relation to *H pylori* infection.

8. Potential Role of *H pylori* in the ITP Pathogenesis

The prevalence of *H pylori* infection in ITP patients is similar to that in the general population matched for age and geographical area [6-8], thus reinforcing the hypothesis that *H pylori* is involved in only certain patients developing ITP or that *H pylori* infection is just one of several factors that promote the pathogenic process of ITP. Since *H pylori* infection is usually established in infants with immature immune system, it is presumed that a long incubation period is required to develop ITP involving *H pylori* infection. *H pylori* does not invade the gastric epithelium but is capable of inducing the secretion of soluble inflammatory mediators and cellular apoptosis in the host, leading to local inflammation in the epithelium and subepithelial layers [44]. In addition to this local inflammation, *H pylori* infection also induces systemic immune responses through stimulation of the innate immunity and activation of T- and B-cells. These host immune responses are presumed to contribute to the ITP pathogenesis in *H pylori*–infected individuals, but it is currently unknown what mechanisms trigger the development of ITP. In this regard, the gastric mucosal lesions of *H pylori*–infected ITP patients showed a corpus-predominant pattern of gastritis, which was similar to the lesion observed in non-ITP individuals [45]. In addition, the virulence profile of *H pylori* strains in ITP patients does not differ from that of peptic ulcer patients [45], indicating that no specific gastric lesions or *H pylori* strains induce ITP.

Whether the *H pylori* eradication regimen influences the anti-platelet autoantibody response is still under debate. Several reports describe a reduction in platelet-associated IgG (PAIgG) after the successful eradication of *H pylori* [42,46], although PAIgG is unreliable as an assay to detect pathogenic anti-platelet autoantibodies [47]. One study reported no changes in anti-GPIIb/IIIa antibody levels after successful *H pylori* eradication [48], but our recent study utilizing the enzyme-linked immunospot assay for detection of B-cells producing IgG anti-GPIIb/IIIa antibodies showed that the anti-platelet autoantibody response in ITP patients was suppressed after the successful *H pylori* eradication [20], suggesting that the *H pylori* infection is involved in the process of anti-platelet autoantibody production.

Many hypotheses have been made about the mechanisms by which *H pylori* may induce the development of ITP. One potential mechanism is that antibodies to *H pylori* components cross-react with platelet surface antigens, in a process like the mechanism that induces HIV-mediated ITP [49]. In this regard, molecular mimicry between the *H pylori* polysaccharide antigens and the human Lewis antigens has been postulated as an explanation for why *H pylori* is tolerated in humans [50,51]. Amedei et al reported that *H pylori* infection is capable of activating cross-reactive T-cells that recognize both *H pylori* antigens and H^+, K^+-adenosine triphosphatase via molecular mimicry [52]. Michel and co-workers were the first to investigate this molecular mimicry hypothesis by testing platelet eluates derived from *H pylori*–positive ITP patients, but platelet eluates with capacity to react with GPIIb/IIIa or GPIb failed to recognize *H pylori* antigens [31]. In contrast, another group reported that platelet eluates from *H pylori*–positive ITP patients recognized CagA, but those from *H pylori*–infected nonthrombocytopenic individuals did not [53]. Unfortunately, IgG concentrations in the eluates used in their experiment were not adjusted. To date, pathogenic roles of cross-reacting antibodies against *H pylori* remain obscure.

Another hypothesis is that chronic *H pylori* infection influences the host's immune system in a manner that promotes the emergence of autoreactive B-cells. Yamanishi and coworkers showed that *H pylori* components are able to initiate autoimmune responses via producing autoantibodies through the activation of B-1 cells [54]. However, no significant difference between *H pylori*–positive and *H pylori*–negative individuals was reported in the autoantibody responses, such as anti-nuclear, anti-microsome, and anti-smooth muscle antibodies [55]. Other hypotheses include platelet activation and aggregation induced by forming a complex of *H pylori*, anti–*H pylori* antibody, and von Willebrand factor bound to platelet membranes [56], and a cobalamin deficiency due to *H pylori*–induced gastritis [57].

We are focusing on a quick platelet response observed in some *H pylori*–positive ITP patients after the eradication of *H pylori*. Most previous cohorts evaluated the platelet response 1 to 6 months following the eradication regimen, but several case series suggested that some patients experienced the platelet response within days of completion of the eradication regimen [11,58,59]. We recently reported that nearly half of *H pylori*–positive ITP patients showed a rapid rise in platelet count when the eradication regimen was just completed [20]. To evoke a molecular mimicry hypothesis for *H pylori*–related ITP, a platelet response should take several weeks, to allow for clearance of the pathogenic antibody. Interestingly, circulating anti-GPIIb/IIIa antibody–producing B-cells were not decreased at this time point. Taken together with a lack of correlation between a rapid rise in platelet count and the following platelet response, rapid platelet increase should be mediated by the mechanism independent of suppressing anti-platelet antibody production. In this regard, a quick increase in platelet count without reduction in the number of anti-GPIIb/IIIa antibody–producing B-cells is also observed in ITP patients who responded to intravenous immunoglobulin. One potential mechanism for early platelet response after *H pylori* eradication regimen might be blockade of platelet clearance by reticuloendothelial phagocytes, which is demonstrated as one of major therapeutic actions of intravenous immunoglobulin [60]. These different actions of *H pylori* eradication suggest that multiple processes are responsible for platelet recovery in ITP patients (Figure 1). Further studies evaluating a potential linkage between blockade of platelet clearance and subsequent suppression of anti-platelet antibody response are necessary to elucidate the role of *H pylori* infection in the pathogenesis of ITP.

9. Summary and Future Perspectives

Accumulating evidence indicates efficacy and safety of the *H pylori* eradication regimen in a subset of *H pylori*–infected ITP patients. It is likely that platelet recovery after the *H pylori* eradication regimen results from the eradication of *H pylori* itself, suggesting a direct role for *H pylori* infection in the pathogenesis of ITP. The safety profiles of the *H pylori* eradication therapy compared to standard ITP treatments certainly make this regimen an attractive option, but randomized controlled studies enrolling a large number of patients from various ethnic backgrounds will be necessary to determine the precise response rate and predictors of the response. Elucidating the mechanism responsible for the platelet response in ITP patients after successful eradication of *H pylori* may provide valuable clues to the pathogenesis of ITP and is useful in developing new therapeutic strategies for refractory ITP.

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HEPATOLOGY

Evaluation of platelet kinetics in patients with liver cirrhosis: Similarity to idiopathic thrombocytopenic purpura

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Abstract

Background: Thrombocytopenia is a common manifestation of liver cirrhosis (LC), but its underlying mechanism is not fully understood. The purpose of the present paper was to evaluate the platelet kinetics in LC patients by examining several non-invasive convenient markers.

Methods: Fifty-seven LC patients, 32 patients with idiopathic thrombocytopenic purpura (ITP), 12 with aplastic anemia (AA), and 29 healthy individuals were studied. Plasma thrombopoietin was measured by enzyme-linked immunosorbent assay. Absolute reticulated platelet (RP) count and plasma glycocalicin were used as indices for thrombopoiesis, and the indices for platelet turnover were the RP proportion and the plasma glycocalicin normalized to the individual platelet count (GCI).

Results: There was no difference in thrombopoietin levels between LC patients and healthy controls. The RP proportion and GCI were significantly higher and the absolute RP count and glycocalicin significantly lower in LC patients than in healthy controls. These markers in ITP and LC patients were comparable, but significantly different from those in AA patients. The bone marrow megakaryocyte density in LC and ITP patients was similar, and significantly higher than in AA patients.

Conclusions: Cirrhotic thrombocytopenia is a multifactorial condition involving accelerated platelet turnover and moderately impaired thrombopoiesis. Thrombopoietin deficiency is unlikely to be the primary contributor to cirrhotic thrombocytopenia.

Introduction

Thrombocytopenia is a major hematologic condition commonly observed in patients with liver cirrhosis (LC), and is a risk factor for gastrointestinal bleeding and other life-threatening hemorrhagic events. Historically, cirrhotic thrombocytopenia was attributed to hypersplenism, in which platelets are sequestered in the pathologically enlarged and congested spleen.[1–3] However, we recently reported that autoantibody-mediated platelet destruction, as seen in patients with idiopathic thrombocytopenic purpura (ITP), may also contribute to cirrhotic thrombocytopenia.[4] In these mechanisms, the thrombocytopenia is principally mediated by enhanced platelet clearance in the periphery, resulting in accelerated platelet turnover. However, impaired platelet production due to thrombopoietin (TPO) deficiency was recently proposed as another cause of thrombocytopenia in LC patients. This theory assumes that the level of TPO, a principal regulator of megakaryogenesis and thrombopoiesis predominantly produced by the liver,[5,6] is insufficient in the state of advanced liver failure. This theory is supported by the clinical observation that the reduced circulating TPO level in LC patients is restored in conjunction with an increase in platelet count after orthotopic liver transplantation.[7–9] However, it is still controversial as to whether TPO production is actually suppressed or maintained *in vivo* in LC patients.[10–13]

A platelet kinetics study is useful for discriminating a state of platelet hypoproduction from accelerated platelet turnover in thrombocytopenic patients, but its application is limited because it requires radioisotopes and special equipment. Recently, several non-invasive laboratory tests that provide information about the platelet lifespan have been developed; these include measurements of reticulated platelets (RP) and plasma glycocalicin (GC). Reticulated platelets are young platelets that contain higher levels of nucleic acid components than mature platelets. The absolute RP count is a reliable indicator of the thrombopoiesis rate, analogous to using the erythrocyte reticulocyte count to evaluate erythropoiesis.[14–16] Glycocalicin is a proteolytic fragment of the α-chain of

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glycoprotein (GP)Ib, which is cleaved from the surface of megakaryocytes and platelets.[17] The plasma GC concentration is decreased in patients with aplastic anemia (AA) and greatly increased in patients with essential thrombocythemia, indicating that it is a marker for platelet production.[17–19] Thus, both the absolute RP count and plasma GC level reflect the rate of platelet production. In contrast, the proportion of RP in total platelets (%RP) and the plasma GC level normalized to the individual platelet count (GC index; GCI) have been shown to reflect platelet turnover.[14,18] Here we used these non-invasive tests to evaluate the platelet kinetics in LC patients, and examined whether accelerated platelet turnover or impaired thrombopoiesis due to TPO deficiency is the primary contributor to cirrhotic thrombocytopenia.

Methods

Patients and controls

Fifty-seven LC patients with thrombocytopenia ($<150 \times 10^9$/L) who were followed up at Keio University Hospital were enrolled. The diagnosis of LC was based on clinical history, physical examination, laboratory findings, and ultrasonographic and/or computed tomographic imaging studies with or without liver biopsy.[20] The etiology of the LC was hepatitis B virus (HBV) infection in 15 patients, hepatitis C virus (HCV) infection in 34, and excessive alcohol intake (alcoholic liver disease; ALD) in eight. The demographic and clinical parameters recorded included age at examination, sex, Child's grade, and the presence or absence of hepatocellular carcinoma and/or splenomegaly (defined by an ultrasound splenic index >40 cm^2). Thirty-two patients with ITP served as the disease control for increased platelet turnover status, and 12 patients with acquired AA as the control for impaired platelet production. At the time of blood examination, all patients with ITP or AA had a platelet count $<100 \times 10^9$/L. The ITP was defined as thrombocytopenia persisting for longer than 6 months, with normal or increased numbers of bone marrow megakaryocytes without morphologic evidence of dysplasia, and with no secondary immune or non-immune diseases that could account for the thrombocytopenic state.[21,22] The diagnosis of AA was based on criteria including pancytopenia, the absence of splenomegaly or lymphadenopathy, reduced cellularity in the bone marrow, and no other concurrent disease or therapy that would cause pancytopenia.[23] Twenty-nine laboratory volunteers also served as healthy controls. The study protocol conformed to the ethical principles of the World Medical Association Declaration of Helsinki as reflected in a priori approval from the Keio University Institutional Review Boards, and written informed consent was obtained from each subject.

Sample preparation

Heparinized venous blood was obtained from all subjects. After separation of platelet-rich plasma, the residual cell components were applied to Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient centrifugation to isolate peripheral blood mononuclear cells (PBMC). Freshly isolated PBMC were resuspended in RPMI1640 containing 10% heat-inactivated fetal bovine serum. After 10^6 platelets were used in the RP assay, the remaining platelet-rich plasma was spun again to obtain platelet-poor plasma that was used for the measurement of GC and TPO.

Anti-platelet antibody response

The anti-platelet antibody response was evaluated by detection of circulating B cells producing IgG anti-GPIIb/IIIa antibodies using an enzyme-linked immunospot assay as described previously.[24] Each experiment was conducted in five independent wells, and the results represent the mean of the five values. The frequency of circulating anti-GPIIb/IIIa antibody-producing B cells was presented as the number per 10^5 PBMC, and a cut-off value was defined as 2.0.[24]

Plasma thrombopoietin

The plasma TPO level was measured using a commercially available enzyme-linked immunosorbent assay kit (Quantikine; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. The lower detection limit of the kit was 31.2 pg/mL. All samples were measured in duplicate with the same assay, and the results were calculated as the mean of two values.

Reticulated platelets

Reticulated platelets were detected by staining the platelets with thiazole orange (Retic-Count; BD Biosciences, San Jose, CA, USA) followed by flow cytometric analysis using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences), as described elsewhere.[15,25] Briefly, the fluorescence histogram was analyzed using a linear gate set to capture 1% of the RP count. This standard gate was used for all samples and the percentage of thiazole orange-positive platelets in this gate was the %RP. The absolute number of RP was calculated from the %RP and the circulating platelet count.

Plasma glycocalicin

The plasma GC level was determined using a commercially available enzyme-linked immunosorbent assay kit (Glycocalicin EIA Kit; Takara Bio, Ohtsu, Japan), according to the manufacturer's protocol. The lower detection limit of the kit is 10 ng/mL. All samples were measured in duplicate with the same assay, and the results were calculated as the mean of two values. The GCI was calculated according to the formula: [GC concentration (ng/mL) $\times 250 \times 10^6$] / individual platelet count per L.[18]

Bone marrow evaluation

Bone marrow specimens obtained by either autopsy or biopsy were evaluated for megakaryocyte density. Formalin-fixed paraffin-embedded sections were stained with hematoxylin–eosin, and the number of megakaryocytes was semiquantitatively assessed.[26] Five randomly selected high-power fields were examined by three independent observers, and the results were calculated as the mean of 15 values.

Statistical analyses

All continuous results were expressed as mean ± SD. To assess the clinical characteristics and experimental results, the χ^2, Kruskal–Wallis, or Mann–Whitney *U*-test was used, as appropriate.

Correlation between two parameters was evaluated using a single regression model. $P < 0.05$ was considered significant. All statistical procedures were performed using StatView software (SAS Institute, Cary, NC, USA).

Results

Clinical characteristics of liver cirrhosis patients

Table 1 shows the demographic and clinical characteristics of the cirrhotic patients according to their etiology. Sex distribution and age at examination were significantly different among the HBV, HCV, and ALD subgroups. The LC patients with HBV and ALD were predominantly men, whereas men and women were nearly equally represented among the HCV-infected patients. The platelet counts were also significantly different among subgroups: patients with ALD had a higher platelet count than the HBV-infected subgroup. The other indices were not significantly different among subgroups. Hepatocellular carcinoma was present in 63% of the patients, although none of them was terminally advanced so that they did not have tumor-related coagulopathy, such as disseminated intravascular coagulation. Nearly all the patients had splenomegaly. Anti-GPIIb/IIIa antibody response was frequently detected in LC patients independent of the etiologies.

Thrombopoietin measurement

Figure 1(a) shows the plasma concentration of TPO in LC, ITP, and AA patients, and in healthy controls. The TPO level in LC patients (105.8 ± 70.6 pg/mL) was comparable to that in ITP patients (111.5 ± 73.3 pg/mL), and tended to be higher than in healthy controls (81.7 ± 18.3 pg/mL), but without a statistically significant difference. The plasma TPO concentration in AA patients (746 ± 485 pg/mL) was significantly higher than in the other three groups ($P < 0.05$ for all comparisons). When LC patients were subgrouped according to etiology, the ALD group showed the lowest TPO level, and the difference between the ALD and HBV groups was statistically significant (Fig. 1b; $P = 0.04$), perhaps because of higher platelet counts in the ALD group compared with the HBV group. The TPO levels were not different when LC patients were stratified based on Child's grade, or the presence or absence of hepatocellular carcinoma. There was no significant correlation between TPO levels and platelet counts in all LC patients combined, or in individual LC groups stratified according to etiology.

Platelet turnover studies

The %RP and GCI were used as indices for platelet turnover. Figure 2(a–c) shows representative fluorescence histograms of thiazole orange-stained platelets. The LC and ITP patients had an RP population highly stained with thiazole orange, which was not apparent in the healthy controls. As shown in Fig. 2(d), the %RP in 46 LC patients was $2.4 \pm 2.2\%$, which was significantly higher than that in healthy controls ($1.0 \pm 0.6\%$; $P = 0.0002$), and similar to that in ITP patients ($2.5 \pm 1.5\%$). There was no difference in %RP among LC patients subgrouped according to etiology.

Similarly, the GCI in LC patients was significantly higher than in healthy controls (5.2 ± 7.1 vs 1.2 ± 0.4; $P < 0.0001$) and in AA

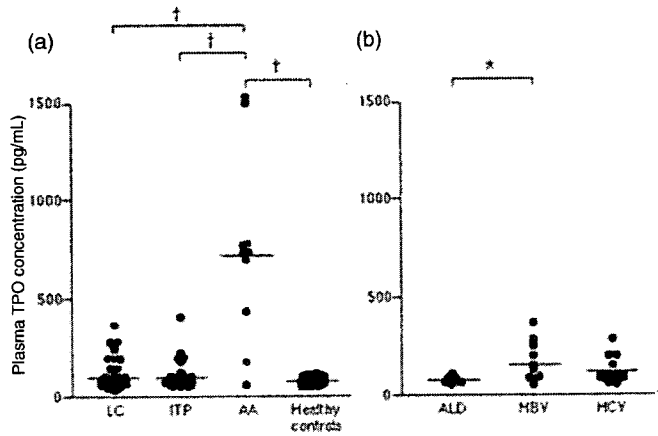

Figure 1 (a) Plasma thrombopoietin (TPO) concentration in liver cirrhosis (LC; $n = 40$), idiopathic thrombocytopenic purpura (ITP; $n = 32$), and aplastic anemia (AA; $n = 10$) patients, and healthy controls ($n = 29$) and (b) in LC patients according to their etiology (ALD, alcoholic liver disease, $n = 8$; HBV, hepatitis B virus, $n = 11$; HCV, hepatitis C virus, $n = 24$). (—), mean level. The TPO levels were compared between two groups using the Mann–Whitney *U*-test. *$P < 0.05$ and †$P < 0.005$.

Table 1 Characteristics of liver cirrhosis patients according to their etiology

	All subjects ($n = 57$)	HBV ($n = 15$)	HCV ($n = 34$)	ALD ($n = 8$)
Age at examination (years)* (mean ± SD)	63.0 ± 8.7	57.2 ± 8.8	66.3 ± 7.5	59.4 ± 6.9
Sex (% male)*	67	80	53	100
Child's grade (%)				
A	33	33	32	13
B	46	53	44	50
C	21	13	24	38
Hepatocellular carcinoma (%)	63	73	62	50
Splenomegaly (%)	95	90	96	100
Platelet count ($\times 10^9$/L)* (mean ± SD)	69 ± 39	59 ± 31	70 ± 33	84 ± 37
Anti-GPIIb/IIIa antibody response (%)	96	100	94	100

ALD, alcoholic liver disease; HBV, hepatitis B virus; HCV, hepatitis C virus.
*$P < 0.05$ among HBV, HCV, and ALD groups. The χ^2 or Kruskal–Wallis test was used as appropriate.

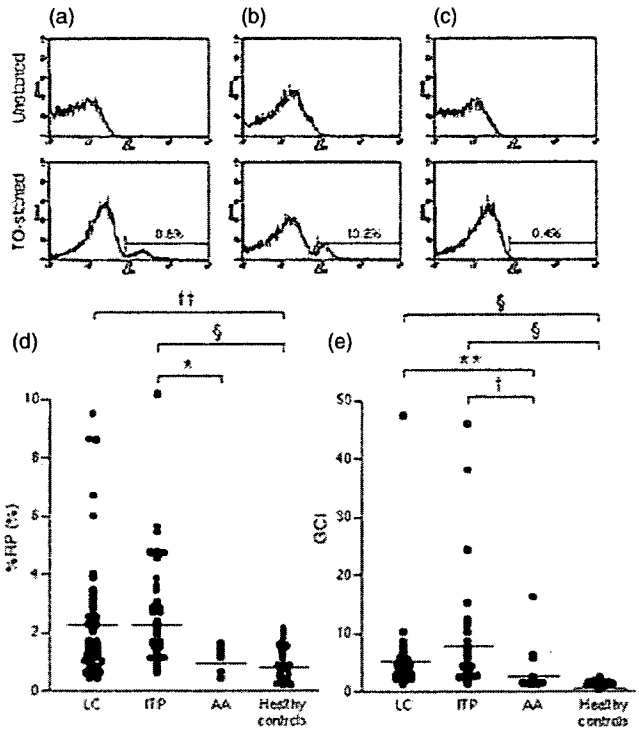

Figure 2 Evaluation of platelet turnover by proportion of reticulated platelets (RP) in total platelets (%RP) and glycocalicin index (GCI). Fluorescence histograms of thiazole orange (TO)-stained platelets in samples obtained from (a) a patient with liver cirrhosis (LC), (b) a patient with idiopathic thrombocytopenic purpura (ITP), and (c) a healthy control. Upper panels show unstained platelets used as a control. The subset of platelets with prominent TO staining was defined as RP, and its proportion (%RP) is shown in individual histograms. (d) The %RP in LC ($n = 45$), ITP ($n = 32$), and aplastic anemia (AA) patients ($n = 7$), and healthy controls ($n = 22$). (e) The GCI in LC ($n = 39$), ITP ($n = 33$), and AA patients ($n = 12$), and healthy controls ($n = 22$). (—), mean level. The %RP and GCI were compared between two groups using the Mann–Whitney *U*-test. $^*P < 0.05$, $^{**}P < 0.01$, $\dagger P < 0.005$, $\dagger\dagger P < 0.0005$, and $\S P < 0.0001$.

patients (3.3 ± 4.4; $P = 0.005$), but was comparable to the level in ITP patients (8.0 ± 10.1; (Fig. 2c). There was no significant difference in the GCI among LC patients subgrouped according to etiology.

Platelet production studies

The absolute RP count and plasma GC level were used as indices for platelet production. As shown in Fig. 3(a), LC patients had a significantly lower RP count than the healthy controls ($17.7 \pm 20.4 \times 10^9$/L vs $23.8 \pm 14.6 \times 10^9$/L; $P = 0.02$), although the value in the LC patients was higher than in the ITP patients ($9.4 \pm 6.5 \times 10^9$/L; $P = 0.03$). The AA patients had the lowest absolute RP count ($3.2 \pm 2.1 \times 10^9$/L), which was significantly lower than that of the LC patients, ITP patients, and healthy controls ($P = 0.0008$, 0.003, and 0.0002, respectively).

The plasma GC level in the LC patients was also significantly lower than in healthy controls (985 ± 284 ng/mL vs

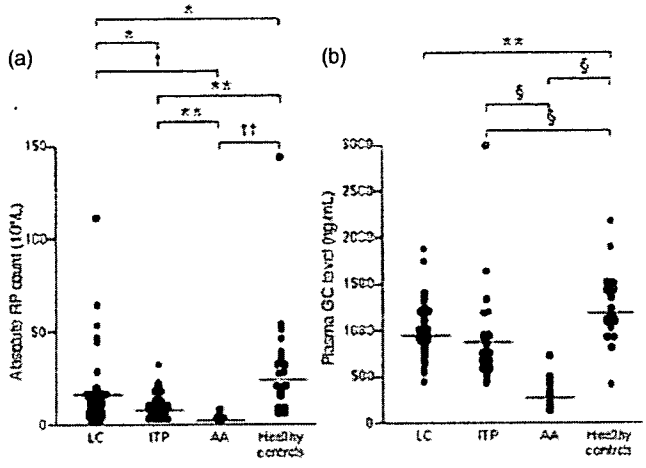

Figure 3 Evaluation of thrombopoiesis by the absolute reticulated platelet (RP) count and plasma glycocalicin (GC) level. (a) Absolute RP count in liver cirrhosis (LC; $n = 41$), idiopathic thrombocytopenic purpura (ITP; $n = 32$), and aplastic anemia (AA; $n = 7$) patients, and healthy controls ($n = 22$). (b) Plasma GC level in LC ($n = 41$), ITP ($n = 32$), and AA ($n = 13$) patients, and healthy controls ($n = 24$). (—), mean level. The absolute RP count and plasma GC concentration were compared between two groups using the Mann–Whitney *U*-test. $^*P < 0.05$, $^{**}P < 0.01$, $\dagger P < 0.005$, $\dagger\dagger P < 0.0005$, and $\S P < 0.0001$.

1225 ± 354 ng/mL; $P = 0.001$) and similar to that in the ITP patients (817 ± 482 ng/mL; Fig. 3b). The AA patients had the lowest plasma GC level (321 ± 161 ng/mL), which was significantly lower than the level in the LC patients, ITP patients, or healthy controls ($P < 0.0001$ for all comparisons).

There was no significant difference in the absolute RP count or plasma GC levels among the LC patients grouped according to etiology. In addition, in the LC patients there was no statistically significant correlation between the plasma TPO concentration and the absolute RP count or GC level.

Bone marrow evaluation

Megakaryocyte density was determined in bone marrow specimens from 10 patients with LC, 11 with ITP, and five with AA. The representative marrow histology results from three patients with LC show preserved megakaryocytes (Fig. 4a, all except the lower right), while the total number of nucleated cells, including megakaryocytes, was markedly decreased in a patient with AA (Fig. 4a lower right). Semiquantitative assessment of the megakaryocyte density (Fig. 4b) revealed no difference between the LC and ITP patients (1.6 ± 0.6 per field and 2.1 ± 1.2 per field, respectively), but their values were significantly greater than in the AA patient group (0.4 ± 0.3 per field; $P < 0.005$ for both comparisons).

Discussion

Recent evidence that inadequate TPO production is a contributory factor for thrombocytopenia has shed new light on the pathogenic process of LC.[7–9] However, this hypothesis is still controversial because, although some reports support it, others do not. For

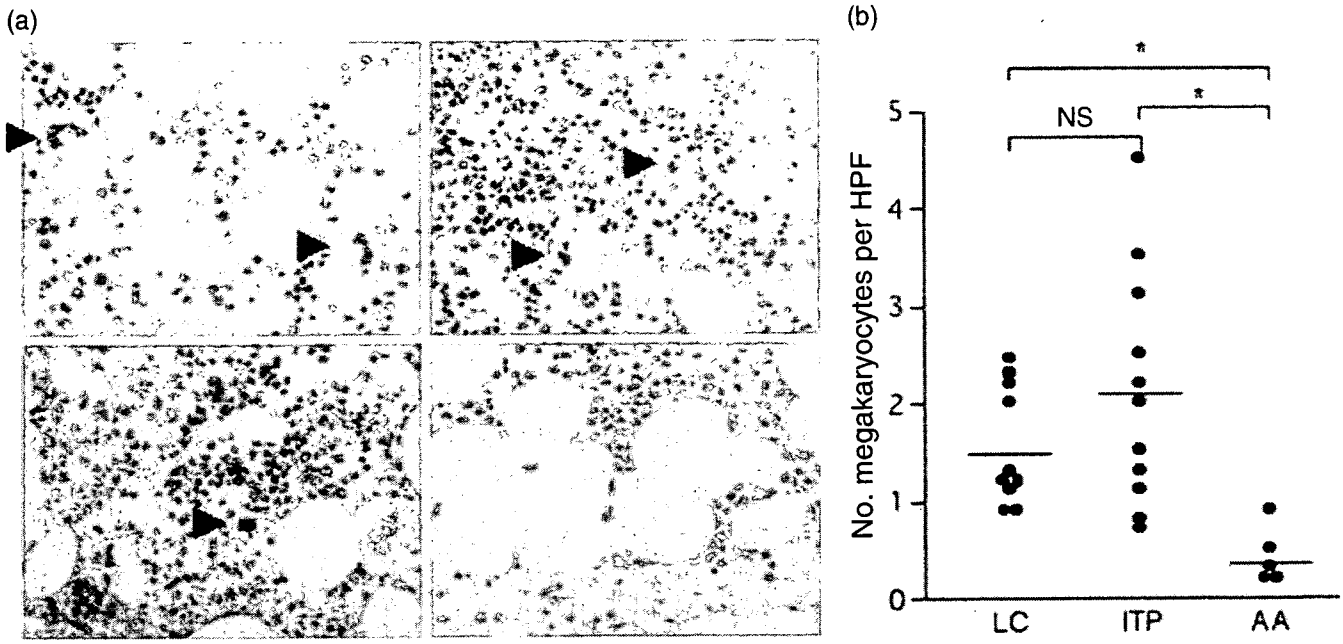

Figure 4 Evaluation of the bone marrow megakaryocyte density in liver cirrhosis (LC), idiopathic thrombocytopenic purpura (ITP), and aplastic anemia (AA) patients. (a) Representative images of hematoxylin–eosin-stained bone marrow sections obtained from LC patients (all except the lower right) and a patient with AA (lower right). Arrowheads indicate multinucleated megakaryocytes. Original magnification ×200. (b) Megakaryocyte density in LC ($n = 10$), ITP ($n = 11$), and AA ($n = 5$) patients. (—), mean level. HPF, high-power field. Results were compared between two groups using the Mann–Whitney *U*-test. $^*P < 0.005$.

example, the mRNA expression of TPO in the liver was shown to decrease during liver failure.[27] In contrast, several other studies found that the circulating TPO level is maintained or even increased in LC patients;[11–13] this observation is supported by the present study. In our series of LC patients, TPO level was not decreased in patients with advanced liver failure (Child's grade C) or was not increased in those with hepatocellular carcinoma. The circulating TPO level is known to be regulated by a 'sponge effect', meaning that it is controlled solely by binding to its receptor, rather than by the upregulation or downregulation of its production.[28,29] That is, TPO is produced constantly by the liver, kidney, and marrow stroma,[5,6] and its circulating level depends on the total amount of TPO receptor, which is mainly expressed on bone marrow megakaryocytes and their precursors. In megakaryocytic hypoplasia, such as occurs in AA, and also in amegakaryocytic thrombocytopenia, the observed increase in circulating TPO is explained by a decreased consumption of TPO, not by upregulated production. In contrast, lack of TPO signaling results in nearly complete loss of megakaryocytes, as shown in children with congenital amegakaryocytic thrombocytopenia, who have mutations in the *c-mpl* (TPO receptor) gene,[30] indicating that megakaryogenesis would be suppressed in the state of TPO deficiency. Therefore, the TPO production status cannot be adequately evaluated simply by measuring circulating TPO levels, as was done in many previous studies.[10–13]

In contrast, this is the first comprehensive study evaluating platelet kinetics and bone marrow megakaryocytes in combination with circulating TPO levels in LC patients. When compared with healthy controls, LC patients presented (i) normal or slightly increased plasma TPO; (ii) accelerated platelet turnover based on elevated %RP and GCI; and (iii) reduced platelet production based on decreased absolute RP count and plasma GC. These findings indicate that cirrhotic thrombocytopenia is a multifactorial condition involving both increased platelet clearance in the periphery and impaired thrombopoiesis. Surprisingly, these platelet kinetic features in LC patients were consistent with those in patients with ITP, a typical disease mediated by antiplatelet autoantibodies, but were apparently different from those in patients with AA, a disease characterized by impaired thrombopoiesis due to a defect in megakaryocytic stem cells and their progenitors. These findings were confirmed by the analysis of marrow megakaryocytes, whose density in LC patients was comparable to that in ITP patients, but higher than that in AA patients. In addition, these features were again not different among LC subgroups stratified according to etiology, although the number of patients in each group was relatively small. The lack of observed differences between LC subgroups suggests a primary role of the pathogenic process shared by all LC patients, rather than etiology-specific mechanisms, in the development of thrombocytopenia.

Liver cirrhosis patients had markedly enhanced platelet turnover, suggesting that cirrhotic thrombocytopenia cannot be explained by impaired thrombopoiesis due to TPO insufficiency alone. In addition, the accelerated platelet turnover in LC patients indicates a primary role of platelet clearance in the periphery, which is likely mediated through hypersplenism[1–3] and/or antiplatelet autoantibody-mediated platelet destruction.[4]

Thrombopoiesis as evaluated by absolute RP count and plasma GC level was suppressed in LC patients compared with healthy subjects; the degree of suppression was comparable to that of ITP patients but was much less prominent than that of AA patients. It

is unlikely that the mild thrombopoiesis impairment in LC patients is solely due to TPO deficiency, because the bone marrow evaluation showed that megakaryogenesis was preserved in LC patients, at least to the same extent as in patients with ITP, in which TPO production is believed to be unaffected. Furthermore, the circulating TPO level in LC patients was not correlated with the absolute RP count or plasma GC level, which reflects the platelet production rate, suggesting that thrombopoiesis is not simply controlled by TPO level in LC patients. In contrast, thrombopoiesis is known to be slightly reduced in ITP patients.[31–33] A recent study by McMillan *et al.* showed that anti-GPIIb/IIIa autoantibodies derived from ITP patients suppressed the production and maturation of megakaryocytes *in vitro*,[34] suggesting that the thrombocytopenia in ITP patients may result not only from Fcγ receptor-mediated platelet clearance in the reticuloendothelial system, but also, at least to some extent, from the suppression of megakaryogenesis. A potential mechanism for such an autoantibody-mediated process involves complement-dependent cytotoxicity to megakaryocytes in the marrow.[35] Liver cirrhosis patients frequently had the anti-GPIIb/IIIa autoantibody response as well,[4] suggesting that LC patients may undergo autoantibody-mediated suppression of megakaryogenesis.

In summary, cirrhotic thrombocytopenia is a multifactorial condition, but accelerated platelet clearance in the periphery, rather than impaired thrombopoiesis due to TPO insufficiency, plays the primary role in the pathogenic process.

Acknowledgments

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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 λ 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 *Hin*dIII-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 800*g* 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 μg/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 $3.0 \times 10^5/\mu L$ 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 μM; Mitsubishi Chemical Corporation, Tokyo, Japan). The fluorescent dye mepacrine (10 μM; 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-μm z-axis intervals within a defined area (156.4 × 119.6 μm) 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 χ^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.

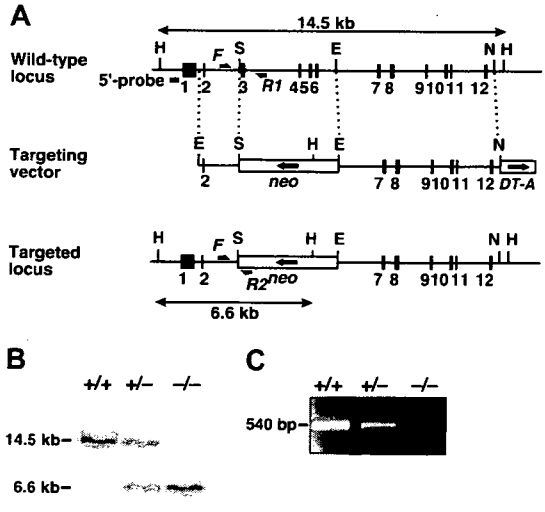

Figure 1. Targeted disruption of the mouse *Adamts13* gene. (A) Structure of the targeted locus in the mouse *Adamts13* gene. Exons are represented by filled boxes. A neomycin-resistance cassette (neo), in the opposite transcriptional orientation, and a forward-oriented diphtheria toxin A fragment expression cassette (DT-A) are indicated. Homologous fragments are indicated by dotted lines; the *Hin*dIII 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 *Hin*dIII; S, *Sal*I; E, *Eco*RI; N, *Nco*I. (B) Southern blot analysis. gDNA from offspring obtained from heterozygous intercrosses was digested with *Hin*dIII 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.

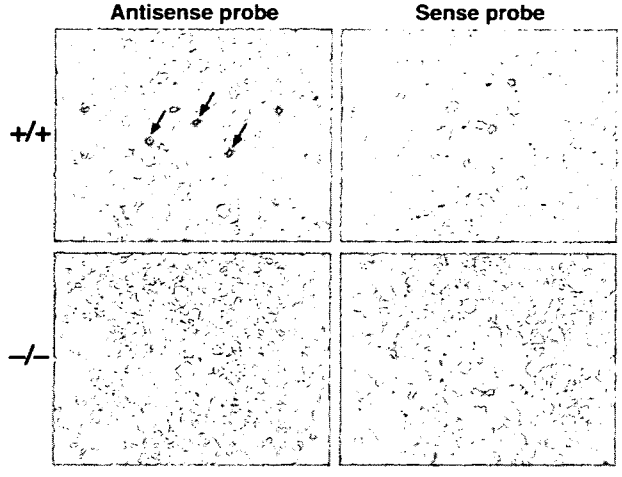

Figure 2. In situ hybridization analysis of ADAMTS13 mRNA. Liver sections from *Adamts13*$^{+/+}$ (top panels) and *Adamts13*$^{-/-}$ (bottom panels) mice were hybridized to the antisense (left panels) or sense (right panels) *Adamts13* RNA probes. The hybridized sections were counterstained with Kernechtrot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

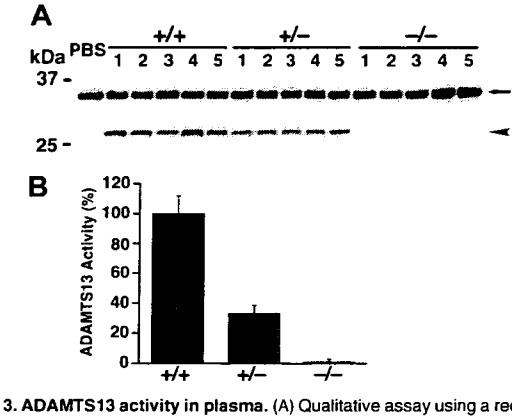

Figure 3. ADAMTS13 activity in plasma. (A) Qualitative assay using a recombinant substrate, GST-mVWF73-H. The substrate and product bands are indicated by arrows and arrowheads, respectively. (B) Quantitative assay using a fluorogenic substrate, FRETS-VWF73. Data are mean ± SD from 4 mice for each genotype.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

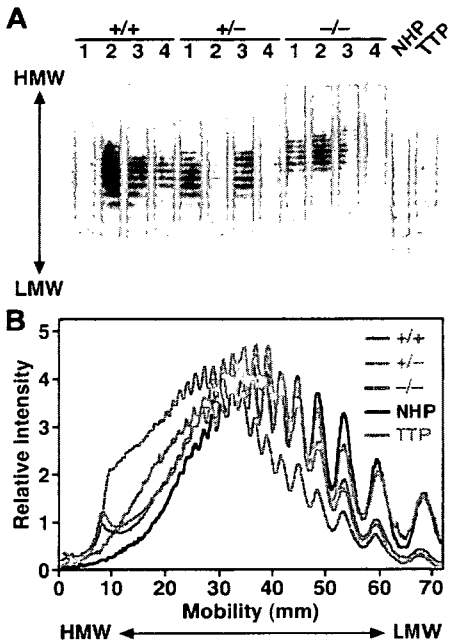

Figure 4. Analysis of plasma VWF multimers. (A) VWF multimer patterns. Plasma samples (1 μL/lane) from *Adamts13*$^{+/+}$, *Adamts13*$^{+/-}$, and *Adamts13*$^{-/-}$ mice were electrophoresed on SDS-agarose gels and transferred to nitrocellulose membranes. VWF multimers were detected with anti-VWF antibodies. Normal human plasma (NHP) and ADAMTS13-deficient TTP patient plasma (TTP) were analyzed in parallel (0.2 μL/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, μL	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.

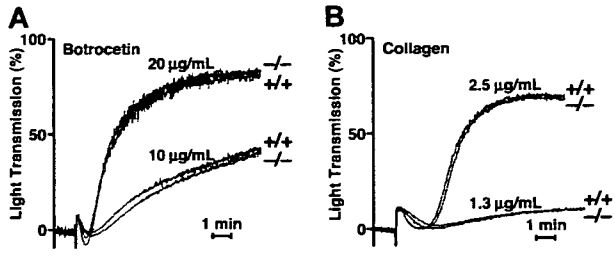

Figure 5. Platelet aggregation under static condition. (A) Botrocetin-induced aggregation. Pooled PRP samples from *Adamts13*$^{+/+}$ or *Adamts13*$^{-/-}$ mice were treated with botrocetin at a final concentration of 10 or 20 μg/mL. Aggregation was measured using an aggregometer at 37°C with constant 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 μg/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 χ^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

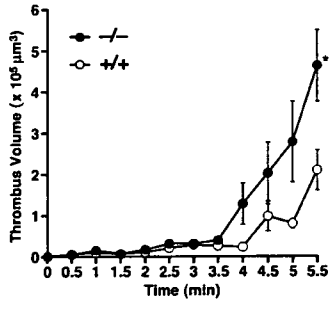

Figure 6. Thrombogenesis on collagen surface under flow. Whole blood from *Adamts13*$^{+/+}$ or *Adamts13*$^{-/-}$ mice containing mepacrine-labeled platelets was perfused over an acid-insoluble type I collagen-coated surface at a wall shear rate of 750 s^{-1}. The cumulative thrombus volume, analyzed 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.

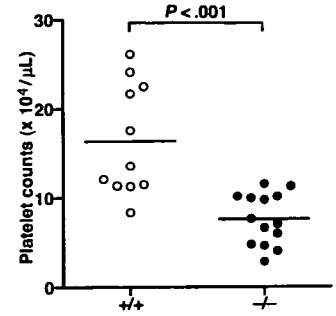

Figure 7. Platelet counts following collagen plus epinephrine challenge. Mice were given injections of 600 ng/g collagen plus 60 ng/g epinephrine via tail vein and platelet counts were measured 15 minutes after injection. Symbols represent platelet counts of a single mouse. Bars represent the mean values 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/μL and 16.4 ± 6.2 × 10^4/μL, respectively (mean ± SD; $P < .001$), whereas platelet counts without challenge were not different between groups (*Adamts13*$^{-/-}$, 86.2 ± 13.2 × 10^4/μL; *Adamts13*$^{+/+}$, 83.7 ± 3.3 × 10^4/μL; 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.

Acknowledgments

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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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ORIGINAL ARTICLE

Plasma protein S activity correlates with protein S genotype but is not sensitive to identify K196E mutant carriers

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Summary. *Background:* Protein S (PS) is an anticoagulant protein that functions as a cofactor for activated protein C (APC), and congenital PS deficiency is a well-known risk factor for the development of deep vein thrombosis (DVT). Recently, we and others identified the K196E missense mutation in the second epidermal growth factor-like domain of PS as a genetic risk factor for DVT in the Japanese population. The incidence of this mutation is high in the Japanese population. *Objectives:* In the present study, we investigated the relationship between plasma PS activity and the presence of the K196E mutation. *Patients and methods:* We measured PS activity as a cofactor activity for APC in 1862 Japanese individuals and determined the PS K196E genotype in this population. *Results:* Individuals heterozygous for the mutant E-allele had lower plasma PS activity than wildtype subjects (mean ± SD, 71.9 ± 17.6%, $n = 34$ vs. 87.9 ± 19.8%, $n = 1828$, $P < 0.0001$). However, the PS activity of several heterozygous individuals ($n = 8$) was greater than the population average. In contrast, multiple wildtype subjects ($n = 26$) had PS activity less than 2 SD below the population mean, indicating that other genetic or environmental factors affect PS activity. *Conclusions:* Plasma PS activity itself is not suitable for identifying PS 196E carriers and other methods are required for carrier detection.

Keywords: deep vein thrombosis, missense mutation, protein S.

Introduction

Protein S (PS) is an important regulator of coagulation that serves as a cofactor for activated protein C (APC), the anticoagulant protease that proteolytically degrades activated factor (F) V and FVIII [1]. Individuals with homozygous or compound heterozygous deficiency for PS develop disseminated thrombosis after birth, and heterozygosity for PS deficiency increases the risk of deep vein thrombosis (DVT) [2,3].

Recently, we and others identified that a PS missense mutation prevalent in the Japanese population, which causes Lys196 to be replaced by Glu (K196E mutation, formerly known as PS Tokushima, and referred to as K155E mutation), is a genetic risk factor for the development of DVT [4,5]. This mutation lies within the second epidermal growth factor-like domain of PS, and, *in vitro*, K196E mutant PS has decreased APC cofactor activity and poorly accelerates prothrombinase inactivation [6–8]. This missense mutation was originally identified in Japanese patients with PS deficiency suffering from DVT [9,10]. However, the plasma PS activity in individuals with this mutation remained controversial. In one report, PS activity was decreased in carriers of the K196E mutation with normal PS levels [9]. In contrast, another study found PS activity within the normal range in affected individuals [10].

We identified 66 heterozygotes and no homozygotes for the mutant PS 196E-allele from a population of 3651 individuals [5]. Therefore, the frequency of the mutant E-allele in the Japanese population was about 0.009. Extrapolating from these values, we estimated that approximately one out of every 55 Japanese individuals is heterozygous for the E-allele [11]. Thus, a substantial number of Japanese carry the E-allele for PS and are at increased risk for the development of DVT. Given the relatively high frequency of this mutation and its strong correlation with DVT, it may be advisable to screen individuals for the presence of this mutation so that carriers can avoid additional environmental risk factors associated with DVT. An appropriate screening test is lacking, however, and we hypothesized that plasma PS activity levels may directly correlate with PS genotype. If this were the case, genetic testing

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