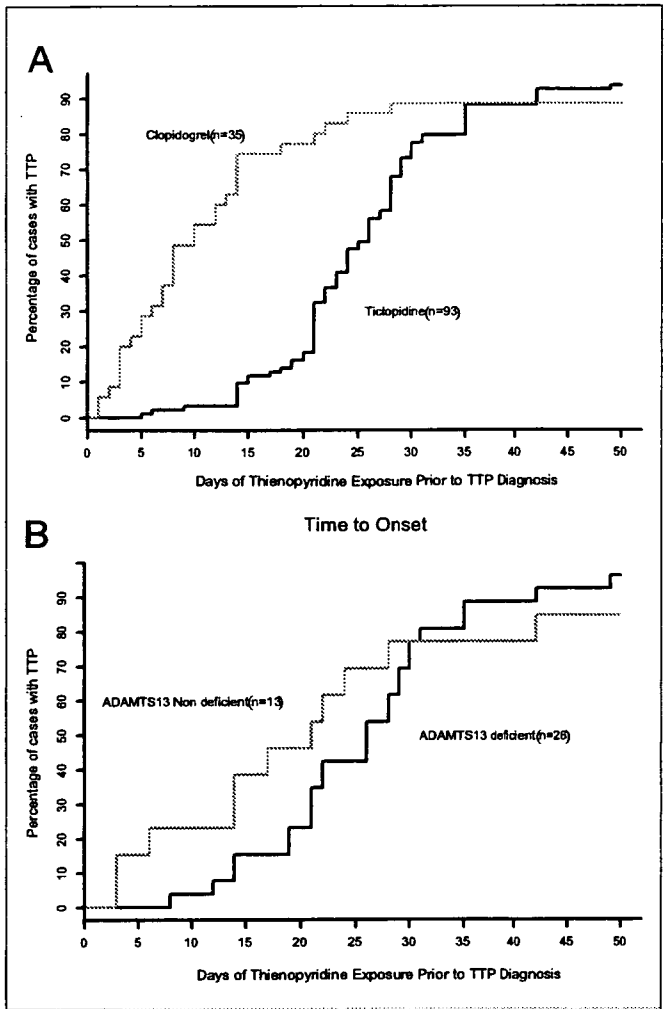

Figure 1 **Duration of Thienopyridine Exposure Prior to TTP Onset**

(**A**) Thienopyridine-associated thrombotic thrombocytopenic purpura (TTP) onset: ticlopidine versus clopidogrel ($p = 0.0016$). (**B**) Thienopyridine-associated TTP onset: ADAMTS13 deficient versus ADAMTS13 nondeficient ($p > 0.05$).

with TPE and 78% without. A multivariate classification tree analysis model revealed that among thienopyridine-associated TTP patients who received TPE, those patients with ADAMTS13 activity levels >15% at the time of diagnosis of TTP were 4-fold more likely to die (41.9% vs. 9.1%, $p < 0.036$).

Discussion

Our study identifies distinct clinical, laboratory, and outcome differences between ticlopidine- and clopidogrel-associated TTP. More than 90% of the ticlopidine-associated TTP cases develop after more than 2 weeks of thienopyridine use. Among these patients, severe thrombocytopenia and preserved renal function at diagnosis is common, ADAMTS13 activity levels are frequently <15%, and survival is 86% if TPE is administered versus 46% if TPE is not used. These findings are similar to those reported previously for idiopathic TTP cases with severely deficient ADAMTS13 activity levels (16,23,25). In contrast, three-quarters of the clopidogrel-associated TTP cases develop after 2 weeks or less of thienopyridine use. These patients are characterized by mild thrombocytopenia and renal insufficiency at diagnosis, ADAMTS13 activity levels >15%, and survival rates that are similar with versus without TPE (72.4% and 66.7%), findings that are similar to those reported previously for TTP cases with ADAMTS13 activity levels >25%. Our findings suggest 2 mechanistic pathways for thienopyridine-associated TTP, an immunologic pathway associated with more than 2 weeks of thienopyridine use and a nonimmunologic pathway associated with 2 weeks or less of thienopyridine use. In interpreting our study, several factors should be considered.

The results for patients with severe ADAMTS13 deficiency and thienopyridine-associated TTP reinforce previous observations for patients with ticlopidine-associated TTP. Tsai et al. (12) reported 7 ticlopidine-associated TTP patients who had severe ADAMTS13 deficiency and inhibitors to ADAMTS13 at diagnosis, all of whom responded rapidly to TPE. The use of TPE in these patients may result in removal of ADAMTS13 inhibitors and ultra-large VWF multimers, replenishment of ADAMTS13 and VWF, and reduction of cytokines that induce endothelial cell damage and platelet activation (26). Our study also describes cases of thienopyridine-associated TTP cases who do not have severe ADAMTS13 deficiency and whose survival was not influenced by TPE. Preservation of ADAMTS13 activity has been described in patients with post-transplantation thrombotic microangiopathy (27,28) who frequently present with renal insufficiency, moderate thrombocytopenia, and high mortality rates despite TPE. Others have described TTP-like findings among persons with factor V Leiden mutation (29).

Our study has implications for patient safety. First, for the rare individual with a drug-eluting coronary artery stent who develops TTP after the administration of clopidogrel and for whom discontinuation of thienopyridine-therapy could be catastrophic, ticlopidine challenge can be consid-

Table 2 **Outcomes for Ticlopidine- and Clopidogrel-Associated TTP Cases**

	Survival With TPE, %	Survival Without TPE, %
All patients (n = 128)*	81.6	50.0
Ticlopidine (N = 93) *	85.5	45.8
Ticlopidine Rx ≤14 days (n = 9)	100.0	100.0
Ticlopidine Rx >14 days (n = 84)	84.1*	38.1*
Clopidogrel (N = 35)	72.4	66.7
Clopidogrel Rx ≤14 days (n = 26)	70.0	66.7
Clopidogrel Rx >14 days (n = 9)	77.8	—
Thienopyridine Rx ≤14 days (n = 35)	76.9	77.8
Thienopyridine Rx >14 days (n = 93)	83.3*	38.1*

*$p < 0.05$ (for comparison of survival with TPE vs. without TPE).
Abbreviations as in Table 1.

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ered. For most patients with clopidogrel-associated TTP, our findings suggest that the toxicity is unlikely to be immunologic in etiology. Patel et al. (30) recently described a case report of a patient with a history of clopidogrel-associated TTP who successfully received ticlopidine therapy following implantation of a drug eluting coronary artery stent. Two years had elapsed between the development of clopidogrel-associated TTP and ticlopidine initiation. Second, the RADAR program has developed new approaches to drug safety that build on close collaborations with referral centers that have developed novel assays (13). We identified a large part of our cohort by querying hematologists or medical directors of TPE centers who were collaborating in a prospective case-control epidemiologic study or who sent plasma samples for possible TTP cases to a referral center for measurement of ADAMTS13 activity. Similar collaborations with a referral center that developed novel assays for detecting antierythropoietin-associated antibodies facilitated the identification of another drug-associated toxicity, erythropoietin-associated pure red cell aplasia (31).

Study limitations. The limitations of our study should be identified. First, thienopyridine-associated TTP is undoubtedly a rare diagnosis, limiting our ability to obtain plasma from large numbers of patients. Second, although clinical information on most of the cases reported herein have been reported previously, these studies did not directly compare TTP cases according to drug (ticlopidine vs. clopidogrel) or the duration of thienopyridine administration (4,5). Also, previous studies included information on ADAMTS13 activity levels and ADAMTS13 inhibitors for only 10 patients with thienopyrindine-associated TTP. Third, the demographic characteristics of the TTP patients in this study differ from those reported in case series of TTP patients. In particular, in comparison with thienopyridine-associated TTP patients, patients in the study of Vesely et al. (24) were younger (mean 35 to 50 vs. 60 to 65 years) and more likely to be female (80% vs. 45%) (16,17,25) and, therefore, there continues to be uncertainty about causal mechanisms for clopidogrel, primarily because clopidogrel-associated TTP occurs markedly less often than ticlopidine-associated TTP (32–35).

Conclusions

Thrombotic thrombocytopenic purpura is a rare complication of thienopyridine treatment. This drug toxicity appears to occur by 2 different mechanistic pathways, characterized primarily by time of onset of $>$ versus <2 weeks of thienopyridine administration. If TTP occurs after 2 weeks of ticlopidine or clopidogrel therapy, TPE must be promptly instituted to enhance the likelihood of survival.

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ORIGINAL ARTICLE

Prophylactic fresh frozen plasma may prevent development of hepatic VOD after stem cell transplantation via ADAMTS13-mediated restoration of von Willebrand factor plasma levels

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We initially conducted a multicenter, randomized trial ($n = 43$), and subsequently a questionnaire study ($n = 209$) of participating hospitals, to evaluate whether infused fresh frozen plasma (FFP) could prevent the occurrence of hepatic veno-occlusive disease (VOD) after stem cell transplantation (SCT). Forty-three patients were divided into two groups: 23 receiving FFP infusions and 20 not receiving it. VOD developed in three patients not receiving FFP. Plasma von Willebrand factor (VWF) antigen levels were lower at days 0, 7 and 28 after SCT in patients receiving FFP than in those not receiving it, whereas plasma ADAMTS13 activity (ADAMTS13:AC) did not differ between them. Plasma VWF multimer (VWFM) was demonstrated to be defective in the high ~ intermediate VWFM during the early post-SCT phase, but there was a significant increase in high VWFM just before VOD onset. This suggests that a relative enzyme-to-substrate (ADAMTS13/high-VWFM) imbalance is involved in the pathogenesis of VOD. To strengthen this hypothesis, the incidence of VOD was apparently lower in patients receiving FFP infusions than in those not receiving it (0/23 vs 3/20) in the randomized trial. Further, the results combined with the subsequent questionnaire study (0/36 vs 11/173) clearly showed the incidence to be statistically significant (0/59 vs 14/193, $P = 0.033$).

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Introduction

Hepatic veno-occlusive disease (VOD) is a life-threatening complication that develops in 1–54% of patients undergoing allogenic stem cell transplantation (SCT).[1–6] Hepatic VOD is clinically characterized by hyperbilirubinemia, painful hepatomegaly and fluid retention.[2,3] Histologically, hepatic VOD is marked by fibrosis of sinusoids, necrosis of pericentral hepatocytes and narrowing of central veins with eventual fibrosis.[7] Recent work suggests that the primary site of toxic injury caused by chemotherapy and/or radiation before SCT is the sinusoidal endothelial cells and that this initial insult is followed by a series of biologic processes that ultimately leads to circulatory compromise of centrilobular hepatocytes, fibrosis and obstruction of liver blood flow.[7] However, the precise pathogenesis of hepatic VOD has yet to be clarified.

von Willebrand factor (VWF) is synthesized in vascular endothelial cells and released into the plasma as 'unusually large' VWF multimers (UL-VWFM), which actively interact with platelets.[8,9] In the normal circulation, UL-VWFM are rapidly degraded into smaller VWFM by ADAMTS13 (a disintegrin-like metalloproteinase with thrombospondin type-1 motifs 13),[10,11] which cleaves the Tyr842–Met843 bond within the VWF A2 domain.[12,13] Deficiency of ADAMTS13 caused either by mutations of the *ADAMTS13* gene[10] or by inhibitory autoantibodies against

ADAMTS13[14,15] increases plasma levels of UL-VWFM, which leads to platelet clumping and/or thrombi under high shear stress, resulting in thrombotic thrombocytopenic purpura (TTP).[16]

We previously demonstrated that plasma ADAMTS13 activity (ADAMTS13:AC) is reduced in hepatic VOD patients after SCT compared to non-VOD patients, even before conditioning therapy,[17] and that its activity could, therefore, be a predictor for the development of hepatic VOD. Additionally, ADAMTS13:AC is significantly low in patients with advanced liver cirrhosis[18] and those with alcoholic hepatitis.[19] Furthermore, we have demonstrated a rapid decrease in ADAMTS13:AC in association with early adverse events including ischemia–reperfusion injury and/or acute graft rejection in living donor-related liver transplantation.[20] Our most recent study demonstrates that ADAMTS13 is produced exclusively in hepatic stellate cells (HSCs) located in the space of Disse adjacent to endothelial cells.[21] Considering that hepatic sinusoidal endothelial cell injury is an important causative factor in the development of hepatic VOD[1] and that ADAMTS13:AC is reduced in hepatic VOD,[17] it is of particular interest to evaluate the effect of fresh frozen plasma (FFP) administration as a supplemental source of ADAMTS13 in patients at high risk for post-SCT hepatic VOD.

In this study, we performed a multicenter, prospective, randomized controlled trial, and a subsequent study of participating hospitals by questionnaires to evaluate whether or not the infusion of FFP could prevent the occurrence of hepatic VOD in high-risk patients who had undergone SCT. Towards elucidating the mechanism that underlies the development of VOD, we sequentially determined plasma levels of ADAMTS13:AC, and its substrates, VWF antigen (VWF:AG) and UL-VWFM in a randomized controlled trial.

Materials and methods

Study design

The study on prevention of hepatic VOD associated with SCT was conducted at 10 hospitals in Osaka, Hyogo and Nara prefecture. The study was performed between April 2001 and March 2003, and was a multicenter, randomized and controlled study. A subsequent study was carried out between January 2004 and December 2006 by questionnaires sent to participating hospitals. With respect to the randomized controlled trial, patients were eligible to participate in this prospective study if they were at high risk for developing VOD after allogenic SCT and fulfilled one of the following criteria: (1) intensified conditioning regimen, (2) second SCT, (3) liver dysfunction and (4) receiving intensified chemotherapy until just before SCT because of the poor situation of the underlying disease. Physicians from the referring hospitals registered patients to our registration office by facsimile, with patient information including age, gender and underlying disease. Patients were divided into a child group (18-year-old or under) and an adult group (over 18-year-old). Patients in each group were then randomly divided into two groups: patients to receive FFP infusion (FFP (+) group) and those not to receive it (FFP (−) group). FFP was infused twice a week during the conditioning regimen and until day 28 after SCT in patients in the FFP (+) group. The volume of FFP infused was based upon body weight and determined as follows: 1 unit (=80 ml) for patients under 10 kg, 2 units for 10–20 kg, 3 units for 20–30 kg, 4 units for 30–40 kg and 5 units for over 40 kg. The amount of FFP infused on this protocol was calculated to contribute about a 10% increase in plasma ADAMTS13:AC, which was enough to eliminate UL-VWFM in patients with congenital TTP, termed Upshaw–Schulman syndrome.[22,23] In our previous study, plasma ADAMTS13:AC was about 20% of the normal control level between pre-conditioning and day 21 in patients with hepatic VOD.[17] We therefore attempted to maintain plasma ADAMTS13:AC at the level of 30% of normal control throughout the study, by FFP infusion. As the half-life of plasma ADAMTS13:AC has been reported to be approximately 3 days,[24] we decided to infuse FFP twice weekly.

A diagnosis of hepatic VOD was made according to the criteria of McDonald *et al.*;[2] VOD patients needed to have at least two of the following clinical features within day 30 after transplantation: (1) jaundice, (2) hepatomegaly and right upper quadrant pain and (3) ascites and/or unexplained body weight gain. Citrated plasma was obtained from patients before the conditioning regimen (pre-SCT) and on days 0, 7, 14, 21 and 28 (post-SCT), and was stored in aliquots at −80°C until use. ADAMTS13:AC, VWF:AG, and UL-VWFM levels were determined using these plasma samples. ADAMTS13 inhibitor was analyzed using plasma from day 28. Other laboratory parameters, including thrombin–antithrombin complex (TAT), D-dimer, thrombomodulin (TM), tissue plasminogen activator/plasminogen activator inhibitor-1 (t-PA/PAI-1) complex and antithrombin (AT), were measured at pre-SCT and on days 0, 7, 14, 21 and 28. The study protocol was approved by the ethics committee of each participating hospital. All patients gave written informed consent before registration in the study.

The subsequent questionnaire was sent to 10 participating hospitals asking whether or not prophylactic FFP infusion to prevent hepatic VOD had been given in high-risk patients who had undergone SCT, and requesting information on the incidence of hepatic VOD in these patients with and without FFP infusion.

Measurements

ADAMTS13:AC was assayed using a highly sensitive, novel enzyme-linked immunsorbent assay (ELISA), recently developed by our laboratory.[25] The normal level of ADAMTS13:AC using this assay in 55 healthy individuals was 99.1±21.5% (mean±s.d.).[25] The ADAMTS13 inhibitor was evaluated using heat-inactivated plasma at 56°C for 30 min.[14,15] One Bethesda unit of inhibitor was defined as the amount of plasma that reduces ADAMTS13:AC to 50% of the control,[26] and its titer was estimated to be significant in more than 0.5 Bethesda U/ml. The UL-VWFM was evaluated by SDS–0.9% agarose electrophoresis followed by western blotting with luminographic detection.[27,28] Multimers were defined as low molecular

weight (corresponding to bands 1–5), intermediate molecular weight (bands 6–10) and high molecular weight (bands >10).[29] Furthermore, the bands corresponding to higher molecular weight, which could never be detected in pooled normal plasma, were defined as UL-VWFM. VWF:AG was measured by a sandwich ELISA using a rabbit anti-human VWF polyclonal antibody (DakoCytomation, Kyoto, Japan). The value obtained from normal individuals ($n=20$, 13 males and 7 females aged 20–40 years) was $102\pm33\%$.[9]

Statistical analysis

All experimental data are presented as means±s.d. Paired and unpaired comparisons between the two groups were performed using the Student's *t*-test and Fisher exact test. A two-tailed *P*-value of less than 0.05 was considered statistically significant. Analyses were carried out using the statistical software Statview (version 5.0, SAS Institute, Cary, NC, USA).

Results

Randomized control study

Patient characteristics. Of 47 patients enrolled, 15 patients belonged to the child group and 32 belonged to the adult group. Of these, a patient belonging to the FFP (+) group and three patients belonging to the FFP (−) group were excluded from this protocol, because they could not undergo SCT because of poor physical condition. Finally, 43 patients consisting of 15 in the child group and 28 in the adult group were investigated. Twenty-three patients were assigned to the FFP (+) group and the remaining 20 patients to the FFP (−) group. There were no statistically significant differences between the two groups with regard to clinical features and laboratory findings. The clinical characteristics and transplant procedures in patients finally enrolled are shown in Table 1.

Clinical features of VOD patients with respect to VWF:AG, ADAMTS13:AC and VWF multimers. Case no. 8 with hepatic VOD was a 53-year-old female diagnosed with leukemic transformation of myelodysplastic syndrome who received a mini-transplant from a human leukocyte antigen (HLA)-matched sibling while she had active disease (Table 1). At day 14 after SCT, this patient developed hyperbilirubinemia (5.1 mg/dl), weight gain and right upper quadrant pain, and was thus diagnosed as having hepatic VOD (Figure 1a). She was treated with steroids from day 14 and FFP since day 28, and she completely recovered at day 40 after SCT. In this case, VWF multimers corresponding to high and intermediate molecular weight, which are usually seen in normal plasma, were lacking pre-SCT before conditioning (Figure 1b). VWF multimers gradually appeared from day 0 to day 7 after SCT. VWF:AG increased from 64% (pre-SCT) to 396% (day 7), and ADAMTS13:AC decreased from 67% (pre-SCT) to 30% (day 7), resulting in an increasing ratio of VWF:AG to ADAMTS13:AC from 1.0 (pre-SCT) to 13.0 (day 7). A week later, VWF:AG decreased to 171%, but ADAMTS13:AC further decreased to 24%, resulting in ratios of VWF:AG to ADAMTS13:AC as high as 7.1 at day 14, when hepatic VOD developed (Figures 1a and b). Thereafter, ADAMTS13:AC increased and VWF:AG remained relatively unchanged. This patient completely recovered by day 40, after the occurrence of hepatic VOD.

Case no. 12 was a 53-year-old male who received a bone marrow transplant from an HLA-matched unrelated donor for refractory acute lymphocytic leukemia (Table 1). At day 28 after SCT, this patient exhibited mild jaundice (1.3 mg/dl), weight gain and painful hepatomegaly, and was then diagnosed as having hepatic VOD (Figure 1c). He was treated with FFP infusions from day 35, but unfortunately died of hepatic failure due to VOD on day 40 after SCT. In this case, VWF multimer patterns were similar to those in case no. 8: VWF multimers of high and intermediate molecular weight were lacking at day 0 (just after SCT), but gradually appeared between days 7 and 21 (Figure 1d). VWF:AG gradually increased from 101% (day 0) to 205% (day 21), and ADAMTS13:AC gradually decreased from 51% (day 0) to 43% (day 21), resulting in increasingly high ratios of 2.0 (day 0) to 4.8 (day 21). A week later, VWF:AG further increased and reached 234%, and ADAMTS13:AC decreased to 32%, resulting in a high ratio of VWF:AG to ADAMTS13:AC of 7.3 at day 28, when hepatic VOD developed (Figures 1c and d).

Case no. 14 was a 41-year-old female with active acute myelocytic leukemia who underwent BMT from an HLA-matched unrelated donor. Soon after SCT, she exhibited weight gain and painful hepatomegaly, and was diagnosed as having hepatic VOD on day 7. This patient was therefore dropped from the study and treated with FFP infusions. She completely recovered from hepatic VOD at day 42 after SCT, and was categorized as a VOD case in the FFP (−) group.

Comparison of plasma VWF:AG, ADAMTS13:AC and VWF multimers between patients receiving and not receiving FFP infusions. We next evaluated the effect of FFP on the clinical parameters of ADAMTS13:AC and VWF:AG in patients receiving and not receiving FFP infusions. There were no differences in TAT, D-dimer, TM, PAI-I and AT III between FFP (−) and (+) groups (Table 2). VWF:AG gradually increased over the time period pre-SCT to day 28 post-SCT in patients belonging to the FFP (−) group (Figure 2). In contrast, in patients belonging to the FFP (+) group, the VWF:AG did not increase at days 0 and 7, but gradually increased thereafter between days 14 and 21, and later decreased at day 28. There was a significant difference in VWF:AG at days 0, 7 and 28 between the groups receiving and not receiving FFP (day 0: 96 ± 39 vs 147 ± 78, $P<0.05$; day 7: 103 ± 62 vs 156 ± 83, $P<0.05$; and day 28: 146 ± 82 vs 212 ± 81, $P<0.05$) (Figure 2). On the other hand, ADAMTS13:AC gradually decreased from pre-SCT to day 28 in both groups. No difference in the levels of ADAMTS13:AC was observed between the groups. The ratio of VWF:AG to ADAMTS13:AC gradually increased from pre-SCT to day 14 in patients in the FFP (−) group, but in patients in the FFP (+) group, this ratio did not increase at days 0 and 7 but gradually increased later at day 14 (Figure 2). Plasma inhibitor of ADAMTS13 at day 28 was detected only in one patient

Table 1 Clinical characteristics of patients with SCT

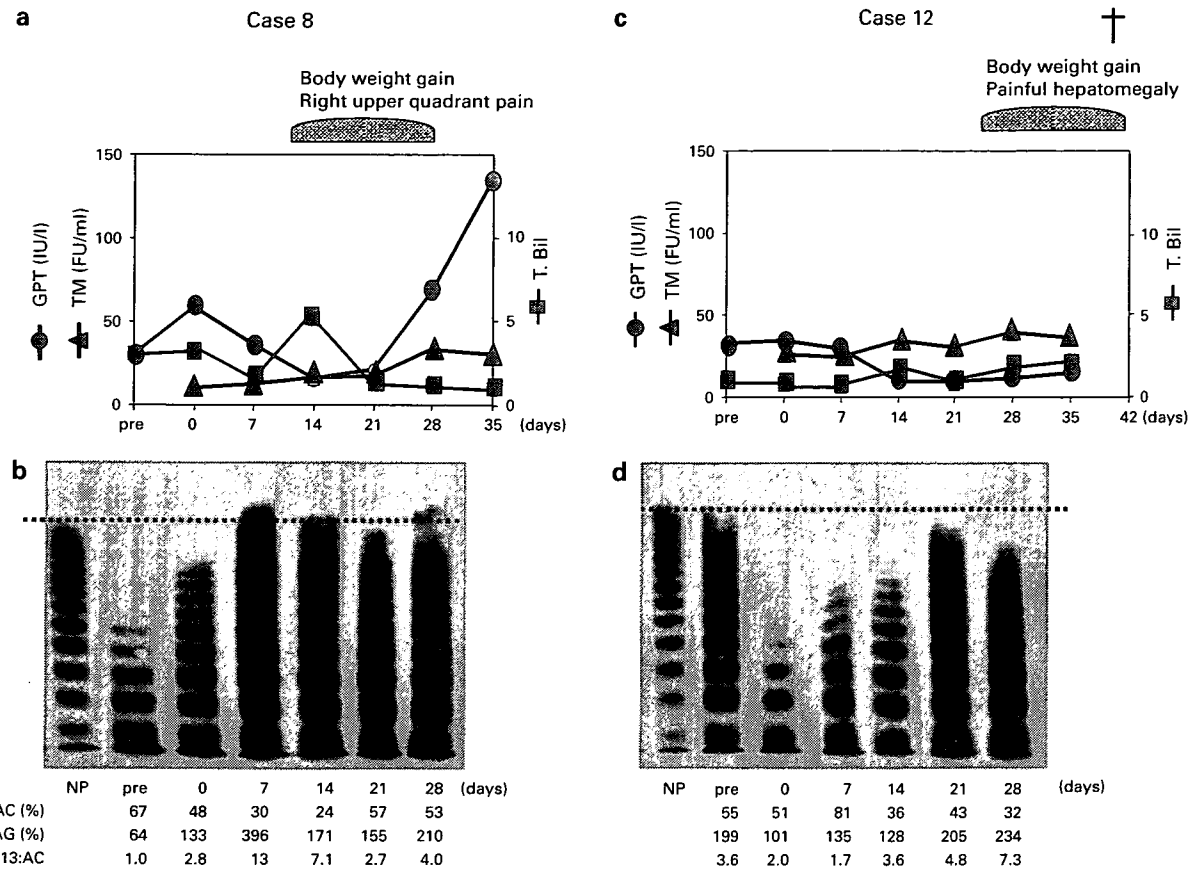
Case number	*Age (years)*	*Gender*	*Underlying disease*	*Disease state*	*Transplant type*	*Related (R) or unrelated donor (N)*	*Conditioning regimen*	*GVHD prophylaxis*	*Acute GVHD grade*
FFP (−)									
1	15	F	ALL	CR2	CBSCT	U	Flu/LPAM/TBI	CsA	I
2	10	M	ALL	CR2	BMT	R	LPAM/TBI	FK/MTX	I
3	13	M	ALL	Refractory	PBSCT	R	BU/Cy/TEPA	–	I
4	9	M	NK-leukemia	CR1	PBSCT	R	VP16/Cy/TBI	FK	II
5	14	M	CAEBV	Refractory	PBSCT	R	Flu/Cy/TBI	FK	II
6	17	M	AML	Refractory	BMT	U	Cy/Flu/TBL	FK/sMTX	II
7	1	M	ALL	CR1	CBSCT	U	BU/Cy/VP16	CsA/MTX	0
8	53	F	MDS	Non-CR	Mini-PBSCT	R	Bu/Flu	CsA/MTX	II
9	64	M	HD	Refractory	Mini-CBSCT	U	Flu/TBI	FK	NE
10	47	M	ALL	Refractory	BMT	U	Cy/TBI	CsA/MTX	II
11	32	F	AML	CR2	CBSCT	U	Flu/TBI	FK	II
12	52	M	ALL	Refractory	BMT	U	Bu/Cy	CsA/MTX	0
13	39	M	CML	BC	BMT	R	Bu/Cy/TBI	CsA/MTX	I
14	41	F	AML	Refractory	BMT	U	Bu/Cy/TBI	CsA/MTX	I
15	27	M	ALL	CR1	CBSCT	U	Flu/TBI	FK	I
16	26	M	ALL	Refractory	Mini-BMT	U	Flu/LPAM	CsA/MTX	II
17	43	M	NHL	Refractory	PBSCT	U	Bu/Cy/TBI	CsA/MTX	0
18	28	M	AML	Refractory	BMT	U	Flu/Bu/TBI	CsA/MTX	III
19	51	M	MDS	Non-CR	BMT	U	Bu/Cy/TBI	CsA/MTX	III
20	25	M	AML	CR1	CBSCT	U	Flu/TBI	CsA/MTX	NE
FFP (+)									
21	5	M	AML	CR2	Mini-CBSCT	U	Cy/TBI	CsA	I
22	5	M	AML	CR2	BMT	U	Cy/TBI	FK/MTX	I
23	3	F	MDS	Non-CR	BMT	R	Flu/LPAM/TBI	CsA	II
24	1	M	MDS	Non-CR	PBSCT	R	Bu/Cy/CA	CsA	III
25	7	M	ALL	CR2	BMT	U	LPAM/TBI	FK/MTX/PSL	II
26	3	M	AML	Refractory	BMT	R	VP16/Cy/Flu/TBI	FK/sMTX	I
27	1	M	WAS	Non-CR	CBSCT	U	Bu/Cy/Flu	CsA/MTX	0
28	6	M	ALL	CR2	BMT	U	CA/Cy/TBI	CsA	II
29	54	F	ALL	Refractory	CBSCT	U	Cy/TBI	CsA/MTX	II
30	35	F	NHL	Refractory	PBSCT	R	VP16/Cy/TBI	CsA/MTX	II
31	37	F	AML	CR2	CBSCT	U	Flu/TBI	FK	NE
32	34	M	AML	Refractory	CBSCT	U	Cy/TBI	CsA	IV
33	47	F	AML	CR2	Mini-CBSCT	U	Flu/TBI	FK/MTX	NE
34	45	F	NHL	Refractory	CBSCT	U	Flu/TBI	FK	IV
35	43	F	AML	CR1	PBSCT	R	Bu/Cy	CsA/MTX	I
36	38	M	MDS	Refractory	CBSCT	U	Flu/LPAM/TBI	FK/MTX	NE
37	34	F	MDS	Refractory	BMT	U	Bu/Cy/TBI	FK/MTX	I
38	40	M	CML	CP2	CBSCT	U	Flu/TBI	FK	II
39	50	M	ALL	Refractory	CBSCT	U	Flu/TBI	FK	II
40	43	M	AML	Refractory	PBSCT	R	Bu/Cy/TBI	FK/MTX	NE
41	19	M	AA	Severe	BMT	R	Flu/Cy/TBI	FK/MTX	II
42	53	M	CML	CP1	Mini-PBSCT	R	Flu/Bu	CsA/MTX	0
43	51	M	CML	Refractory	CBSCT	U	Flu/TBI	CsA	NE

Abbreviations: AA = aplastic anemia; ALL = acute lymphoblastic leukaemia; BC = blast crisis; BMT = bone marrow transplantation; BU = busulfan; CA = cytosine arabinoside; CAEBV = chronic active EBV infection; CBSCT = cord blood stem cell transplantation; CML = chronic myeloblastic leukemia; CR1 = 1st complete remission; CR2 = 2nd complete remission; CsA = cyclosporin; Cy = cyclophosphamide; FK = tacrolimus; Flu = fludarabine; HD = Hodgkin's disease; LPAM = melphalan; MDS = myelodysplastic syndrome; MTX = methotrexate; NE = not evaluated; NHL = non-Hodgkin lymphoma; PBSCT = peripheral blood stem cell transplantation; PSL = predonisolone; SCT = stem cell transplantation; TBI = total body irradiation; VP16 = vepeside; WAS = Wiskott–Aldrich syndrome.
The patients with shaded area developed hepatic VOD.

overall (patient no. 26), who belonged to the FFP (+) group and exhibited an inhibitor level of 1.3 Bethesda U/ml.

We further investigated VWF multimer patterns in patients from the FFP (−) and (+) groups because VWF multimers of high and intermediate molecular weight were specifically lacking pre-SCT in case 8 and at day 0 post-SCT in case 12, but thereafter gradually increased in these cases (Figures 1b and d). Representative VWF multimer patterns are shown in Figure 3. In patients from the FFP (−) group, VWF multimers corresponding to high and/or intermediate molecular weight were less or absent at pre-SCT and on days 0 and 7 in case 1 (Figure 3a); at pre-SCT and on days 0, 14 and 21 in case 5 (Figure 3b); and at pre-SCT and on days 0, 7 and 21 in case 10 (Figure 3c). In contrast, in patients from the FFP (+) group, no apparent changes in VWF multimer patterns were found throughout SCT, including during the preconditioning period when they compared them to VWF multimer levels found in normal control plasma.

The incidence of hepatic VOD occurrence by randomized controlled trial. Out of the 20 patients belonging to the



	NP	pre	0	7	14	21	28	(days)
ADAMTS13:AC (%)		67	48	30	24	57	53	
VWF:AG (%)		64	133	396	171	155	210	
VWF:AG/ADAMTS13:AC		1.0	2.8	13	7.1	2.7	4.0	

	NP	pre	0	7	14	21	28	(days)
		55	51	81	36	43	32	
		199	101	135	128	205	234	
		3.6	2.0	1.7	3.6	4.8	7.3	

Figure 1 von Willebrand factor (VWF) multimeric analysis in patients with hepatic veno-occlusive disease (VOD). Patient no. 8 (left panel) was diagnosed with hepatic VOD on day 14 (**a**). VWF multimer analysis showed absence of high and intermediate molecular weight VWF multimer (VWFM) before stem cell transplantation and on day 0. At day 7, VWF:AG was increased and unusually large VWF multimers (UL-VWFM) were detected (**b**). Patient no. 12 (right panel) was diagnosed with hepatic VOD on day 28 (**c**). VWF multimer analysis showed absence of high and intermediate molecular weight VWFM on days 0, 7 and 14. VWF:AG was increased at day 21 (**d**). The multimers over the dotted line indicate UL-VWFM, which can be never detected in normal plasma.

FFP (−) group, three (15%) patients (patient no. 8, 12 and 14) developed hepatic VOD. In contrast, none of the 23 patients in the FFP (+) group did so (Table 3). The incidence of VOD was clearly higher in the FFP (−) group than in the FFP (+) group, but the difference between the FFP (−) group and (+) group did not reach statistical significance ($P=0.092$) because of the small number of patients in the study.

As for risk factors for the development of hepatic VOD, all three patients with VOD came from the 29 patients who had been treated with intensified chemotherapy until just before SCT. Both case 8 and 12 came from the 11 patients with liver dysfunction, and case 14 was one of 21 patients who received an intensified conditioning regimen. There was thus no relationship between these risk factors for developing hepatic VOD. Further, we compared ADAMTS13:AC and VWF:AG among four criteria of risk factors as described in Materials and methods. However, no significant difference was found in either ADAMTS13:AC and VWF:AG among these criteria (data not shown).

The incidence of VOD occurrence by subsequent questionnaire study

According to the questionnaire, two out of 10 participating hospitals gave prophylactic FFP infusions to prevent hepatic VOD in high-risk patients who had undergone SCT, but others did not. Hepatic VOD developed in 11 of 173 high-risk patients who did not receive prophylactic FFP infusions (Table 3). In contrast, hepatic VOD never occurred in 36 high-risk patients who had prophylactic FFP infusion. Based on the result under this questionnaire, the difference in the incidence of hepatic VOD did not reach statistical significance between patients receiving and not receiving prophylactic FFP infusions ($P=0.120$). However, when we analyzed all the patients who underwent SCT between 2001 and 2006, the incidence of hepatic VOD was significantly lower in patients receiving prophylactic FFP infusions (0/59) than in those who did not (14/193) (0 vs 7.3%, $P=0.033$) (Table 3).

Discussion

In this study, we performed a randomized controlled trial, and a subsequent study by questionnaire, to evaluate whether prophylactic FFP infusions could prevent the occurrence of hepatic VOD in high-risk patients receiving SCT. As for the incidence of hepatic VOD in the randomized controlled study, 3 (15%) of 20 patients belonging to the FFP (−) group developed hepatic VOD,

Table 2 Differences in clinical parameters between patients with and without FFP infusions

	WBC ($\times 10^9$/l)	*RBC* ($\times 10^{12}$/l)	*Hb* (g/l)	*Ret* (‰)	*Plt* ($\times 10^9$/l)	*T.Bil* (mg/dl)	*GOT* (IU/l)	*GPT* (IU/l)	*LDH* (IU/l)	*ALP* (IU/l)	*BUN* (mg/dl)	*Cre* (mg/dl)	*CRP* (mg/dl)	*TAT* (ng/l)	*D-dimer* (ng/ml)	*TM* (FU/ml)	*t-PA/PAI-1* (ng/ml)	*AT* (%)	*VWF: AG* (%)	*ADAMTS13: AC* (%)	*VWF:AG/ ADAMTS13: AC*
Pre																					
FFP (−)	7.9±19.9	2.9±0.5	95±19	14±15	96±91	1.1±1.5	30±30 5	37±41	336±444	414±396	11±5.0	0.6±0.3	3.4±5.6	2.9±3.2	2.2±3.2	15±9	11±10	95±17	121±44	65±26	2.1±1.0
FFP (+)	4.2±4.4	3.2±0.7	101±23	9.9±8.2	101±78	0.6±0.3	30±2	48±60	211±60	441±315	10±3.9	0.5±0.3	1.1±2.6	5.6±10.1	0.7±0.5	11±8	22±22	101±16	103±59	63±26	1.9±1.4
Day 0																					
FFP (−)	1.3±1.8	2.7±0.5	89±15	5.2±7.1	48±40	1.2±1.2	18±10	27±30	206±75	354±326	13±7.7	0.5±0.3	2.5±4.4	7.1±8.2	3.5±6_3	14±8	14±12	104±23	147±78	55±23	2.9±1.8
FFP (+)	0.5±0.7	3.0±0.6	93±18	2.6±3.8	53±62	0.6±0.3	20±12	34±48	179±52	297±144	12±6.3	0.4±0.3	2.4±5.1	4.8±7.9	1.1±1.1	8.9±7	20±14	100±18	96±39	62±24	1.9±1.2
Day 7																					
FFP (−)	0.2±0.7	2.9±0.5	91±13	3.5±5.8	24±10	1.0±1.0	23±29	28±44	175±78	339±269	15±5.2	0.5±0.3	3.2±3.2	6.4±9.1	1.7±2.4	11±7	15±10	87±16	156±83	54±20	3.5±3.1
FFP (+)	0.1±0.2	2.9±0.6	89±15	2.3±3.5	23±19	0.8±0.5	17±10	25±18	170±55	280±119	14±6.7	0.4±0.2	5.8±8.8	8.1±24	1.1±0.9	8.7±6	13±6.5	85±16	103±62	55±23	2.0±1.2
Day 14																					
FFP (−)	1.3±2.2	2.9±0.5	91±14	3.9±5.2	32±23	1.3±1.4	46±83	56±62	199±88	339±187	25±14	0.7±0.4	6.1±7.5	4.8±7.9	2.3±2.4	15±11	18±13	80±17	165±73	42±15	4.5±2.5
FFP (+)	2.3±2.9	3.0±0.4	91±14	19±53	39±32	1.6±3.2	54±76	73±71	360±172	381±175	22±19	0.5±0.4	6.1±7.7	4.9±4.4	3.1±3.2	16±15	23±14	86±24	169±91	49±26	3.8±2.4
Day 21																					
FFP (−)	3.5±2.2	3.0±0.5	93±15	5.4±5.4	40±30	1.1±1.6	40±31	78±76	266±120	414±214	20±9.1	0.5±0.2	1.3±1.8	5.6±9.0	1.3±0.9	17±10	19±15	102±21	157±68	53±17	3.2±1.7
FFP (+)	3.2±2.5	3.1±0.5	93±14	33±54	52±51	2.1±4.6	86±75	92±81	424±339	500±385	30±30	0.6±0.6	3.8±4.9	7.7±14	1.5±1.6	16±12	39±53	90±22	178±87	51±20	4.3±4.3
Day 28																					
FFP (−)	3.7±3.4	2.9±0.5	91±15	13±8.6	50±48	1.2±2.0	53±55	96±92	251±92	441±359	22±11	0.7±0.4	2.2±3.9	6.9±11	1.2±1.0	20±11	21±20	105±27	212±81	57±22	4.4±2.9
FFP (+)	3.5±2.0	2.9±0.6	88±17	39±57	45±42	2.3±3.8	51±45	83±80	317±164	545±393	29±33	0.7±0.7	2.3±3.5	4.6±4.9	1.9±2.8	16±13	28±19	93±23	146±82	49±23	3.8±3.9

Abbreviations: ALP = alkaline phosphate; AT = antithrombin; BUN = blood urea nitrogen; Cre = creatine; CRP = C reactive protein; FFP = fresh frozen plasma; GOT = glutamic oxaloacetic transaminase; GPT = glutamic-pyruvate transaminase; Hb = hemoglobin; LDH = lactic dehydrogenase; Plt = platelet; RBC = red blood cell; Ret = reticulocyte; TAT = thrombin–antithrombin complex; T.Bil = total bilirubin; TM = thrombomodulin; t-PA/PAI-1 = tissue plasminogen activator/plasminogen activator inhibitor-1; VWF:AG = von Willebrand factor antigen; WBC = white blood cell.
Values are mean ± s.d.
The values in shaded area denote statistically significant differences between FFP (−) and FFP (+) groups ($P<0.05$).

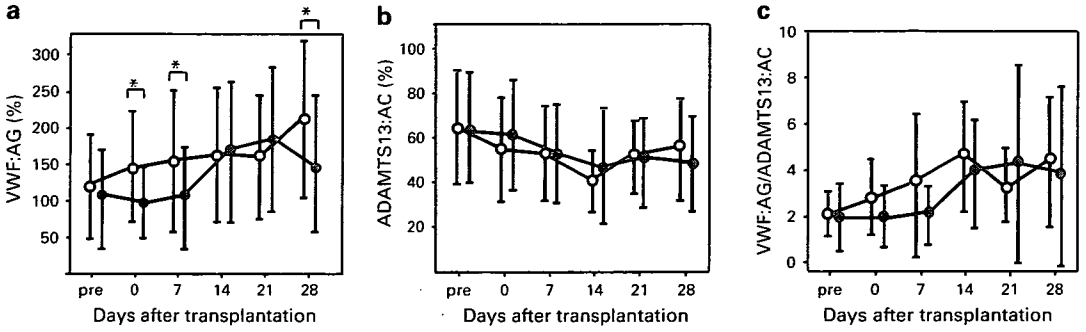


Figure 2 Changes in von Willebrand factor (VWF):AG, ADAMTS13:AC and VWF:AG/ADAMTS13:AC after stem cell transplantation. Open circles represent patients from the fresh frozen plasma (FFP) (−) group. Closed circle represents patients from FFP (+) group. (**a**) VWF:AG in patients from the FFP (−) group was significantly higher than in patients from FFP (+) group on days 0, 7 and 28. (**b**) No difference in the level of ADAMTS13:AC was observed between the groups. (**c**) The ratio of VWF:AG/ADAMTS13:AC in FFP (−) group was higher than in FFP (+) group, but this difference was not statistically significant. $*P<0.05$.

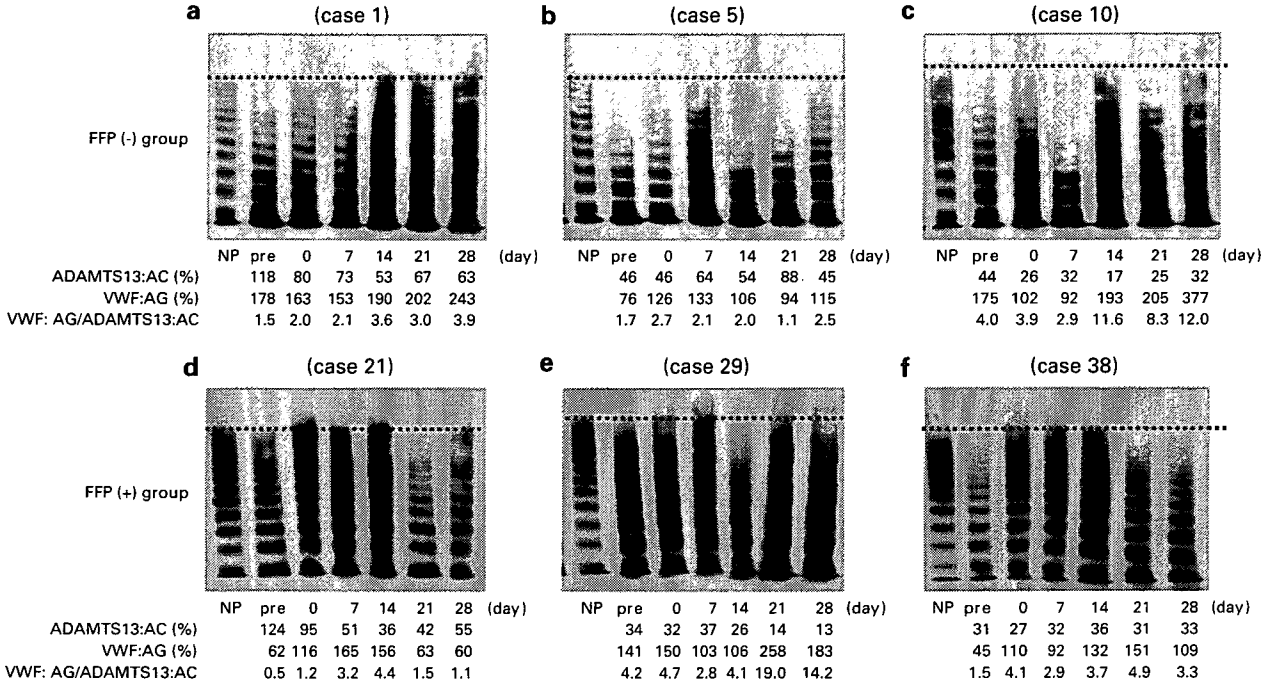


Figure 3 von Willebrand factor (VWF) multimer analysis in patients without hepatic veno-occlusive disease (VOD). VWF multimer analyses of six representative cases, three fresh frozen plasma (FFP) (+) and three FFP (−), without hepatic VOD are shown. In patients from FFP (−) group, VWF multimers of high and intermediate molecular weight were less or absent before stem cell transplantation (SCT) and on days 0 and 7 in case 1 (**a**); at pre-SCT and on days 0, 14 and 21 in case 5 (**b**) and at pre-SCT and on days 0, 7 and 21 in case 10 (**c**). In contrast, in patients from FFP (+) group (case 21, 29, 38), no apparent changes in VWF multimer patterns were found throughout SCT including the preconditioning period, when compared to patterns found in normal control plasma (**d**, **e**, **f**). The multimers over the dotted line indicate unusually large VWF multimers (UL-VWFM), which can never be detected in normal plasma.

whereas none of the 23 patients belonging to the FFP (+) group did so. The incidence between patients receiving and not receiving FFP did not reach statistical significance because of the small numbers of enrolled patients. However, when analysis involving all the patients examined both the randomized controlled trial and the subsequent study by questionnaire, the incidence of hepatic VOD was significantly lower in patients receiving prophylactic FFP infusion than in those not doing so (Table 3). These results suggest that prophylactic FFP infusions may be instrumental in preventing the development of hepatic VOD after SCT.

In the patients who developed VOD (cases 8 and 12) in the randomized controlled study, VWF:AG progressively increased and ADAMTS13:AC gradually decreased, resulting in ratios of 13.0 at day 7 in case 8, and 4.8 at day 21 in case 12, which were notably higher than that found in normal subjects (1.0). Hepatic VOD developed a week later, when the ratio of VWF:AG to ADAMTS13:AC showed values as high as 7.1 in case 8, and 7.3 in case 12. An inverse correlation between decreased ADAMTS13:AC and increased VWF:AG was thus seen in our VOD patients. These findings were consistent with previous

Table 3 The incidence of VOD occurrence in SCT patients with and without FFP infusion

	FFP (−)	*FFP (+)*	P-*value*
Randomized study	3[a]/20[b] (15%)	0/23 (0%)	0.092
Study by the questionnaire	11/173 (6.4%)	0/36 (0%)	0.120
Total	14/193 (7.3%)	0/59 (0%)	0.033

Abbreviations: FFP = fresh frozen plasma; SCT = stem cell transplantation; VOD = veno-occlusive disease.
[a]Number of patients with hepatic VOD.
[b]Number of high-risk patients to lapse into hepatic VOD.

findings in pathological conditions including liver cirrhosis, chronic renal insufficiency, acute inflammatory states and major surgery.[18] Furthermore, our previous study[17] demonstrated that the mean value of ADAMTS13:AC pre-SCT was 32% in seven patients with hepatic VOD. In this study, ADAMTS13:AC was, however, 67 and 55% pre-SCT in cases 8 and 2, respectively. The activity thereafter decreased from 67 to 24% on day 14 in case 8, and from 55 to 32% on day 28 in case 12. From these results, there appeared to be a possibility that hepatic VOD could develop even in the patient whose plasma ADAMTS13:AC pre-SCT did not drop below 30%, indicating that the imbalance of VWF:AG to ADAMTS13:AC before and throughout SCT may be more important for the development of hepatic VOD than the decrease of ADAMTS13:AC.

Surprisingly, VWF multimers corresponding to high and intermediate molecular weight, which are usually seen in normal plasma, were absent before SCT in case 8 and on day 0 in case 12 (Figures 1b and d), but thereafter gradually appeared. These results suggest that the initial absence of high and intermediate VWF multimers at preconditioning or just after SCT, and subsequent appearance of these VWF multimers in combination with the increase in VWF:AG and decrease in ADAMTS13:AC may play an important role in the development of VOD after SCT. It remains unclear why high and intermediate VWF multimers were lacking at preconditioning and/or during the early period after SCT but thereafter gradually appeared in VOD patients. We speculate, however, that the target lesion caused by intensive chemotherapy and/or total body irradiation given in the setting of SCT is to the sinusoidal endothelial cells. Indeed, chemotherapy including cyclophosphamide and busulfan before SCT is a regimen associated with a high incidence of hepatic VOD,[4] and total body irradiation causes radiation-induced liver disease.[30] The amount of VWF released from injured endothelial cells may be increased at first, but may decrease thereafter because the endothelial cells are extensively damaged. After SCT, as damaged endothelial cell gradually regenerate, the release of VWF may increase, resulting in the appearance of high and intermediate VWF multimers. Under these circumstances, plasma ADAMTS13 may be consumed to degrade the large amounts of VWF derived from damaged hepatic endothelial cells. Moreover, ADAMTS13 that is exclusively generated in the HSCs may be decreased owing to the liver injury itself. Our previous report that plasma ADAMTS13:AC is significantly reduced in patients with hepatic VOD even before and throughout SCT[17] supports the hypothesis that ADAMTS13:AC decreases may be an indicator of impending development of VOD. This imbalance of decreased activity of ADAMTS13 vs 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 easily damaged by hypoxia.[1] Whether or not patients develop VOD after SCT may depend upon the degree of imbalance between VWF:AG and ADAMTS13:AC at preconditioning and/or just after SCT.

We then compared clinical parameters between patients receiving and not receiving FFP infusions to evaluate the effect of FFP on the prevention of VOD. VWF:AG was significantly lower at days 0, 7 and 28 in patients receiving FFP than in those not doing so. The reciprocal relationship between gradually decreased ADAMTS13:AC and gradually increased VWF:AG after SCT was seen in the FFP (−) group, but not in the FFP (+) group (Figures 2a and b). No difference in the levels of ADAMTS13:AC was found between the two groups (Figure 2b). The ratio of VWF:AG to ADAMTS13:AC tended to be lower at days 0 and 7 in the FFP (+) group (Figure 2). These results indicated that the FFP infusions can suppress the increase in plasma VWF:AG in the early stages after SCT. Furthermore, in the FFP (−) group, high and/or intermediate molecular weight VWF multimers were lacking in the early stages and even in the later stage after SCT (Figure 3). In the FFP (+) group, however, no apparent changes in VWF multimer patterns were found throughout SCT (Figure 3). These results indicate that the supplementation of ADAMTS13 achieved by FFP administration may suppress the increase in VWF:AG that is extensively released from damaged endothelial cells after chemotherapy and/or total body irradiation. ADAMTS13 may be consumed to degrade a large amount of UL-VWFM released from damaged endothelial cells throughout SCT. We therefore were unable to observe any increase in ADAMTS13:AC supplemented by the administration of FFP in patients with FFP infusion. From this vantage point, FFP administration as a source of ADAMTS13 affords a compellingly effective means of preventing hepatic VOD.

In conclusion, hepatic VOD developed only in patients not receiving FFP infusions, probably because of increased VWF production relative to decreased ADAMTS13:AC throughout SCT, although other mechanisms may have played a role. Prophylactic FFP infusions should therefore be considered in patients at high risk of developing of hepatic VOD after SCT.

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Brief report

Functional imaging of shear-dependent activity of ADAMTS13 in regulating mural thrombus growth under whole blood flow conditions

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The metalloprotease ADAMTS13 is assumed to regulate the functional levels of von Willebrand factor (VWF) appropriate for normal hemostasis in vivo by reducing VWF multimer size, which directly represents the thrombogenic activity of this factor. Using an in vitro perfusion chamber system, we studied the mechanisms of ADAMTS13 action during platelet thrombus formation on a collagen surface under whole blood flow conditions. Inhibition studies with a function-blocking anti-ADAMTS13 antibody, combined with immunostaining of thrombi with an anti-VWF monoclonal antibody that specifically reflects the VWF-cleaving activity of ADAMTS13, provided visual evidence for a shear rate–dependent action of ADAMTS13 that limits thrombus growth directly at the site of the ongoing thrombus generation process. Our results identify an exquisitely specific regulatory mechanism that prevents arterial occlusion under high shear rate conditions during mural thrombogenesis. (Blood. 2008; 111:1295-1298)

Introduction

The adhesive protein von Willebrand factor (VWF) plays a major role in platelet thrombogenesis, a process crucial for hemostasis. However, the excessive function of VWF is thought to increase the risk of fatal arterial thrombosis.[1,2] The thrombogenic activity of VWF is strictly dependent upon its multimeric structure, which is thought to be regulated in vivo by the metalloprotease ADAMTS13 through its cleavage of the A2 domain of the VWF subunit.[3,4] Indeed, patients with congenital deficiency of ADAMTS13 suffer repeated thrombotic complications attributed to excessive function of the ultra-large VWF (ULVWF) multimer, which is not found in normal blood circulation.[3-6] This concept was recently confirmed by knock-out mouse studies, in which ADAMTS13$^{-/-}$ mice exhibited enhanced thrombogenicity in the ex vivo or in vitro experimental blood flow conditions tested.[7,8]

The mechanisms by which ADAMTS13 regulates VWF remain poorly understood. However, recent studies showing that ADAMTS13 under flow conditions can rapidly cleave ULVWF secreted from and anchored to cultured endothelial cell layers[9,10] have raised the possibility that blood flow is critical in activating ADAMTS13.[11] Indeed, the VWF-cleaving activity of ADAMTS13 cannot be reproduced in vitro under static conditions unless the substrate VWF molecule is somewhat modified (eg, denatured by guanidine-HCl or urea).[3,4] Further, the question arises of whether ADAMTS13, in addition to its known action on ULVWF freshly released from endothelial cells, might also act directly at the local sites of thrombus generation to regulate thrombus growth.

To address these issues, we analyzed the role and mechanisms of ADAMTS13 action in mural platelet thrombogenesis on a collagen-coated glass surface in an in vitro perfusion chamber system. Our visual evidence demonstrates that ADAMTS13 cleaves VWF and down-regulates mural thrombus growth at the site of ongoing thrombus generation in a shear rate–dependent manner under whole blood flow conditions.

Methods

Blood collection

The present work was approved by the institutional review board of Nara Medical University, and informed consent was obtained in accordance with the Declaration of Helsinki. Using 200 μM argatroban as an anticoagulant, blood was collected from 10 nonsmoking healthy volunteers who had not taken any medications in the previous 2 weeks.

Monoclonal antibodies

A function-blocking anti-ADAMTS13 monoclonal antibody (A10), which completely inhibits plasma ADAMTS13 activity at the concentration of 20 μg/mL,[12] was used as a divalent $(ab')_2$ fragment in inhibition studies. An anti-VWF monoclonal antibody (N10) was used that reacts with an epitope within the VWF A2 domain (10-amino acid VWF peptide; D^{1596}REQAPNLVY^{1605}) only after cleavage by ADAMTS13 exposes the epitope; thus, reactivity of antibody N10 specifically reflects the VWF-cleaving activity of ADAMTS13, as described.[13]

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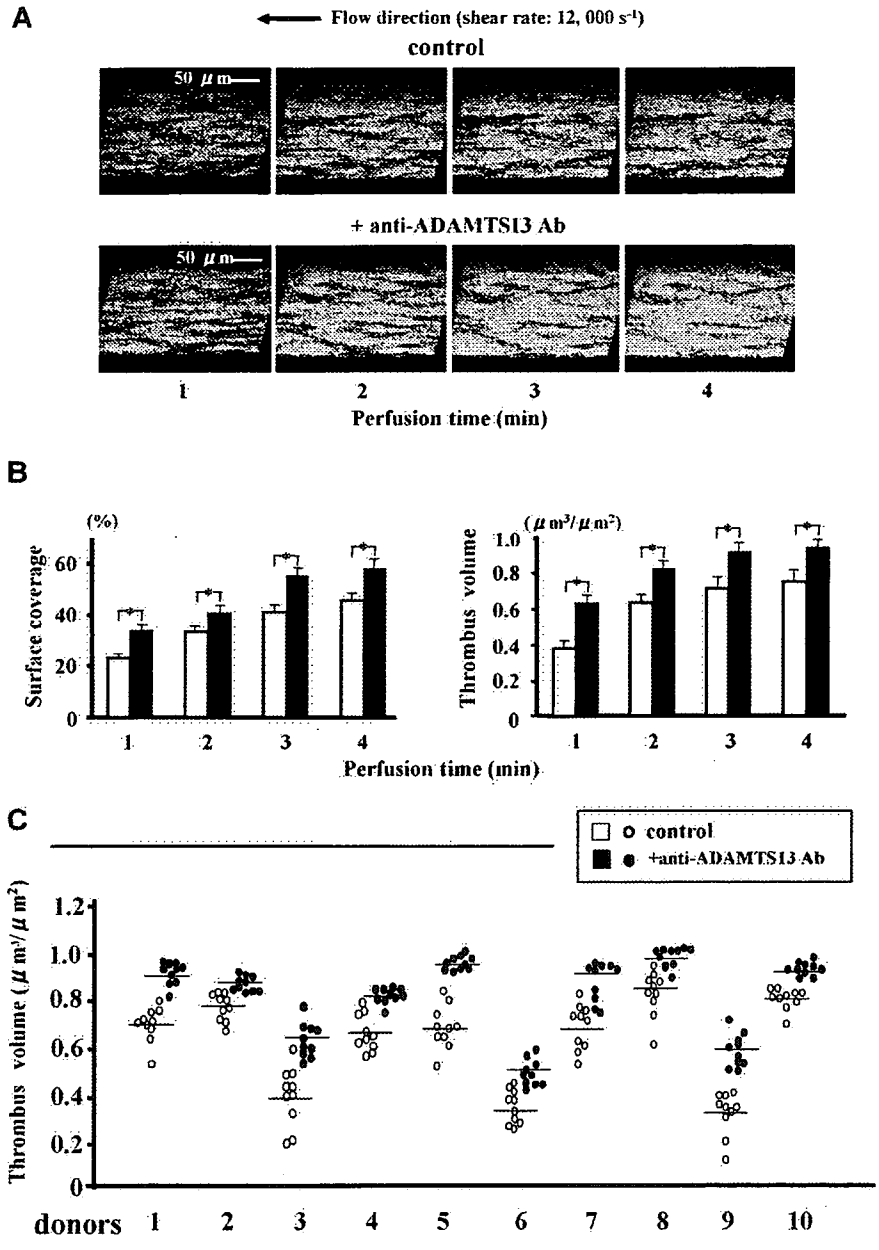

Figure 1. Effects of a function-blocking anti-ADAMTS13 monoclonal antibody (A10) on mural thrombus generation under very high shear rate conditions. Whole blood from healthy volunteers containing DiOC6 (1 μM)-labeled platelets, anticoagulated with argatroban, was perfused over a type I collagen-coated glass surface under very high shear rate (12 000 s^{-1}) with anti-ADAMTS13 antibody A10 or with control mouse IgG (each 20 μg/mL). (A) Time-course changes of 3-dimensional images of thrombi (original magnification, ×600), which were constructed by the image-analyzing system of confocal laser scanning microscopy (CLSM) based on successive horizontal slices at identical portions, are representative of 10-pair flow experiments using blood from 10 independent donors. (B) Statistical analyses corresponding to the above images; bars represent mean (+ SD) surface coverage or total thrombus volume in 10 areas (each 133 × 100 μm) randomly selected in each perfusion using a single donor blood (donor number 1 in panel C). Note that thrombus generation is significantly (*; $P < .01$) accelerated in the presence of the anti-ADAMTS13 antibody. (C) Thrombus volume at 3 minutes' perfusion in 10-pair flow experiments using 10 independent donors; data points represent values of 10 areas randomly selected in each perfusion with (●) or without (○) anti-ADAMTS13 antibody, and transverse lines indicate mean values for each group. Note also that thrombus volumes generated in the presence of anti-ADAMTS13 antibody are significantly ($P < .01$; asterisks not included in the figure) greater than control thrombi in all 10-pair experiments.

In vitro perfusion studies

Thrombus generation on a type I collagen-coated (Sigma-Aldrich, Tokyo, Japan) glass surface was studied under various shear rates in a parallel plate flow chamber system as described.[14-17] Surface coverage and volume of thrombi generated at the indicated time points during whole blood perfusion were evaluated based on images obtained by confocal laser scanning microscopy (CLSM; FV300; Olympus, Tokyo, Japan), as described.[15-17] Immunohistochemical staining of thrombi using anti-VWF antibodies was performed as described.[15-17] Briefly, thrombi on a glass surface were fixed with paraformaldehyde and incubated with a mixture of anti–whole VWF rabbit polyclonal antibody (30 μg/mL; DAKO Cytomation, Kyoto, Japan) and N10 antibody (60 μg/mL) or with the negative control IgG mixture (rabbit; 30 μg/mL, mouse; 60 μg/mL; DAKO Cytomation) for 90 minutes at 37°C. Samples were then stained with a mixture of fluorescein isothiocyanate (FITC)–conjugated anti–rabbit IgG (3.3 μg/mL; BioSource International, Camarillo, CA) and Cy3-conjugated anti–mouse IgG (3.3 μg/mL; Sigma-Aldrich) as secondary fluorescent antibodies for 90 minutes at 37°C, and viewed by CLSM. These conditions were determined in preliminary experiments to confirm the sufficient infiltration of both primary and secondary fluorescent antibodies into thrombi.

Results and discussion

To address the potential role of ADAMTS13 in the ongoing process of mural thrombus generation, we compared the size of thrombi generated in the presence or absence of a function-blocking antibody against ADAMTS13 in a perfusion chamber system, using blood from the same donor. This relatively simple experimental approach is able to precisely evaluate ADAMTS13 function in uniform blood conditions, avoiding the individual heterogeneity of sample blood conditions including VWF and platelets that might otherwise seriously affect the size of thrombi generated in this type of flow experiment.

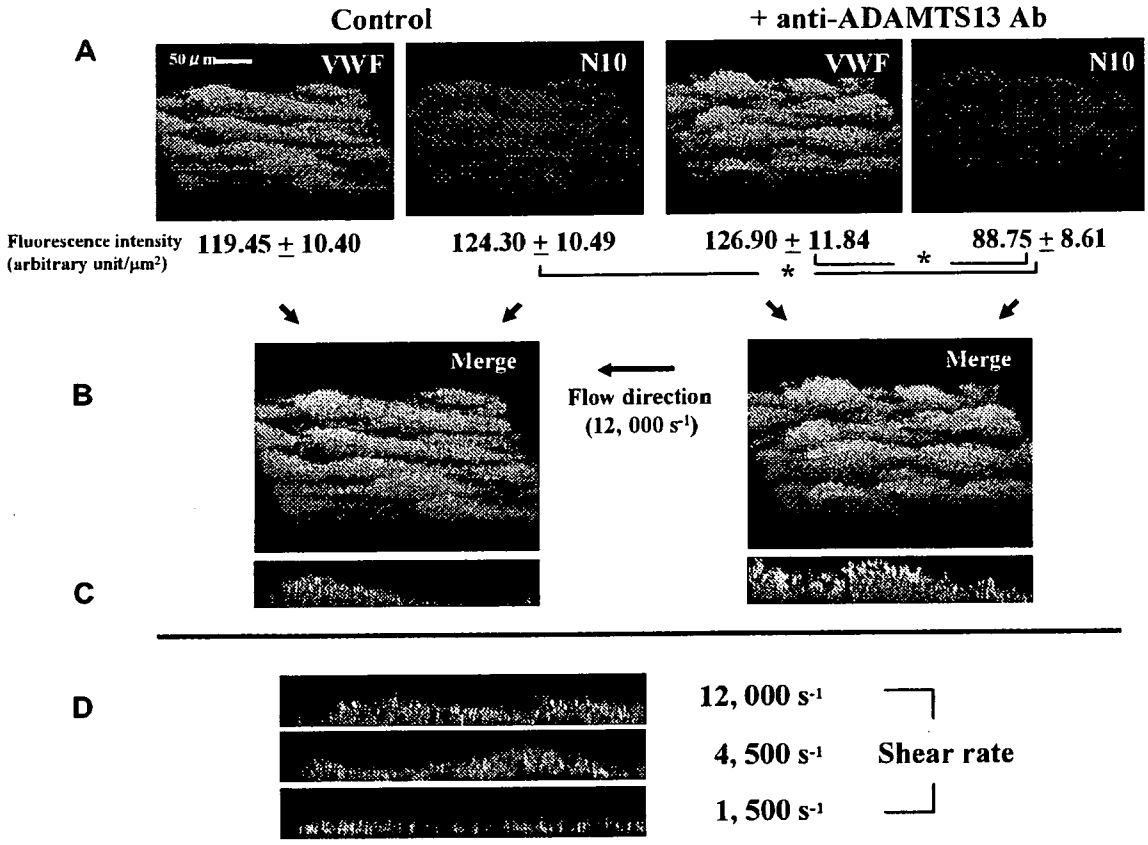

Figure 2. Visual evaluation of ADAMTS13 activity within thrombi generated under high shear rate conditions using a monoclonal antibody (N10) that specifically detects ADAMTS13-cleaved VWF. Experimental conditions were as described in Figure 1, except that platelets were not labeled. Thrombi generated on a collagen-coated glass surface at 3 minutes' perfusion with or without an anti-ADAMTS13 antibody under 12 000 s^{-1} shear were fixed, reacted with both N10 antibody and anti–whole VWF antibody, double-stained with Cy3 (red)- and FITC (green)-fluorescence, and viewed by CLSM. (A) Three-dimensional images of thrombi, representative of 5 independent flow experiments, and the corresponding fluorescence intensity (mean ± SD of 5 areas randomly selected in a single perfusion) corrected for background value (negative control IgGs), indicate that VWF cleavage by ADAMTS13 (red color) within thrombi is significantly (*: $P < .01$) reduced in the presence of anti-ADAMTS13 antibody as compared with control thrombi (original magnification; ×600). (B) Merged 3-dimensional images and (C) the corresponding longitudinal views of thrombi; in the merged images, portions stained with both green and red fluorescence basically show the color of the higher pixel value, whereas a yellowish color is seen when both pixel values are nearly equal. Thus, the predominantly reddish appearance of the surface portions of control thrombi suggests that ADAMTS13 is more active on the surface of thrombi forming under very high shear rate conditions, while this tendency is barely visible in the presence of anti-ADAMTS13 antibody. (D) Longitudinal views of thrombi, generated at 3 minutes' perfusion without an anti-ADAMTS13 antibody under various shear rates and double-stained, are representative of 5 separate experiments. Note the prominent red color, especially at the surface portions of thrombi, indicating higher ADAMTS13 activity under higher shear rates.

Under a very high shear rate of 12 000 s^{-1}, thrombus growth was significantly accelerated by addition of anti-ADAMTS13 antibody (Figure 1). This enhanced thrombogenesis most likely reflects a block in ADAMTS13 activity rather than nonspecific effects of antibody on platelets, since immunostaining of thrombi with N10 antibody, which reacts only with VWF cleaved by ADAMTS13, visually confirmed the reduced VWF cleavage within thrombi by the anti-ADAMTS13 antibody (Figure 2A). Thus, these results clearly point to the regulatory role of ADAMTS13 during thrombus generation.

While the preceding observations were made under a much higher shear rate than the high shear rate typically used in platelet functional studies (ie, 1500 s^{-1} in our laboratory[14-16]), similar observations, although less pronounced, were confirmed under lower shear rates (Figure 2D). In addition, longitudinal views of thrombi revealed the preferential VWF-cleavage activity of ADAMTS13 at the surface portions of forming thrombi during thrombogenesis (Figure 2B,C). The thrombus surface is thought to directly encounter blood flow with the highest shear rate under such flow circumstances, where the wall shear rate can increase as the flow path narrows in parallel with thrombus growth.[15] Together, these observations strongly suggest a shear rate–dependent property of ADAMTS13 function.

Shear forces are thought to transform the globular conformation of the immobilized VWF multimer observed under static conditions to a shape resembling a spreading bird wing, consistent with the shear rate–dependent acceleration of the VWF-glycoprotein Ib interaction under high shear.[18] By analogy, a stretching of the VWF multimeric structure by shear forces may also be critical for the action of ADAMTS13 in exposing the latent reactive site on the VWF molecule. In this regard, increased tensile strength of the VWF multimeric structure on binding to platelets might augment the stretching effects of shearing forces, resulting in up-regulated ADAMTS13 activity.[19,20] This possibility seems compatible with recent findings indicating that even under low shear rate conditions, ADAMTS13 can cleave ULVWF released from endothelial cells,[9,10] because a greater number of platelets can bind spontaneously to ULVWF as compared with normal-sized VWF without shearing forces.

The mechanisms described here represent an exquisite orchestration by platelets, VWF, and ADAMTS13 under high shear to properly regulate the final size of mural thrombi in vivo and

prevent excessive thrombogenesis from occluding the vessel lumen. Because ADAMTS13 activity appears to be triggered in response to the increased local shear rate associated with the development of thrombi, our results may provide a novel avenue toward strategies that prevent arterial thrombosis without bleeding complications.

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Authorship

Contribution: Y.S. performed most of the flow experiments, data analysis, and the manuscript preparation; T.M. and M.H. helped perform the flow experiments and data analysis; S.K., M.M., and Y.F. produced and characterized monoclonal antibodies; A.Y. and K.O. provided direction throughout the work and helped prepare the manuscript; and M.S. and K.N. provided the overall experimental designs and direction of this work, and prepared the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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REGULAR ARTICLE

Reduced Cd4$^+$Cd25$^+$ T cells in patients with idiopathic thrombocytopenic purpura

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KEYWORDS
ITP;
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Foxp3;
Platelet count

Abstract Immunoregulatory CD4$^+$CD25$^+$ T cells play an important role in the induction and maintenance of peripheral self-tolerance. These professional regulatory cells prevent the activation and proliferation of potentially autoreactive T cells that have escaped thymic deletion. Therefore, CD4$^+$CD25$^+$ T cells are believed to possibly play an important role in pathogenic autoimmune diseases. We measured the count of CD4$^+$CD25$^+$ T cells in 44 patients with idiopathic thrombocytopenic purpura (ITP), and the number of CD4$^+$CD25$^+$ T cells and clinical features were then analyzed. By using a flow cytometric analysis, the number of CD4$^+$CD25$^+$ T cells in the patients with ITP showed a very wide distribution in comparison to healthy volunteers. The number of CD4$^+$CD25$^+$ T cells was significantly lower in the ITP patients in the severe phase, and in patients positive for anti-glycoprotein IIb-IIIa antibody. However, the number of those cells increased in the patients at the complete remission phase, especially after a splenectomy. The Foxp3 mRNA levels of peripheral blood mononuclear cells (PBMC) of ITP patients were higher with an improved platelet count than in those with a low platelet count. In addition, the Foxp3 mRNA levels closely correlated with the number of CD4$^+$CD25$^+$ cells. These mechanisms remain to be fully elucidated, however, the count of CD4$^+$CD25$^+$ T cells is considered to possibly be related to the severity of ITP.

Introduction

Some autoimmune diseases such as Grave's disease, myasthenia gravis and immune cytopenias have

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been recognized to be mediated by pathogenic autoantibodies. It is evident that the autoantibody production by B cells requires the presence of autoantigens-specific T cells [1]. Indeed, autoreactive T cells have been identified in patients with various systemic or organ-specific autoimmune diseases [2-4]. On the other hand, autoreactive T cells are also present in peripheral blood (PB) of a healthy individual without any evidence of autoimmune disease [5]. These findings thus suggest that some mechanisms exist which regulate these autoreactive T cells in order to thereby prevent autoimmune disease.

Naturally arising $CD4^+CD25^+$ T cells, which regulate autoreactive T cells, have been described in rodent models [6-8]. These professional regulatory cells prevent the activation and proliferation of potentially autoreactive T cells that have escaped thymic deletion. In animal models, the elimination or inactivation of $CD4^+CD25^+$ T cells is known to result in various severe autoimmune diseases [8,9]. In humans, $CD4^+CD25^+$ T cells have also been reported to have a similar regulatory function to that of the murine population as they were anergic to stimulation by T cell receptor cross-linking (TCR) in the absence of exogenous IL-2 and also regarding their ability to suppress the activation of other T cells in a cell-contact dependent manner that could not be inhibited by blocking such cytokines as IL-10. An increased number of $CD4^+CD25^+$ cells was found in patients with autoimmunity, cancer, or chronic infection [10-13]. It has recently been shown that the effector function of $CD4^+CD25^+$ had a marked decrease in multiple sclerosis (MS) [14].

Idiopathic thrombocytopenic purpura (ITP) is one of the autoimmune diseases characterized by an increased platelet clearance caused by anti-platelet autoantibodies. Although Kuwana et al. [15] reported that autoreactive $CD4^+$ T cells to glycoprotein on platelet membrane (GpIIb–IIIa, GpIb) mediate anti-platelet autoantibody production in patients with ITP, the role of $CD4^+CD25^+$ T cells in ITP remain to be elucidated.

In this study, we defined regulatory CD4 T cells as high $CD4^+CD25^+$ T cells, and we studied the number of $CD4^+CD25^+$ T cells in patients with ITP, and analyzed the relationship of the regulatory cells and clinical features.

Materials and methods

Patients

ITP was defined as thrombocytopenia (platelet count $<100\times10^9$/L), normal or increased bone marrow (BM) megakaryocytes without any morphological evidence for dysplasia, and no secondary immune or nonimmune diseases that account for the thrombocytopenic state. In this study, the PBMC from 44 patients with ITP and 21 healthy volunteers was examined. The study protocol was approved by the Human Ethics Review Committees of Mie University Graduate School of Medicine and a signed consent form was obtained from each subject. Among these ITP patients, 7 were newly diagnosed while 37 were in the chronic phase (CP) when this study was done. The patients with ITP were classified into three groups according to their platelet count, as follows: Group I; platelet $<50\times10^9$/L, Group II; platelet $5\text{-}10\times10^9$/L, Group III; platelet $>10\times10^9$/L.

Flow cytometry

PBMC were isolated by Ficoll density gradient centrifugation, then the $CD4^+CD25^+$ T cell population was measured using a flow cytometric analysis. The cells were washed with PBS with 2% FCS, and then were stained for 20 min at 4 °C with an optimal dilution of each antibody for Cy-Chrome labeled anti-CD4 and FITC labeled anti-CD25 (BD Biosciences). The cells were washed again and analyzed by flow cytometry (FACScalibur™ and CELLQuest™ software; Becton Dickinson).

ELISA assay for anti-platelet antibodies

PAIgG was measured using alkaline phosphatase-conjugated anti-human IgG (Sigma Co, St Louis, Missouri, USA) by a competitive enzyme immunoassay. Antibody to platelet glycoproteins (GP) IIb-IIIa was detected using a platelet antibody screening kit (GTI PAKPLUS, Wisconsin, USA). The serum was incubated in microtiter wells which had been precoated with platelet GPIIb-IIIa. An alkaline phosphatase labeled anti-human globulin reagent was added to the wells. After a brief incubation period, the microtiter wells were washed, and the enzyme substrate PNPP (*p*-nitrophenyl phosphate) was added. The optical density of the color produced was measured in an ELISA reader at a wavelength of 405 or 410 nm.

Quantitative real-time polymerase chain reaction analysis

The mRNA was extracted using an MACS mRNA Isolation Kit (Miltenyi Biotec, Auburn, Calfornia, USA) according to the manufacturer's instructions, and cDNA was prepared with 2.5 μM random hexamers

Table 1 Character of patients with ITP

	ITP (n=44)
Sex	16
Male	28
Female	48 (20-80)
Median age (range)	56 (2-403)
Median platelet count ($\times 10^9$/L)	
Medication	
(−)	14
Prednisolone	23
Immune suppressive therapy	5
Others	2
Splenectomy	
(+)	8
(−)	36

(Applied Biosystems Inc., Foster City, California, USA). The massage level was quantified by real-time PCR using the iCycler iQ Real-Time PCR Detection System (Applied Bio-Rad Laboratories Inc.) Amplification was carried out in a total volume of 20 μl for 50 cycles of 15 s at 95 °C, 1 min at 60 °C, and the product was detected using SYBR Green I dye (Molecular Probes Inc., Eugene, Oregon, USA). Samples were run in triplicate, and their relative expression was determined by normalizing the expression of each target to GAPDH, and then comparing this normalized value to the normalized expression in a reference sample to calculate a fold-change value. The primers were designed so that amplicons spanned intron/exon boundaries to minimize the amplification of genomic DNA. The primer sequences were as follows: GAPDH: sense, 5′-CCC ATG TTC GTC ATG GGT GT-3′ and anti-sense, 5′-TGG TCA TGA GTC CTT CCA CGA TA-3′; Foxp3: sense, 5′-CCC AGG AAG GAC AGC ACC CTT-3′ and anti-sense, 5′-TTC TCA CAT CCG GGC CAC TTG-3′.

Statistical analysis

All data were expressed as the mean ± SD or median values. Differences between the groups were examined for statistical significance using the Mann-Whitney's U test while the correlation between two variables was tested by Pearson's correlation analysis. A P value of less than 0.05 denoted the presence of a statistically significant difference.

Results

Patients

In this study, 44 cases with ITP were studied. Among these patients, the median age at examination was 48 years (range, 20-80) consisting of 16 males and 28 females. Median platelet count at this study was 56×10^9/L (range, 2-403 $\times 10^9$/L). Thirty patients had been taking various medications for ITP until this examination, specifically, 23 patients had been taking prednisolone, 5 patients had been taking immunosuppressive therapy, and 2 patients had been taking other drugs. A splenectomy was performed in 8 cases for the treatment of ITP (Table 1).

Number of CD4⁺CD25⁺ cells in the patients with ITP

In each case, the percentage of CD4⁺CD25⁺ cells in the PBMC was examined by flow cytometry, and then the number of CD4⁺CD25⁺ cells was calculated. The mean number of CD4⁺CD25⁺ cells in healthy volunteers was $69.2 \pm 13.6 \times 10^6$/L and that of 44 patients with ITP was $73.8 \pm 38.3 \times 10^6$/L. In comparison to the healthy volunteers, the number of CD4⁺CD25⁺ cells in the patients with ITP showed a very wide distribution (Fig. 1) (to analyze the relationship between the platelet count and the number of CD4⁺CD25⁺ cells, ITP were classified into three groups according to their platelet count; Group I $< 50 \times 10^9$/L, 50×10^9/L ≤ Group II ≤ 100×10^9/L, Group III $> 100 \times 10^9$/L). All patients in Group III were treated by some type of medication and their platelet count thus improved. As shown in Fig. 2, the number of CD4⁺CD25⁺ cells of the patients in Group I ($59.7 \pm 27.5 \times 10^6$/L) were closely similar to those of the healthy volunteers. On the other hand, the number of CD4⁺CD25⁺ cells of the patients in Group III ($100.8 \pm 37.3 \times 10^6$/L) was

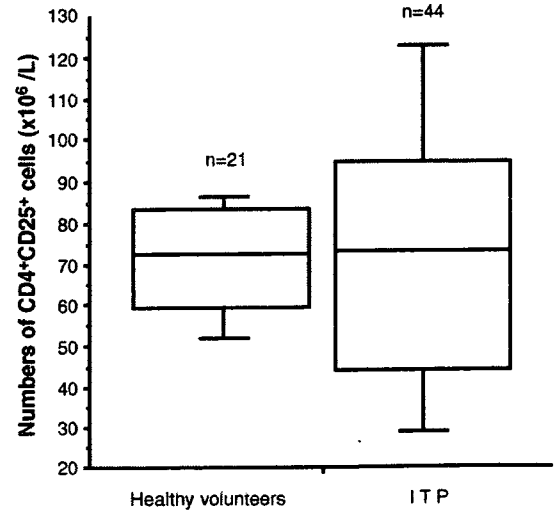

Figure 1 The number of CD4⁺CD25⁺ cells in the patients with ITP and healthy volunteers. The mean number of CD4⁺CD25⁺ cells was $69.2 + 13.6 \times 10^6$/L in healthy volunteers and $73.8 \pm 38.3 \times 10^6$/L in the patients with ITP.

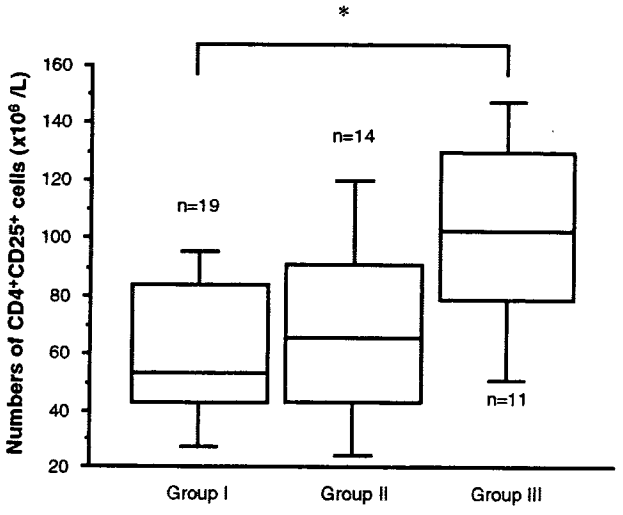

Figure 2 The number of $CD4^+CD25^+$ cells in patients with ITP for various platelet counts. I; less than 50×109/L of platelet count, II; 50-100×109/L of platelet count, III; more than 100×109/L of platelet count. *P=0.018.

significantly higher than that of healthy volunteers. All together, the number of the $CD4^+CD25^+$ cells in the patients before treatment was similar to that of the healthy volunteers (data not shown).

Association between the number of $CD4^+CD25^+$ cells and a splenectomy

A splenectomy is one of the effective treatments for ITP, therefore, the number of $CD4^+CD25^+$ cells was investigated in the ITP patients who had undergone a splenectomy (Fig. 3). In 8 patients treated with a splenectomy, the platelet counts improved in 5 patients. It is very interesting that the number of $CD4^+CD25^+$ cells in the patients who had undergone a splenectomy was significantly higher (108.2± 33.4×10^6/L) than in either those without a splenectomy (P=0.038) or healthy volunteers (data not shown). Furthermore, 8 patients who had their

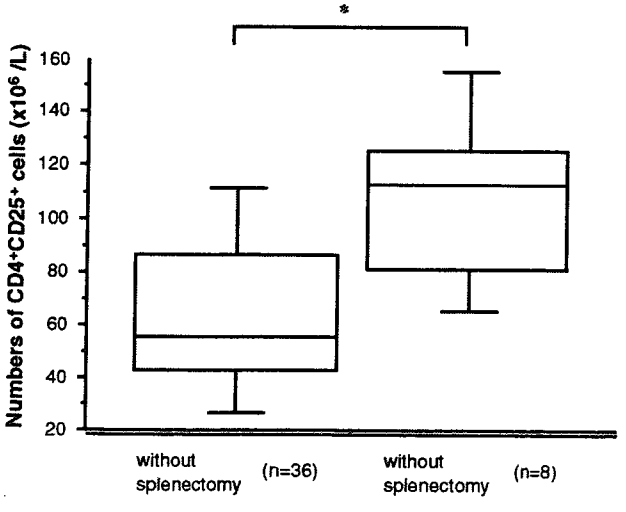

Figure 3 The number of $CD4^+CD25^+$ cells in the ITP patients with or without a splenectomy. The number of $CD4^+CD25^+$ cells was 66.2±35.3×106/L in the patients without a splenectomy and 108.2±33.4×106/L in those with a splenectomy. *; P=0.038.

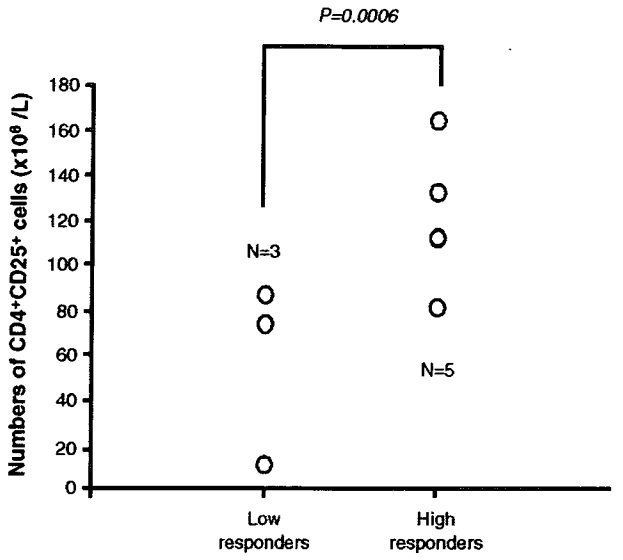

Figure 4 Relationship between the effect of spelenectomy and the number of $CD4^+CD25^+$ cells. Eight patients with ITP treated splenectomy were classified into two groups according to the effect of a splenectomy. In low responders, the increase in the platelet count was less than 10×10^9/L after a splenectomy. In high responders, the increase in the platelet count was more than 10×10^9/L after a splenectomy.

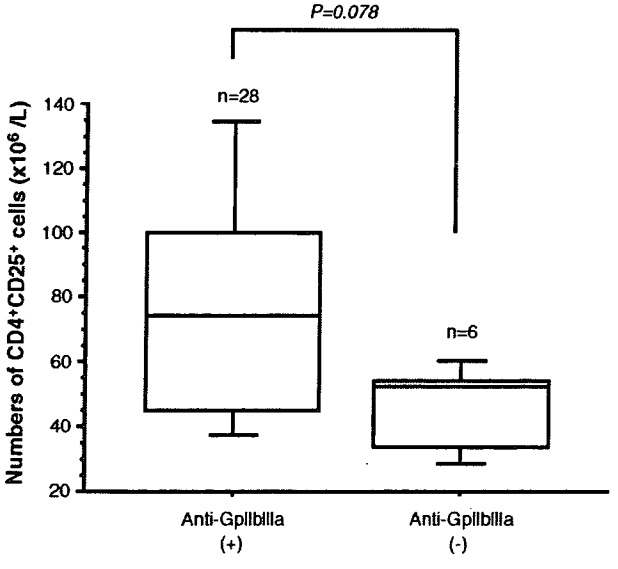

Figure 5 Relationship of between the anti-platelet antibody and the number of $CD4^+CD25^+$ cells.