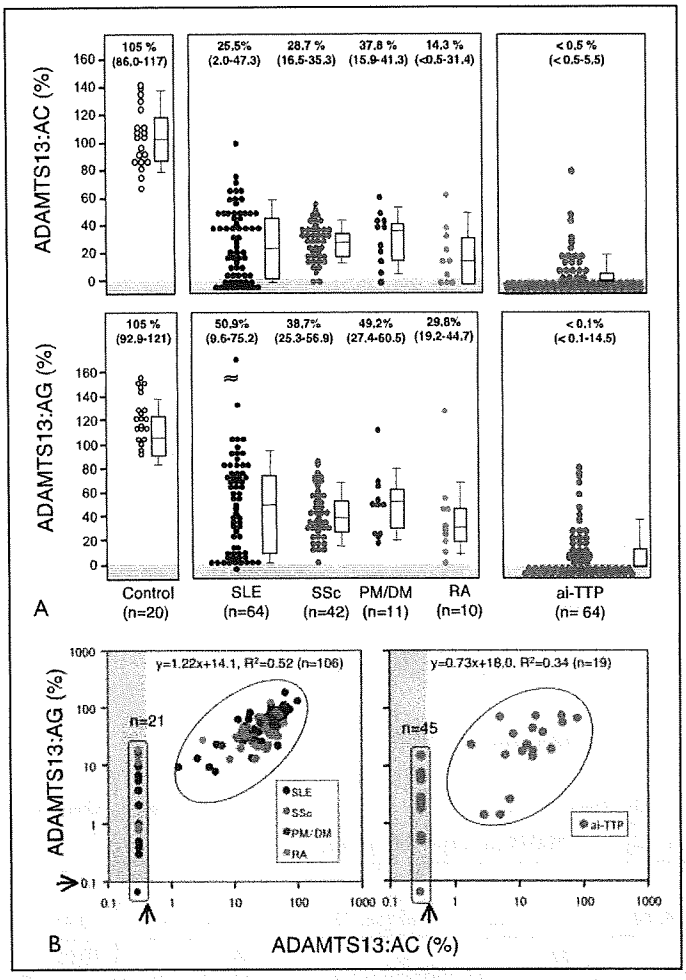

Figure 1: Plasma levels of ADAMTS13:AC and ADAMTS13:AG in patients with CTD-TMA or ai-TTP. A; top) Plasma levels of ADAMTS13:AC measured by chromogenic ADAMTS13-act-ELISA in patients with CTD-TMA or ai-TTP. The median values (25, 75 percentiles) of ADAMTS13:AC in these patients are shown at the top of the figure. A; bottom) Plasma levels of ADAMTS13:AG measured by ag-ELISA. The median values (25, 75 percentiles) of ADAMTS13:AG in these patients are shown at the top of the figure. B) Relationship between plasma levels of ADAMTS13:AC (x) and ADAMTS13:AG (y).

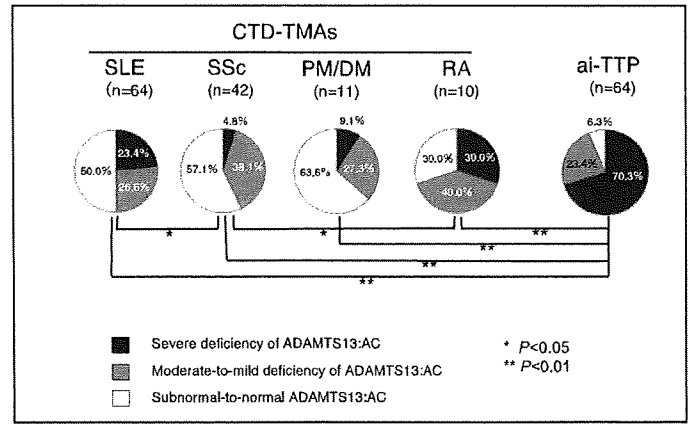

Figure 2: The proportion of CTD-TMA or ai-TTP patients grouped by three levels of plasma ADAMTS13:AC. The proportion of severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal plasma ADAMTS13:AC is illustrated in this figure. Comparison among five patient groups was tested for statistical significance using chi-square tests with Yates' correction for 3 x 5 tables. Significant differences between five groups (overall $p < 0.01$) were found. Comparison between pairs of groups was further investigated by chi-square test.

ai-TTP. Serum creatinine levels of patients with SLE were significantly lower in patients with RA. Interestingly, the plasma levels of VWF:Ag in patients with PM/DM and RA were significantly higher than in ai-TTP patients. PE was conducted in the majority of patients in all groups. This therapeutic approach resulted in a high remission rate in patients with SLE and ai-TTP, but was less effective in patients with SSc and RA.

Plasma levels of ADAMTS13:AC and ADAMTS13:AG

The average plasma levels of ADAMTS13:AC in each category of CTD-TMA and ai-TTP were significantly decreased compared to the levels in normal controls ($p<0.01$) (Fig. 1A top). On the other hand, plasma levels of ADAMTS13:AC in patients with CTD-TMAs were increased compared to the levels in ai-TTP patients. The distribution of ADAMTS13:AC was quite different depending on the category of CTD (Fig. 2). The proportion of severe ADAMTS13:AC deficiency in SSc patients was lower than that in SLE, PM/DM, and RA patients. In contrast, 70.3% of the 64 patients with ai-TTP had severe ADAMTS13:AC deficiency, higher than that of any individual category of CTD-TMA ($p<0.01$).

Plasma levels of ADAMTS13:AG in each category of CTD-TMA patients were also significantly decreased relative to the levels in normal controls ($p<0.01$). Further, plasma levels of ADAMTS13:AG in patients with CTD-TMA were also increased relative to the levels in ai-TTP patients (Fig. 1A bottom).

Next, we examined the relationship between the plasma levels of ADAMTS13:AC and ADAMTS13:AG in both the CTD-TMA and ai-TTP patient groups (Fig. 1B). Twenty-one CTD-TMA patients with severe deficiency ADAMTS13:AC had ADAMTS13:AG levels ranging from less than 0.1% to 19.1% of normal control. On the other hand, 106 patients with detectable ADAMTS13:AC (≥0.5%) had a good correlation between plasma levels of ADAMTS13:AC and ADAMTS13:AG levels ($R^2=0.52$, $p<0.01$). However, the plasma levels of ADAMTS13:AG were almost always higher than the levels of ADAMTS13:AC (Fig. 1B left). This is probably because the presence of circulating anti-ADAMTS13 antibody and ADAMTS13 antigen complexes. These observations were comparable to ai-TTP, in which 45 patients with undetectable ADAMTS13:AC had ADAMTS13:AG levels ranging from less than 0.1% to 14.8% of normal control, whereas 19 patients with detectable ADAMTS13:AC had a good correlation between plasma levels of ADAMTS13:AC and levels of ADAMTS13:AG ($R^2=0.34$, $p<0.01$) (Fig. 1B right).

Severe deficiency of ADAMTS13:AC associated with autoantibodies to ADAMTS13

To investigate the role of anti-ADAMTS13 autoantibodies in severe deficiency of ADAMTS13:AC, we evaluated plasma levels of ADAMTS13:INH in the CTD-TMA and ai-TTP patient groups (Fig. 3A). In 65 of 127 (51.2%) CTD-TMA patients,

plasma levels of ADAMTS13:INH were greater than 0.5 BU/ml. Interestingly, a significant variation was observed, depending on the underlying disease: 60% of RA patients, 54.5% of PM/DM patients, 50% of SLE patients, and 50% of SSc patients had ADAMTS13:INH levels greater than 0.5 BU/ml. In contrast, 55 (85.9%) ai-TTP patients had plasma levels of ADAMTS13:INH greater than 0.5 BU/ml.

Twenty-one CTD-TMA patients with undetectable ADAMTS13:AC had ADAMTS13:INH levels ranging from 0.7 to 125 BU/ml. However, the patients with detectable ADAMTS13:AC had ADAMTS13:INH levels between <0.5 and 1.0 BU/ml, and no correlation between these two parameters was found (R^2=0.021) (Fig. 3B left). Similarly, in ai-TTP patients with undetectable ADAMTS13:AC, ADAMTS13:INH levels ranged from 0.5 to 20 BU/ml, and those patients with detectable ADAMTS13:AC had ADAMTS13:INH levels between <0.5 and 1.5 BU/ml. No correlation between these two parameters was found (R^2=0.058) for this patient group (Fig. 3B right).

As shown in Figure 3C, 21 CTD-TMA patients with undetectable ADAMTS13:AC were analysed for IgG-autoantibodies to ADAMTS13 by immunoblotting. A total of 18 of 21 (86%) patient plasmas displayed a 170 kD-band, indicating the presence of IgG-autoantibodies reacting with the purified ADAMTS13 under non-reducing conditions.

Plasmas from CTD-TMA patient groups with detectable ADAMTS13:AC were analysed by immunoblotting, and positive IgG-autoantibodies were detected in only 2/40 (5%) patients with moderate-to-mild ADAMTS13:AC deficiency, and 0/66 (0%) patients with subnormal-to-normal activity (data not shown).

Thus, in CTD-TMA patients with severe deficiency of ADAMTS13:AC, the presence of ADAMTS13:INH apparently had a high correlation with the appearance of IgG-autoantibodies reacting with purified ADAMTS13.

Clinical features and therapeutic outcomes of patients evaluated by plasma levels of ADAMTS13:AC

The clinical features and laboratory findings as well as therapy and outcome in the CTD-TMA and ai-TTP patient groups have been evaluated and categorised relative to three plasma levels of ADAMTS13:AC: severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal activity (Table 2). In CTD-TMA patients, gender disparity (female predominance) was not remarkable among the three groups of CTD-TMA patients. The frequency of renal involvement was apparently lower in patients with severe deficiency than in other CTD-TMA patients ($p<0.01$). Both platelet counts and serum creatinine levels of patients with severe ADAMTS13:AC deficiency tended to be lower than those in patients with moderate-to-mild deficiency or subnormal-to-normal activity. Plasma levels of VWF:Ag were in almost the same ranges in all three groups. These clinical features associated with the levels of ADAMTS13:AC were also observed in ai-TTP patients. But, there was no apparent difference in response to individual therapeutic regimens. Further, the tendency of higher remission and lower mortality rates was observed in patients with severe deficiency than in those with moderate-to-mild deficiency and with subnormal–to-normal activity ($p=0.053$).

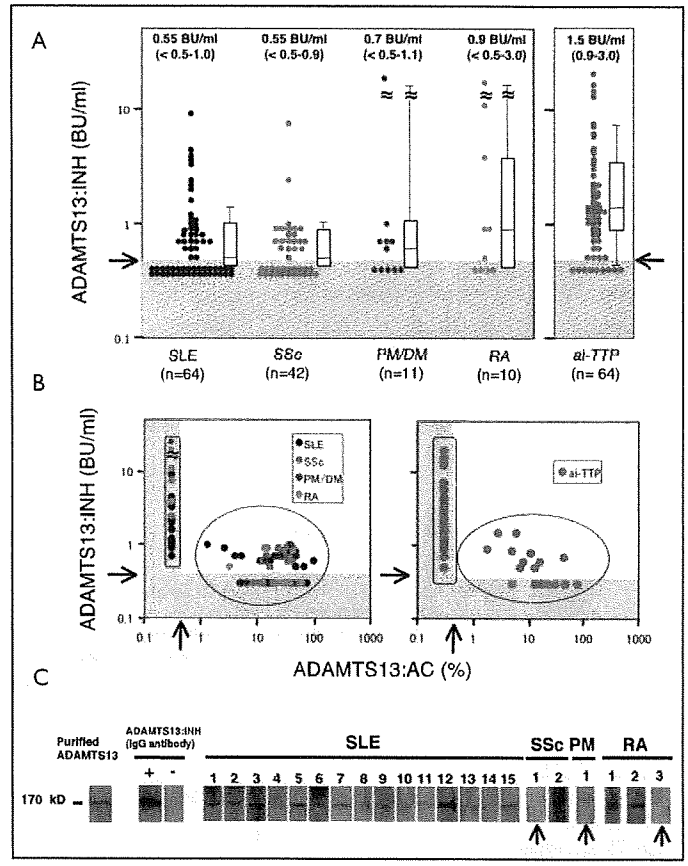

Figure 3: Plasma levels of ADAMTS13:INH and immunoblot analysis of IgG-autoantibodies to ADAMTS13. A) Plasma levels of ADAMTS13:INH in both the CTD-TMA and ai-TTP patient groups. The median values (25, 75 percentiles) of ADAMTS13:INH in these patients are shown at the top of the figure. B) Relationship between plasma levels of ADAMTS13:AC (x) and ADAMTS13:INH (y). C) Detection of IgG-autoantibodies specific to purified ADAMTS13 by immunoblot under non-reducing conditions. A total of 18 of 21 (86%) patient plasmas displayed a 170 kD-band, indicating the presence of IgG-autoantibodies reacting with purified ADAMTS13. The samples with an arrow indicate the IgG-autoantibodies negative (see *Results* for detail). In the left lane, SDS-5% PAGE analysis of purified pd-ADAMTS13 revealed a 170kD-band before reduction. In the next lane, heated plasma from ai-TTP patient with IgG inhibitors against ADAMTS13 (+) displayed a 170kD-band as a positive control, while that from normal individual without ADAMTS13:INH (-) showed no band (a negative control).

Discussion

By analysing 127 CTD-TMA patients, we have shown heterogeneous pathogenic processes of the TMAs, with a minor population defined by severe deficiency of ADAMTS13:AC due to the presence of IgG-inhibitors, and a major population consisting of two subgroups categorised by plasma levels of ADAMTS13:AC: moderate-to-mild deficiency with or without inhibitors, and subnormal-to-normal activity without inhibitors. The percentage of severe deficiency of ADAMTS13:AC in each patient group showed significant variation depending on the underlying disease: severe deficiency was more frequent in patients associated with RA or SLE, and was much less frequent in patients with SSc or PM/DM. These results contrast sharply with

64 ai-TTP patients, which showed a major population (70.3%) with severe deficiency of ADAMTS13:AC due to IgG-inhibitors, and two minor populations of 23.4% with moderate-to-mild deficiency and 6.3% with subnormal-to-normal activity.

Among the clinical features of CTD-TMA patients prior to treatment, serum creatinine levels and platelet counts appeared to be lower in the group with severe deficiency of ADAMTS13:AC than in the moderate-to-mild and subnormal-to-normal subgroups. However, the levels of haemoglobin and plasma VWF:Ag were almost indistinguishable among these three patient groups. Similarly, for ai-TTP patients, the levels of serum creatinine in the group with severe deficiency of ADAMTS13:AC tended to be lower than those of the other two subgroups. These results were in agreement with a previous report of ai-TTP by Vesely et al. (39).

CTD-TMA patients with severe deficiency of ADAMTS13:AC seemed to have better outcomes than patients with moderate-to-mild deficiency (p=0.078). Similarly, the therapeutic outcomes of ai-TTP patients were also likely to be better in patients with severe deficiency of ADAMTS13:AC than in those with moderate-to-mild deficiency (p=0.094), as previously reported (39–41). Thus, except for the differences in proportion of the CTD-TMA with severe ADAMTS13:AC deficiency (16.5%) and ai-TTP with severe deficiency (70.3%), the clinical features and therapeutic outcomes were comparable in these two patient groups.

Pathogenesis of CTD-TMA with severe deficiency of ADAMTS13:AC due to IgG-autoantibodies can be explained in a manner similar to typical ai-TTP: in the absence of ADAMTS13:AC the UL-VWFMs produced in vascular en-

Table 2: Clinical features and therapeutic outcomes according to the levels of plasma ADAMTS13:AC.

	CTD-TMAs (n=127)			ai-TTP (n=64)		
	Severe deficiency (n=21)	Moderate-to-mild deficiency (n=40)	Subnormal-to-normal activity (n=66)	Severe @deficiency (n=45)	Moderate-to-mild deficiency (n=15)	Subnormal-to-normal activity (n=4)
ADAMTS13:AC (%)	< 0.5	0.5 ~ <25	25 ~	< 0.5	0.5 ~ <25	25 ~
Clinical features						
Median age at onset of TMAs, years (25, 75 percentile)	44 (30, 56)	60 (54, 68)	49 (22, 55)	46 (37, 62)	69 (54, 75)	77 (74, 82)
Female (%)	86	90	88	71	53	25
Renal involvement (%)	71*	100*	97*	78	93	100
CNS involvement (%)	71	73	53	67	87	50
Laboratory findings at TMA diagnosis						
Median platelet count, 10^9/l (25, 75 percentile)	8 (6, 14)	37 (20, 55)	41 (22, 55)	15 (8, 16)	19 (10, 24)	19 (8, 47)
Median haemoglobin, g/dl (25, 75 percentile)	7.6 (6.8, 9.2)	7.3 (6.6, 8.0)	7.8 (6.4, 9.2)	7.8 (6.1, 9.0)	8.1 (7.2, 9.8)	7.5 (6.1, 10.3)
Median serum creatinine, mg/dl (25, 75 percentile)	0.9 (0.6, 1.6)	2.7 (1.3, 4.2)	2.3 (1.2, 3.1)	1.2 (0.7, 1.2)	2.2 (1.4, 3.0)	5.0 (3.6, 11.3)
Median VWF:Ag , % (25, 75 percentile)	207 (169, 316)	248 (190, 362)	273 (164, 380)	143 (115, 199)	147 (104, 213)	171 (119, 248)
Therapies						
Plasma exchange (%)	81	70	73	82	53	100
Plasma infusion without plasma exchange (%)	24	23	27	15	40	75
Steroid therapy without pulse therapy (%)	100	80	76	60	60	50
Steroid pulse therapy (%)	100	80	76	11	20	25
Rituximab (%)	0	0	0	11	0	0
Immunosuppressants (%)	33	18	23	7	13	0
Therapeutic response	(n=21)	(n=31)	(n=41)	(n=45)	(n=13)	(n=3)
Remission of TMAs (%)	76	45	61	87	54	67
Death due to TMAs (%)	24	55	39	13	46	33

All comparisons among three groups (severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal ADAMTS13 activity in both CTD-TMAs and ai-TTP) were tested for statistical significance using the Kruskal-Wallis H test or chi-square tests with Yates' correction for 2 x 3 tables. Significant differences between 3 groups (overall $P < 0.05$) were further analyzed by Mann-Whiteney U-test or chi-square test. *Significant difference ($P<0.05$) was only found in renal involvement between severe deficiency and moderate-to-mild deficiency, subnormal-to-normal activity of CTD-TMAs ($P<0.01$).

dothelial cells and released into circulation aggregate platelets excessively under high shear stress, which is exclusively generated at microvasculature networks with luminal narrowing, hyperviscosity, and high flow rate of blood (10, 42).

On the other hand, for the patient subgroups of CTD-TMA with moderate-to-mild deficiency and subnormal-to-normal ADAMTS13:AC, which represent the major population here, it is hard to address the underlying mechanisms. It is well known that congenital deficiency of ADAMTS13:AC (Upshaw-Schulman syndrome) may have mild or absent clinical signs during childhood (23, 24, 43). However, once a rapid increase of plasma VWF, mediated by inflammation, pregnancy, or DDAVP (1-deamino-8-D-arginine vasopressin) administration occurs, TMA bouts are consistently induced (23, 24, 43). These observations indicate that the appearance of TMA bouts depends on the equilibrium between the amount of enzyme (ADAMTS13) and substrate (UL-VWFMs), and may be induced by an extremely low enzyme-to-substrate ratio in circulation (37, 44). In fact, the frequency of TMA has been reported to be 1–6% in the CTD patient population (45), whereas the annual incidence of ai-TTP-HUS has been reported to be 0.00037%-0.00 0446% (3.7–4.46 per million) per year (46, 47). We assume that a high prevalence of TMA bouts associated with CTD might be closely related to high VWF plasma levels (207–339%), together with deficient or moderate-to-mild decrease of plasma ADAMTS13:AC (Table 2). Anatomical changes of the microvasculature in CTD-TMA patients are another point of interest. These patients have narrowed vessel cavities due to the proliferation of vascular endothelial cells, and this generates higher shear stress. Under these circumstances, platelets tend to aggregate more extensively and elevated plasma levels of VWF with or without UL-VWFMs accelerate this reaction, causing platelet thrombi to form. Impaired vascular endothelial function due to vasculitis caused by CTDs may also prevent the efficient cleavage of UL-VWFMs by ADAMTS13. This speculation is derived from recent observations, in which ADAMTS13 most efficiently works as a solid-phase enzyme, after binding to cell surfaces such as vascular endothelial cells and platelets via hitherto unrecognised anchoring systems (48). We therefore speculate that anatomical changes such as luminal narrowing of blood vessel walls alter haemodynamics and generate high shear stress, and the impaired vessel walls fail to bind ADAMTS13 for the efficient cleavage of UL-VWFMs. Coexistence of these two factors apparently accelerates platelet clumping in the presence of high plasma levels of VWF, leading to TMA. High prevalence of CTD-TMA, despite lower frequency of severe deficiency of ADAMTS13:AC, may be explained by these mechanisms.

Since this study was conducted at Nara Medical University on behalf of patients and physicians across Japan, we do not have data on plasma levels of ADAMTS13:AC in CTD-patients without TMA. However, such a control study was previously reported by Mannucci et al. (49) in 2003, who showed that SLE-patients without TMA had slightly but significantly reduced plasma levels of ADAMTS13:AC in comparison to normal individuals (Mean ± standard deviation [SD]: 89 ± 33% vs. 107 ± 27, p=0.013). In fact, none of these CTD patients without TMA had severe deficiencies in ADAMTS13:AC or detectable ADAMTS13:INH. Although we are presently unable to address the difference in plasma levels of ADAMTS13:AC between Japanese and Europeans, Rieger et al. (50) confirmed the results of Mannucci et al. (49) by analysing 40 SLE-patients without TMA, and finding that five of their 40 (13%) patients had non-neutralising IgG-autoantibodies against ADAMTS13. Thus, it is also possible that non-inhibitory autoantibodies to ADAMTS13 may play an important role in the pathogenesis of CTD-TMA without severe deficiency of ADAMTS13:AC, possibly through enhanced clearance of ADAMTS13 from the circulation or by blocking the enzyme from the cell surface for efficient cleavage of UL-VWFMs.

We have demonstrated the existence of at least two phenotypic TMAs related to CTDs: a minor population caused by deficient ADAMTS13:AC with neutralising autoantibodies, as typically shown in ai-TTP, and a major population without these autoantibodies. Interestingly, the former group has had better therapeutic outcomes than the latter.

What is known about this topic?

- Acquired idiopathic thrombotic thrombocytopenic purpura (ai-TTP) is typically defined as 'severe' deficiency of ADAMTS13 activity due to its IgG-autoantibodies, by the previous assays but with a limited sensitivity (3–5% of normal controls).
- However, the pathogenesis of connective tissue disease-associated thrombotic microangipathies (CTD-TMA) has been largely unknown, because only few reports have shown to have 'severe' deficiency of ADAMTS13 activity, in spite of its quite high prevalence (1–6% of patients with CTDs).

What does this paper add?

- By analysing 64 patients with ai-TTP and 127 patients with CTD-TMA, using a highly sensitive ELISA assay for ADAMTS13 activity, frequency of true 'severe' deficiency of ADAMTS13 activity (<0.5% of normal) was identified in 70% in the former and 17% in the latter.
- CTD-TMAs have been categorised into the following three groups; severe, moderate-to-mild, and subnormal-to-normal activity of ADAMTS13. These 3 groups were comparatively analysed on their clinical and therapeutic outcomes.
- Clinical and therapeutic outcome of patients of CTD-TMA with 'severe' deficiency was almost comparable to that of ai-TTP, but the remaining two groups of CTD-TMAs had apparently poor results. Differential mechanistic pathogenesis of these three groups has been extensively discussed.

Acknowledgements

The authors would like to thank Dr. Friedrich Scheiflinger of Baxter BioScience (Vienna) for his helpful discussion at the beginning stage of preparation of this manuscript.

Conflict-of interest disclosure

Y. F. is a member of clinical advisory boards for Baxter BioScience.

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Int J Hematol (2009) 90:157–165
DOI 10.1007/s12185-009-0361-y

ORIGINAL ARTICLE

A phase II, open-label, sequential-cohort, dose-escalation study of romiplostim in Japanese patients with chronic immune thrombocytopenic purpura

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Received: 16 March 2009 / Revised: 4 May 2009 / Accepted: 25 May 2009 / Published online: 20 June 2009

Abstract This phase II, multicenter, open-label, sequential-cohort, dose-escalation study was designed to evaluate the safety and efficacy of romiplostim, a novel peptibody that increases platelet production, in Japanese patients with chronic immune thrombocytopenic purpura (ITP). Sequential cohorts of four patients each received romiplostim (1, 3, or 6 μg/kg) subcutaneously on days 1 and 8 of the dose-escalation phase. Patients who achieved platelet responses (doubling of baseline platelet counts to $\geq 50 \times 10^9$/L) continued romiplostim weekly during the treatment-continuation phase. Romiplostim produced dose-dependent increases in mean and peak platelet counts. Five patients received romiplostim during the treatment-continuation phase, with platelet counts $\geq 50 \times 10^9$/L maintained in approximately half of the weekly assessments. Romiplostim was well tolerated. No severe, serious, or life-threatening adverse events were reported. No binding antibodies to romiplostim or thrombopoietin were detected. Romiplostim is safe and well tolerated in Japanese patients with chronic ITP and is effective in producing platelet count increases, consistent with the results from studies in non-Japanese patients. On the basis of these findings, a starting dose of 3 μg/kg was recommended for phase III evaluation of romiplostim in Japanese patients with chronic ITP.

Keywords Immune thrombocytopenic purpura · Romiplostim · Thrombopoiesis · Platelet production

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1 Introduction

Chronic immune thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by antibody-mediated platelet destruction and suboptimal platelet production [1–4]. Initial therapy typically consists of corticosteroids or intravenous immunoglobulins (IVIg), followed by low-dose corticosteroids (prednisone, 1–2 mg/kg/day) if thrombocytopenia persists. Either repeated administrations of IVIg or splenectomy can be used for intolerance or insufficient response to low-dose corticosteroids [1, 3]. These treatments as well as others used in refractory patients, such as rituximab and cyclophosphamide, suppress the rate of platelet destruction [5–7]. However in many cases they are either transiently effective, insufficiently effective, or poorly tolerated. In addition to the platelet destruction, the rate of platelet production is

inadequate in a majority of patients with chronic ITP [4, 8–11]. Accordingly, treatments that increase platelet production may offer the potential for improved control and outcomes in chronic ITP [12]. Platelet production is primarily regulated by thrombopoietin (TPO), which binds to the TPO receptor (c-Mpl) to increase the megakaryocytopoiesis and thrombopoiesis.

The potential clinical benefit of using a thrombopoietic growth factor to treat chronic ITP was initially demonstrated using pegylated recombinant human megakaryocyte growth and development factor (MGDF), a truncated form of human TPO [13]. Administration of MGDF for 7 days increased platelet counts in 3 of 4 Japanese ITP patients and bleeding was decreased. The production of antibodies against MGDF in healthy volunteers that cross-reacted with endogenous TPO [14] resulted in the discontinuation of MGDF clinical studies and led to the development of novel TPO mimetics. Romiplostim (AMG 531) is an Fc-peptide fusion protein (peptibody) that increases platelet production via the same mechanism as endogenous TPO [15, 16]. However, romiplostim does not share sequence homology with TPO. This lack of sequence homology reduces the probability that antibodies to romiplostim, if produced, would cross-react with endogenous TPO and cause further thrombocytopenia [14]. Initial clinical trials with romiplostim in the United States and Europe showed that romiplostim increases platelet counts in healthy volunteers and during short-term use by patients with chronic ITP [17–19]. Recent phase III studies conducted in the United States and Europe showed that romiplostim raised and sustained platelet counts in splenectomized and non-splenectomized patients with chronic ITP during treatment for 24 weeks [20]. In these studies, platelet responses were defined as durable or transient and required that rescue medication had not been administered in the preceding 8 weeks. Durable platelet responses, a very rigorous end point that required platelet counts $\geq 50 \times 10^9$/L for at least 6 of the last 8 weeks of treatment without a need for rescue medication, were achieved significantly more often with romiplostim than with placebo in splenectomized patients (38 vs. 0%; $P = 0.0013$) and non-splenectomized patients (61 vs. 5%; $P < 0.001$). The overall platelet response rate (i.e., either transient or durable responses with platelet counts $\geq 50 \times 10^9$/L for 4 weeks or more) was also significantly higher with romiplostim than placebo in splenectomized patients (79 vs. 0%; $P < 0.0001$) and non-splenectomized patients (88 vs. 14%; $P < 0.0001$). Romiplostim treatment also allowed many patients to reduce or discontinue concomitant ITP therapies and was well tolerated in each of the phase II and III trials in Western countries.

The incidence of chronic ITP in adults in Japan is estimated to be 500–2000 cases annually, a rate of incidence similar to that seen in Western countries [1, 21, 22]. The safety, pharmacodynamics, and pharmacokinetics of romiplostim in Japanese adult patients were demonstrated in a phase I study and were consistent with those seen previously in healthy non-Japanese subjects [21]. In the phase I Japanese study, romiplostim increased platelet counts in a dose-related manner, with four of eight patients who received a dose of 1 μg/kg and seven of eight patients who received a dose of 2 μg/kg having platelet increases ≥ 1.5 times above baseline. The present phase II study was conducted to evaluate the safety and tolerability of romiplostim and its effect on platelet counts in Japanese patients with chronic ITP, and to identify an appropriate starting dose for a phase III study of romiplostim for the treatment of chronic ITP in adult Japanese patients.

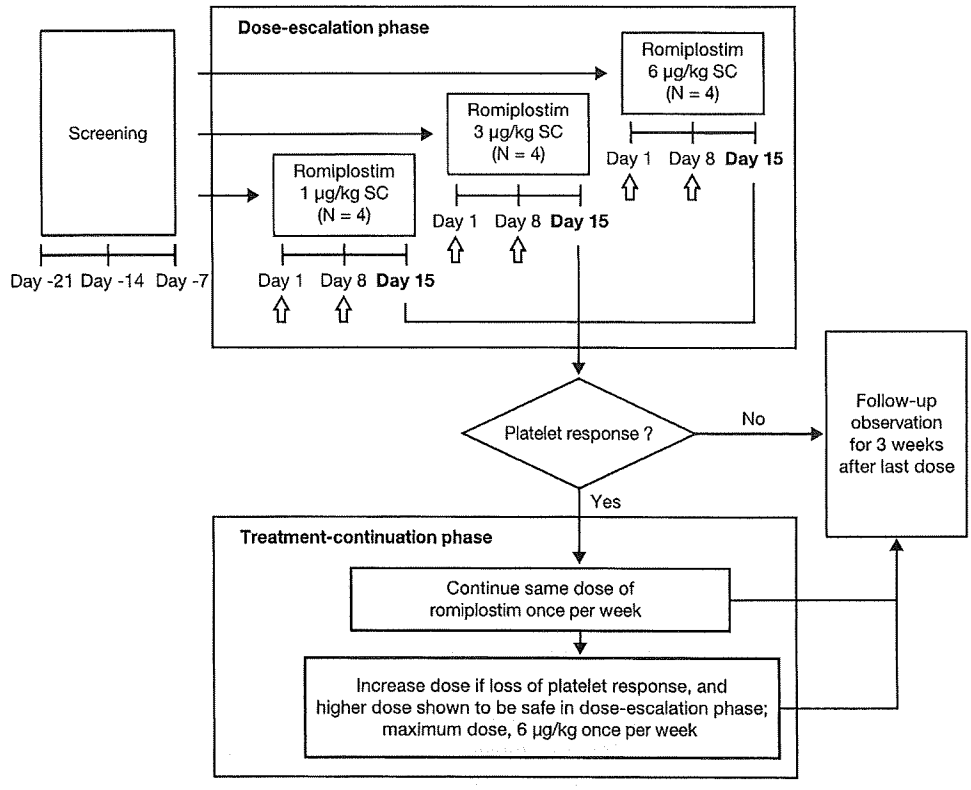
2 Methods

2.1 Study design

This phase II, open-label, sequential-cohort, dose-escalation study was conducted at six centers in Japan and consisted of a 3-week screening period, a 2-week dose-escalation phase and subsequent treatment-continuation phase, and a 3-week follow-up observation period (Fig. 1). It was conducted in accordance with the principles of the Japanese Ministry of Health, Labor and Welfare and International Conference on Harmonization guidelines of Good Clinical Practice. The study protocol and informed consent form were approved by the institutional review board at each study site before any patients were enrolled.

Patients were screened for eligibility over a 3-week period, during which platelet counts were determined each week (days −21, −14, and −7). The baseline platelet count necessary for enrollment was based on the mean of these determinations. Physical examination, vital signs, and laboratory testing were performed at one visit during the screening period. In addition, bone marrow testing was done in all patients aged over 60 years who had not had such testing within the previous 5 years. For the dose-escalation phase, four patients were to be enrolled at each of four sequential dose levels of romiplostim (1, 3, 6, 10 μg/kg). Each dose was administered subcutaneously once weekly for 2 weeks (i.e., days 1 and 8), with no dose adjustments allowed. According to the study protocol, the dose-escalation phase was to be stopped if at least three subjects in a cohort had platelet counts $>450 \times 10^9$/L; at least two subjects in a cohort had platelet counts $>700 \times 10^9$/L; at least one subject in a cohort had platelet counts $>1000 \times 10^9$/L; or if two or more subjects in a cohort had drug-related serious adverse events.

Fig. 1 Study design



Patients who achieved a platelet response, defined as a doubling of the baseline platelet counts to a level $\geq 50 \times 10^9$/L, during the dose-escalation phase were eligible to continue into the treatment-continuation phase. Patients continued to receive romiplostim once weekly at the original dose, with the option of adjusting the dose to achieve platelet counts in a target range of 50–200 $\times 10^9$/L. The treatment-continuation phase ended when the final cohort of the dose-escalation phase was completed. At this point, patients entered the follow-up observation phase and had their end of study (EOS) visit 3 weeks after receiving their last dose of romiplostim. Patients entered the observation phase when (1) they failed to respond to romiplostim during the dose-escalation phase, (2) they lost their response during the treatment-continuation phase (defined by two consecutive platelet counts dropping to baseline), or (3) the final dose-escalation cohort was completed. At the end of the study, platelet responders and non-responders were eligible to enter an open-label extension study.

Rescue medications were permitted in the study for a tendency for severe bleeding or if the investigator thought the patient was at immediate risk. These medications were to be given with the intended purpose of raising platelet counts and included IVIg, platelet transfusion, corticosteroids, or an increase in dose or frequency of concurrent corticosteroids. Romiplostim was to be continued in patients who received rescue medications.

2.2 Patients

Japanese patients aged 20–70 years with a diagnosis of ITP for at least 6 months before the first screening visit were eligible if their mean platelet count measured at the three screening visits was $<30 \times 10^9$/L while not receiving any ITP therapy or $<50 \times 10^9$/L while receiving a stable dose of corticosteroids. Eligible patients had received at least one previous treatment for ITP and had Eastern Cooperative Oncology Group (ECOG) performance status 0–2, adequate renal and hepatic function, and a hemoglobin level ≥ 10 g/dL. Patients who were positive for antibodies to *Helicobacter pylori* had to complete one course of *H. pylori* eradication therapy at least 12 weeks before the first screening visit. All patients provided written informed consent.

Patients with a known history of a bone marrow stem cell disorder or abnormal bone marrow findings other than ITP were excluded as were those with arterial thrombosis within the past year, history of venous thrombosis who were receiving anticoagulation therapy, uncontrolled

cardiac disease, uncontrolled hypertension with diastolic blood pressure above 100 mmHg, high risk of thromboembolic events, active malignancy, or major surgery within the past 8 weeks. Patients were excluded if they were currently receiving any treatment for ITP except a stable dose of oral corticosteroids, or if they had received IVIg, high-dose corticosteroid pulse therapy, any drug administered to increase platelet counts, or hematopoietic growth factors within 4 weeks before the first screening visit, or had undergone splenectomy within 12 weeks.

2.3 Assessments

In the safety assessment, all adverse events observed by the investigator or reported by the patient were recorded, and their severity and relationship to study drug were determined by the investigator. Safety was also assessed by clinical laboratory testing (hematology, clinical chemistry, and coagulation), by the measurement of vital signs and electrocardiogram and by physical examination. In addition, blood samples were collected on day 1, at week 7, and then once every 8 weeks during the treatment-continuation phase, and at the EOS visit to test for induction of serum antibodies. A biosensor immunoassay was used initially to detect antibodies against romiplostim, the biologically active peptide portion of romiplostim or TPO. If a sample tested positive, a cell-based bioassay was to be used to test for the presence of neutralizing antibodies against romiplostim or TPO activity on cell growth.

Efficacy was assessed by measuring platelet counts. Parameters measured included platelet response (as defined above), peak platelet count, time to peak platelet count, absolute change from baseline to peak platelet count, and ratio of the peak platelet count to the baseline platelet count (i.e., fold change from baseline to peak) in each cohort of the dose-escalation phase.

2.4 Statistics

The planned sample size was four patients per dose-level cohort. The safety and efficacy analyses included all patients who received at least one dose of romiplostim, and they were conducted separately for the dose-escalation and treatment-continuation phases. The primary end point was the incidence of adverse events, including the presence of antibodies against romiplostim. Adverse events were categorized according to the Medical Dictionary for Regulatory Activities (MedDRA), summarized by severity and relationship to study drug, and evaluated using descriptive statistics. Secondary end points included the proportion of patients achieving a platelet response; proportion of patients with various peak platelet counts (including doubling of baseline counts, absolute counts $\geq 50 \times 10^9$/L, $\geq 100 \times 10^9$/L, and $\geq 450 \times 10^9$/L, and increases $\geq 20 \times 10^9$/L over baseline); and the peak platelet count, time to peak, and absolute and fold change from baseline to peak platelet count in the dose-escalation phase. For the efficacy evaluation, the baseline platelet count was defined as the average of four scheduled determinations (days −21, −14, and −7 during the screening period and day 1 pre-dose). Each efficacy end point was evaluated using descriptive statistics.

3 Results

3.1 Patient disposition and demographics

Four patients were enrolled at each of the first three dose levels (1, 3, 6 μg/kg). One patient in the 6-μg/kg cohort had an excessively high-platelet count (980×10^9/L), and consequently dose escalation to 10 μg/kg in a new cohort of patients was not performed. All 12 patients who were enrolled completed the dose-escalation phase, and five patients entered and completed the treatment-continuation phase (Fig. 2).

The study cohort of 12 patients, all of whom were Japanese per the study protocol, had a mean age of 55.6 years, and included eight females (66.7%) (Table 1). Overall, the mean duration since ITP diagnosis was 10.3 years, and the mean baseline platelet count was 11.8×10^9/L. All patients had ECOG performance status 0, except for one patient in the 3-μg/kg cohort with a performance status of 1. Eleven patients (91.7%) had a history of purpura/petechiae, and nine patients (75.0%) had a history of epistaxis and oral bleeding. Seven patients (58.3%) received stable corticosteroid therapy concomitantly with study drug, and three patients had previously undergone splenectomy. Prior medications for ITP included corticosteroids (91.7%), IVIg (58%), and danazol (42%).

3.2 Dose-escalation phase

The mean platelet count increased with romiplostim dose when measured on days 8, 11, and 15 (Fig. 3). The proportion of patients with platelet responses by day 8, the first time platelet counts were assessed following treatment, increased with romiplostim dose from 0% at 1 μg/kg to 50% at 3 μg/kg and 100% at 6 μg/kg (Table 2). By day 11, one of four patients (25%) treated with romiplostim 1 μg/kg also had a platelet response. Overall, seven of the 12 patients (58.3%) achieved platelet responses, including six of eight patients (75.0%) treated with doses of 3 or 6 μg/kg. As shown in Table 2, romiplostim produced dose-related increases in the other efficacy measures. The mean peak platelet count ranged from 44×10^9/L at 1 μg/kg to 374×10^9/L at 6 μg/kg,

Fig. 2 Patient disposition

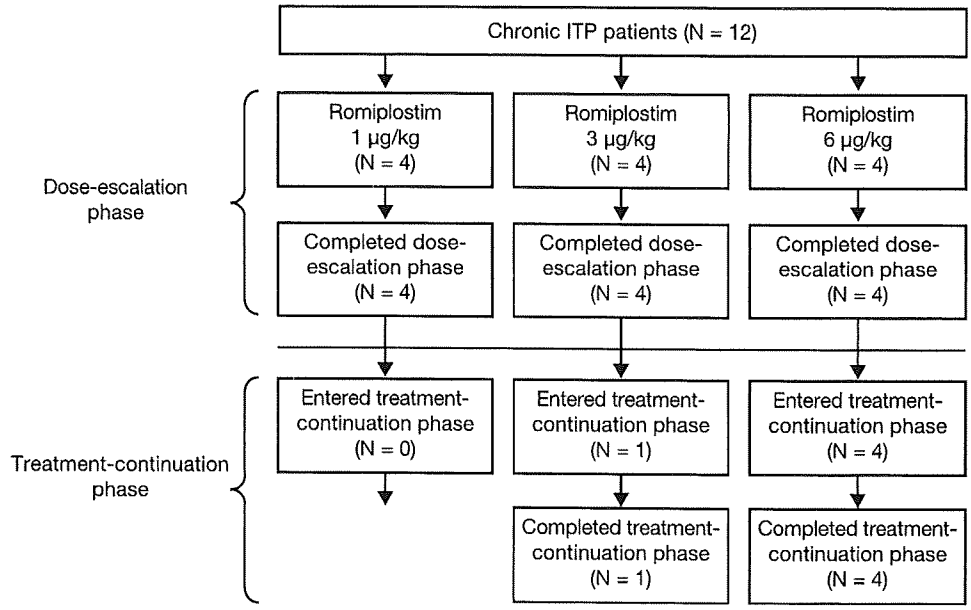


Table 1 Patient demographics and baseline characteristics

Characteristic	Dose cohort			
	1 μg/kg ($n = 4$)	3 μg/kg ($n = 4$)	6 μg/kg ($n = 4$)	Total ($N = 12$)
Sex, *n* (%)				
Female	2 (50.0)	3 (75.0)	3 (75.0)	8 (66.7)
Age (years), mean (SD)	61.5 (1.3)	52.0 (13.7)	53.3 (7.2)	55.6 (9.2)
Years since diagnosis, mean (SD)	11.1 (7.1)	9.0 (4.7)	10.9 (5.9)	10.3 (5.5)
Baseline platelet counts $\times 10^9$/L				
Mean (SD)	9.8 (5.8)	8.8 (9.4)	16.8 (13.0)	11.8 (9.7)
Range	4–15	3–23	5–31	3–31
Corticosteroids, *n* (%)				
Prior use	3 (75.0)	4 (100)	4 (100)	11 (91.7)
Concurrent use	2 (50.0)	3 (75.0)	2 (50.0)	7 (58.3)
Previous splenectomy, *n* (%)	1 (25.0)	0 (0)	2 (50.0)	3 (25.0)
Number of prior ITP therapies[a], mean (SD)	4.5 (3.3)	4.0 (2.1)	3.5 (2.3)	4.0 (2.6)

[a] Excluding splenectomy

with the proportion of patients achieving various cut points for response, including platelet count $\geq 20 \times 10^9$/L above baseline and platelet count $\geq 50 \times 10^9$/L or $\times$ 100 $\times 10^9$/L, also increasing with romiplostim dose. All four patients in the 6-μg/kg cohort achieved these levels of peak response. When compared with the platelet count at baseline, the absolute change ranged from 34 $\times 10^9$/L at 1 μg/kg to 357 $\times 10^9$/L at 6 μg/kg, with the peak values representing a mean 4.1- to 26.2-fold increase above baseline. The mean time to peak response was approximately 13 days and did not differ by dose.

All 12 patients received both scheduled doses of romiplostim. The mean (SD) total dose of romiplostim was 116 ± 18 μg in the 1-μg/kg cohort, 360 ± 70 μg in the 3-μg/kg cohort, and 718 ± 116 μg/kg in the 6-μg/kg cohort. None of the patients received rescue medication.

Romiplostim was well tolerated. Overall, eight patients (66.7%) experienced at least one adverse event, most of which were mild in severity (Table 3). No severe, serious, or life-threatening adverse events were reported, and no patient withdrew due to an adverse event. Six patients (50.0%) had treatment-related adverse events, most commonly headache ($n = 3$; 25.0%). There was no apparent relationship between the romiplostim dose and the incidence of treatment-related adverse events. Other than changes in platelet counts, there were no clinically significant changes in other serum chemistry, hematology, or coagulation laboratory values during the course of the

dose-escalation phase. Similarly, no clinically significant changes in vital signs were observed.

Four patients had adverse events of bleeding. One patient in the 1-μg/kg cohort experienced epistaxis on day 21 (i.e., 13 days after the last dose of romiplostim). The platelet count was not recorded on the day of epistaxis, but was 9×10^9/L on the following day when the patient also experienced mouth hemorrhage. Another patient in the 1-μg/kg group had purpura and tongue hematoma on day 8, which was the day of the second romiplostim dose. This patient never achieved a response to romiplostim and had a platelet count of 2×10^9/L at the time of the bleeding event. One patient in the 3-μg/kg cohort experienced epistaxis on day 12. This subject had platelet counts of 199×10^9/L and 361×10^9/L when measured 2 days before and 3 days after the bleeding event, respectively. Finally, one patient in the 6-μg/kg cohort experienced purpura on day 31 (23 days after the second romiplostim dose); the platelet count was 17×10^9/L on the day of the bleeding event and 38×10^9/L when measured 5 days later.

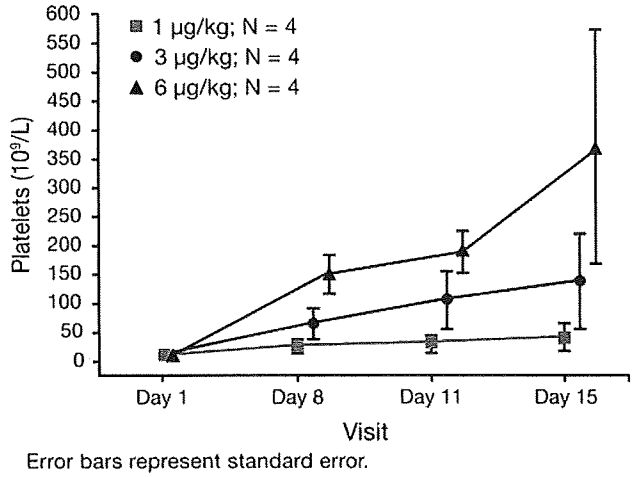


Fig. 3 Mean platelet counts in the dose-escalation phase by romiplostim dose

All patients had a baseline sample and at least one post-baseline sample for testing for the presence of antibodies induced by romiplostim exposure. All tested samples were negative for binding antibodies to romiplostim, the active peptide portion of romiplostim, and TPO in the immunoassay, and therefore no samples were evaluated in the cell-based bioassay.

3.3 Treatment-continuation phase

Five patients entered the treatment-continuation phase, including one patient from the 3-μg/kg cohort and all four patients from the 6-μg/kg cohort. The patient who received romiplostim 3 μg/kg had a baseline platelet count of 6×10^9/L and values ranging from 19 to 115×10^9/L during the treatment-continuation phase. Approximately, half of these weekly assessments showed platelet counts of $>50 \times 10^9$/L (Fig. 4). Mean platelet counts in the four patients who received romiplostim 6 μg/kg generally remained $\geq 100 \times 10^9$/L through week 13, although values varied widely among the individual patients. By week 14, mean platelet counts had declined to 24×10^9/L, and no additional doses of romiplostim were given under the study

Table 2 Platelet responses to romiplostim in the dose-escalation phase

Efficacy measure	Dose cohort			
	1 μg/kg ($n = 4$)	3 μg/kg ($n = 4$)	6 μg/kg ($n = 4$)	Total ($N = 12$)
Platelet response, *n* (%)	1 (25.0)	2 (50.0)	4 (100)	7 (58.3)
Day 8	0 (0)	2 (50.0)	4 (100)	6 (50.0)
Day 11	1 (25.0)	2 (50.0)	4 (100)	7 (58.3)
Day 15	1 (25.0)	2 (50.0)	4 (100)	7 (58.3)
Platelet count $\geq 20 \times 10^9$/L over baseline, *n* (%)	2 (50.0)	3 (75.0)	4 (100)	9 (75.0)
Platelet count $\geq 50 \times 10^9$/L, *n* (%)	1 (25.0)	2 (50.0)	4 (100)	7 (58.3)
Platelet count $\geq 100 \times 10^9$/L, *n* (%)	1 (25.0)	2 (50.0)	4 (100)	7 (58.3)
Platelet count $\geq 450 \times 10^9$/L, *n* (%)	0 (0)	0 (0)	1 (25.0)	1 (8.3)
Peak platelet count $\times 10^9$/L				
Mean (SE)	44.0 (24.6)	145.8 (80.8)	374.3 (202.1)	188.0 (78.1)
Range	5–116	8–361	153–980	5–980
Change from baseline in peak platelet count				
Fold increase, mean (SE)	4.1 (1.6)	15.9 (6.3)	26.2 (7.2)	15.4 (4.0)
Absolute change $\times 10^9$/L, mean (SE)	34.3 (23.0)	137.0 (76.5)	357.4 (199.4)	176.2 (76.5)
Time to peak platelet count—days, mean (SE)	13.3 (1.8)	11.5 (1.4)	14.0 (1.0)	12.9 (0.8)

Table 3 Summary of safety during the dose-escalation phase

Safety parameter, *n* (%)	Dose cohort			
	1 μg/kg ($n = 4$)	3 μg/kg ($n = 4$)	6 μg/kg ($n = 4$)	Total ($N = 12$)
At least 1 adverse event	3 (75.0)	3 (75.0)	2 (50.0)	8 (66.7)
Serious or severe adverse event	0 (0)	0 (0)	0 (0)	0 (0)
Treatment-related adverse event	1 (25.0)	3 (75.0)	2 (50.0)	6 (50.0)
Headache	1 (25.0)	1 (25.0)	1 (25.0)	3 (25.0)
Fatigue	1 (25.0)	0 (0)	0 (0)	1 (8.3)
Back pain	0 (0)	1 (25.0)	0 (0)	1 (8.3)
Muscle tightness	0 (0)	1 (25.0)	0 (0)	1 (8.3)
Flushing	0 (0)	0 (0)	1 (25.0)	1 (8.3)
Withdrawals due to adverse events	0 (0)	0 (0)	0 (0)	0 (0)
Rescue medication use	0 (0)	0 (0)	0 (0)	0 (0)
Anti-romiplostim neutralizing antibodies	0 (0)	0 (0)	0 (0)	0 (0)

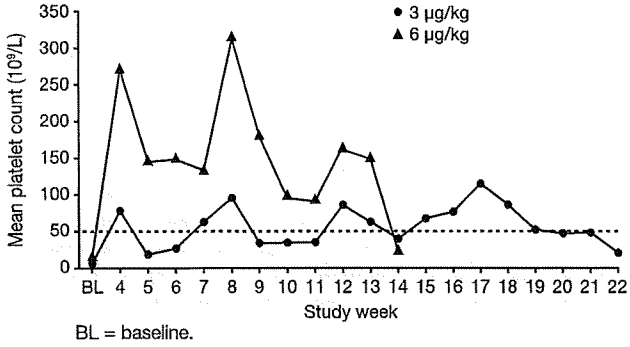


Fig. 4 Mean platelet counts in the treatment-continuation phase by romiplostim dose

protocol due to the completion of the study. However, all five patients in the treatment-continuation phase subsequently entered an open-label extension study to continue romiplostim treatment.

The patients who participated in the treatment-continuation phase received a mean of 10 ± 4.8 doses of romiplostim. The patient in the 3-μg/kg cohort received 18 doses of romiplostim (all doses at 3 μg/kg; total dose 3240 μg). The four patients in the 6-μg/kg cohort received a mean of 8 ± 2 doses (one patient had all doses at 6 μg/kg; three patients had doses adjusted ranging from 0 to 5 μg/kg; mean total dose 2581 μg; range 1775–3030 μg). None of the subjects received rescue medication during the treatment-continuation phase.

The safety and tolerability of romiplostim was comparable to that observed during the dose-escalation phase. No severe, serious, or life-threatening adverse events were reported, and no patient withdrew due to an adverse event. Three patients (60%) had adverse events, most frequently contusion in two subjects receiving the 6-μg/kg dose. Two of the five patients had treatment-related adverse events, with malaise, arthralgia, and contact dermatitis each occurring in one patient treated with 6 μg/kg (Table 4). There were no adverse events of bleeding. Other than the changes in platelet counts, no clinically significant changes were noted in other serum chemistry, hematology, or coagulation laboratory values or clinically significant changes in vital signs during the treatment-escalation phase. Similarly, binding antibodies were not detected by immunoassay in any of the patients.

Table 4 Summary of safety during the treatment-continuation phase

Safety parameter, *n* (%)	3 μg/kg ($n = 1$)	6 μg/kg ($n = 4$)	Total ($N = 5$)
At least 1 adverse event	0 (0)	3 (75.0)	3 (60.0)
Serious or severe adverse event	0 (0)	0 (0)	0 (0)
Treatment-related adverse event	0 (0)	2 (50.0)	2 (40.0)
Malaise	0 (0)	1 (25.0)	1 (20.0)
Arthralgia	0 (0)	1 (25.0)	1 (20.0)
Contact dermatitis	0 (0)	1 (25.0)	1 (20.0)
Withdrawals due to adverse events	0 (0)	0 (0)	0 (0)
Rescue medication use	0 (0)	0 (0)	0 (0)
Anti-romiplostim neutralizing antibodies	0 (0)	0 (0)	0 (0)

4 Discussion

The results of this phase II study show that romiplostim treatment appears to be safe and well tolerated by Japanese patients with chronic ITP. Moreover, this study provides evidence that romiplostim is effective in stimulating platelet count increases in these Japanese patients. All four patients treated with a dose of 6 μg/kg and one half of

those treated with 3 μg/kg achieved platelet responses, which were defined as a doubling of the platelet count above the baseline to a level $\geq 50 \times 10^9$/L. Romiplostim produced dose-dependent effects on other measures of platelet production, including mean and maximum platelet counts, and the fold increase over baseline in platelet counts. None of the patients required rescue medication during the course of this study, and none had severe, serious, or life-threatening adverse events. Importantly, neutralizing antibodies to romiplostim or to endogenous TPO were not detected.

The dosing schedule used in this study was based on the results from the phase II study in Western populations, which evaluated whether a weekly dose of romiplostim (1, 3, or 6 μg/kg) would produce platelet counts within the target range of 50–450 $\times 10^9$/L [17]. This target platelet range was achieved by 10 of 16 patients who received romiplostim 1 or 3 μg/kg weekly for 6 weeks, although two patients had peak platelets counts above the target range. In the present study, romiplostim was administered weekly for 2 weeks producing platelet responses in one of four patients treated with 1 μg/kg, two of four patients treated with 3 μg/kg, and all four patients treated with 6 μg/kg. In the continuation phase of the present study, romiplostim was administered weekly at a dose of 3 μg/kg in one patient and 6 μg/kg in four patients, and generally maintained platelet counts within the desired range on most weeks. There were no bleeding events or rescue medications used in the continuation phase.

On the basis of the Western phase I/II studies, two parallel 24-week phase III trials of romiplostim were conducted in splenectomized and non-splenectomized patients [20]. Romiplostim was started at a dose of 1 μg/kg weekly, and then dose adjustment rules were used to achieve and maintain platelet counts within the range of 50–200 $\times 10^9$/L. Most patients, whether splenectomized or not, achieved overall platelet responses with romiplostim, with platelet counts $\geq 50 \times 10^9$/L for an average of 14–15 weeks of the 24-week treatment period. The primary study end point, durable platelet responses, which were defined by platelet counts $\geq 50 \times 10^9$/L for at least 6 of the last 8 weeks of treatment without need for rescue medication, were achieved significantly more often with romiplostim than with placebo in both splenectomized patients (38 vs. 0%; $P = 0.0013$) and non-splenectomized patients (61 vs 5%; $P < 0.001$). These findings illustrate the importance of individualizing the romiplostim dose in order to maintain platelet responses in patients with ITP. Dose adjustments were not made in the current study.

The most common treatment-related adverse event during dose escalation in this study was headache, which was reported by 3 of 12 patients (25%), whereas no treatment-related adverse event was reported by more than one patient during the treatment-continuation phase. Moreover, there were no discontinuations due to adverse events. These findings are consistent with the safety profile reported for romiplostim in the clinical studies conducted in Western ITP patient populations [17, 18, 20, 21].

The ability to draw conclusions from the present study is limited by the small number of patients treated. Phase III and extension studies are currently in progress to more fully evaluate the long-term safety and efficacy of romiplostim in Japanese patients with chronic ITP. The results from an open-label study of romiplostim in ITP patients from the United States and Europe have recently been published, with some patients treated for up to 3 years [23].

In summary, this phase II study shows that romiplostim is safe and well tolerated in Japanese patients and produces platelet responses over the dose range of 1–6 μg/kg, which is consistent with the results in Western populations. Based on these findings, a starting dose of 3 μg/kg was recommended for the phase III study of romiplostim in Japanese patients.

Acknowledgments This study was sponsored by Amgen KK (Japan). The authors would like to thank Yoshiaki Ogawa, Tokai University; and Kiyoshi Kitamura for providing for study support. M. Ohkura and Y. Sonehara, former employees of Amgen KK, provided biostatistical and study management support, respectively. Editorial support for the manuscript was provided by B. Weichman, PhD, and PAREXEL, and was funded by Amgen Inc.

Conflict of interest statement D. P. Berger is an employee of Amgen Inc. T. Ohtsu is a former employee of Amgen KK. Y. Shirasugi, K. Ando, S. Hashino, T. Nagasawa, Y. Kurata, Y., Kishimoto, and K. Iwato received research funding from Amgen.

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Journal of Thrombosis and Haemostasis, 7: 1373–1383 DOI: 10.1111/j.1538-7836.2009.03510.x

ORIGINAL ARTICLE

Presence of platelet-associated anti-glycoprotein (GP)VI autoantibodies and restoration of GPVI expression in patients with GPVI deficiency

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To cite this article: Akiyama M, Kashiwagi H, Todo K, Moroi M, Berndt MC, Kojima H, Kanakura Y, Tomiyama Y. Presence of platelet-associated anti-glycoprotein (GP)VI autoantibodies and restoration of GPVI expression in patients with GPVI deficiency. *J Thromb Haemost* 2009; **7**: 1373–83.

Summary. *Background:* Glycoprotein (GP)VI deficiency is a rare platelet disorder with a mild bleeding tendency. However, its pathophysiology remains unclear. *Objectives:* We characterized a novel GPVI-deficient patient with immune thrombocytopenic purpura and searched for the presence of anti-GPVI autoantibodies in this and another patient with GPVI deficiency. *Methods and results:* A 12-year-old Japanese girl (case 1) with moderate thrombocytopenia and mild bleeding showed selectively impaired collagen-induced platelet aggregation. Flow cytometric analysis indicated that the patient had a defect in the expression of GPVI–FcRγ. An eluate of her platelet-associated IgG contained anti-$\alpha_{IIb}\beta_3$ autoantibodies. Moreover, using GPVI–FcRγ-transfected cells, we unexpectedly identified anti-GPVI antibodies against the soluble ectodomain of GPVI in the eluate, despite the patient's GPVI deficiency. In contrast, anti-GPVI antibodies were not detectable in her plasma. In another case of GPVI deficiency (case 2) without detectable plasma anti-GPVI antibodies, we again detected platelet-associated anti-GPVI antibodies. In a 2-year follow-up of case 1, the platelet count increased to within the normal range and the bleeding tendency improved. Interestingly, GPVI was again expressed on her platelets, in association with a decrease in the relative amount of anti-GPVI antibodies. *Conclusions:* This is the first demonstration of platelet-associated anti-GPVI antibodies in GPVI-deficient subjects, in one case with spontaneous restoration of GPVI expression. These results strongly suggest an autoimmune mechanism in GPVI deficiency.

Keywords: GPVI deficiency, immune thrombocytopenic purpura, platelet-associated autoantibody.

Introduction

Glycoprotein (GP)VI is a 62-kDa transmembrane protein that plays a key role in platelet adhesion to collagen and subsequent platelet activation. GPVI consists of two immunoglobulin-like domains, a short mucin-like domain, a transmembrane domain, and a 51 amino acid cytoplasmic domain. GPVI is non-covalently associated with the immunoreceptor tyrosine-based activation motif (ITAM)-containing FcRγ-chain in the platelet membrane. Ligand binding to the GPVI–FcRγ-chain complex induces signals via the Syk/SLP-76/PLCγ2 pathway to activate integrins $\alpha_2\beta_1$ (also known as GPIa–IIa) and $\alpha_{IIb}\beta_3$ (GPIIb–IIIa), leading to thrombus formation [1–3].

GPVI deficiency is a rare platelet disorder with a mild bleeding tendency. Twelve cases have been reported to date since the first Japanese case in 1987 [4–15]. Although preliminary reports showed molecular defects in the GPVI gene in two cases of congenital GPVI deficiency [13,14], an immunologic mechanism has been suggested as the more frequent etiology for the depletion of GPVI [16]. In fact, plasma anti-GPVI antibodies have been demonstrated in five patients with GPVI deficiency [4,8,10,12,15], and injection of anti-GPVI monoclonal antibodies such as JAQ1 induces the selective downregulation of GPVI from platelets in mice [17,18] and monkeys [19]. Human GPVI can also be downregulated in a NOD/SCID mouse model by injection of human platelets and an anti-GPVI antibody [20]. Recent reports suggest that GPVI downregulation by anti-GPVI antibodies occurs through metalloprotease-mediated ectodomain shedding and/

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Received 9 December 2008, accepted 27 April 2009

or internalization/degradation pathways [18,19]. However, the pathophysiologic cause of GPVI deficiency in humans still remains unclear, because no anti-GPVI antibodies were demonstrated in the remaining five cases.

In this study, we have characterized a novel case of GPVI deficiency (case 1) and demonstrated the presence of platelet-associated anti-GPVI autoantibodies despite GPVI deficiency. Similar findings were obtained in a previously described patient with unknown etiology (case 2) [11]. Moreover, in a 2-year follow-up of case 1, we observed the spontaneous restoration of GPVI expression with a reduction in platelet-associated anti-GPVI antibodies, indicating that GPVI deficiency in our patient was an acquired abnormality. Our present data provide new evidence for an autoimmune mechanism in the pathophysiology of GPVI deficiency.

Materials and methods

Reagents

Mouse monoclonal anti-GPVI antibody 204-11 and rabbit polyclonal anti-GPVI cytoplasmic tail antibody have been described previously [21,22]. Biotinylated convulxin was made as previously described [23]. 293 cells stably expressing $\alpha_{IIb}\beta_3$ were established as previously described [24]. Chinese hamster ovary (CHO) cells stably expressing GPVI and FcRγ-chain (GPVI–FcRγ CHO cells) and the soluble form of recombinant human GPVI (amino acids 24–219; rhGPVI) were generously provided by J. Kambayashi (Otsuka USA) [25]. Rabbit polyclonal anti-FcRγ antibody and goat polyconal anti-FcγRIIA antibody were obtained from Upstate Biotechnology (Lake Placid, NY, USA) and R&D Systems (Minneapolis, MN, USA), respectively. Mouse monoclonal anti-α_2 antibody Gi9 and anti-β_3 antibody VL-PL2 were obtained from Immunotech International (Marseille, France) and BD Pharmingen (San Jose, CA, USA), respectively.

Platelets, mononuclear cells, and platelet-associated IgG eluate preparation

This study was approved by the Institutional Review Board at Osaka University Hospital, and blood samples were obtained with written informed consent. Platelets were obtained by differential centrifugation from blood anticoagulated with either Na_2-EDTA or sodium citrate [26]. Mononuclear cells were obtained using a Ficoll–Paque gradient [27]. Platelet-associated antibodies were eluted from washed platelet suspensions at a concentration of $200 \times 10^3\ \mu L^{-1}$ by adding an equal amount of diethyl ether as previously described [26,28].

Platelet aggregation study

Platelets in platelet-rich plasma were stimulated with various agonists, and platelet aggregation was monitored using a model PAM-6C platelet aggregometer (Mebanix, Tokyo, Japan).

Flow cytometry

Platelet-associated IgG and surface expression of platelet GPs were analyzed by flow cytometry as previously described [28,29]. In the case of the 204-11 anti-GPVI monoclonal antibody, prostaglandin E_1 was always added to the platelet suspension to prevent aggregation. Specific binding of 204-11 was calculated by subtraction of mean fluorescence intensity (MFI) of the control from that of 204-11, and relative surface expression was estimated by comparison of the specific binding of 204-11 of the patients' platelets with that of normal platelets. To detect autoantibodies in eluates, GPVI–FcRγ CHO cells and $\alpha_{IIb}\beta_3$-expressing 293 cells were used. Cells were suspended in phosphate-buffered saline (PBS) at a concentration of $5 \times 10^6\ \mu L^{-1}$. Fifty-microliter aliquots of the eluates were incubated with an equal volume of cell suspension for 30 min on ice, and this was followed by incubation with Alexa488-conjugated anti-human IgG (Molecular Probes, Eugene, OR, USA) for 20 min. In the case of GPVI–FcRγ CHO cells, the cells were incubated simultaneously with 10 $\mu g\ mL^{-1}$ 204-11 and with phycoerythrin-conjugated anti-mouse $F(ab')_2$ antibody (Serotec, Oxford, UK) to monitor the expression of GPVI. After washing, the cells were suspended in propidium iodide-containing PBS, and multicolor flow cytometric analysis was performed.

For the detection of anti-GPVI antibodies in plasma, platelet-poor plasma was incubated with wild-type CHO cells for 1 h to remove non-specific binding, and then incubated with GPVI–FcRγ CHO cells.

Western blotting

Western blotting was performed as previously described [30]. Twenty-microgram aliquots of platelet lysates were electrophoresed on 5–20% gradient sodium dodecylsulfate polyacrylamide gel electrophoresis gels (Pagel; ATTO korp, Tokyo, Japan) under non-reducing conditions for 204-11 and biotinylated convulxin binding, and under reducing conditions for the anti-GPVI cytoplasmic tail antibody. After transfer to poly(vinylidene difluoride) membranes (Immobilon; Millipore, Bedford, MA, USA), the membranes were incubated with either antibody or convulxin, and then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody or HRP-conjugated streptoavidin (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA, USA). The optical density of the bands was measured using Scion Image software (Scion Corp., Frederick, MD, USA).

N-ethylmaleimide (NEM) treatment of platelets

NEM treatment of platelets was performed as previously described [31]. In brief, $500 \times 10^3\ \mu L^{-1}$ of washed platelets were incubated with 2 mM NEM (Calbiochem, La Jolla, CA, USA) for 15 min at room temperature, and the reaction was terminated by addition of an equal amount of 10 mM EDTA/PBS.

Competition assay for 204-11 binding to rhGPVI by anti-GPVI autoantibodies

Two hundred and fifty nanograms of rhGPVI was coated per microtiter well. After blocking with bovine serum albumin (BSA), 50-μL aliquots of the patient's platelet eluates were added to each well and incubated for 1 h at room temperature. After washing, 1 μg mL^{-1} of 204-11 or MOPC was added to each well and incubated for another 1 h at room temperature, and this was followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG (Sigma, St. Louis, MO, USA). Alkaline phosphatase activity was measured using disodium phenylphosphate as substrate (Sanko Jun-yaku, Tokyo, Japan).

Dot blot analysis

One hundred nanograms of rhGPVI was dotted onto a nitrocellulose membrane. After blocking with BSA, the membrane was incubated with the patient's platelet eluates for 1 h. The binding of anti-GPVI antibodies was detected by incubation with biotinylated anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and this was followed by incubation with HRP-conjugated streptoavidin and a chemiluminescence reaction. As a control for blotting of rhGPVI, biotinylated convulxin was incubated with the membrane, and this was followed by incubation with HRP-conjugated streptoavidin.

RNA preparation, reverse transcription polymerase chain reaction (RT-PCR), and sequence analysis

Platelet RNA was extracted using Trizol solution (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RT-PCR and direct sequencing were performed as previously described [29]. Sequences for the primers were as described previously [11].

Results

Cases

Case 1 A 12-year-old Japanese girl suffering from cutaneous ecchymosis and recurrent epistaxis since May 2006 was referred to Osaka University Hospital in August 2006. There was no family history of a bleeding tendency or consanguinity. Physical examination revealed several petechiae in the upper and lower limbs and chest. Her platelet count was 133×10^3 μL^{-1}, with normal white blood cell and red blood cell counts. There were no abnormalities in blood chemistry and coagulation. Her platelet count decreased to $\sim 80 \times 10^3$ μL^{-1} in the next 2 months. It recovered spontaneously, but remained consistently low, ranging from 100×10^3 μL^{-1} to 120×10^3 μL^{-1} over a 12-month period. The bleeding tendency was unchanged in this period. However, after February 2008, the platelet count increased to more than 150×10^3 μL^{-1}, with improvement of the bleeding tendency (Fig. 1A).

Case 2 A 31-year-old Japanese GPVI-deficient female without any detectable anti-GPVI antibodies or genetic aberrations has been described previously [11].

Platelet aggregation study

The case 1 patient had a prolonged bleeding time (> 10 min, Duke's method), despite a platelet count of more than 100×10^3 μL^{-1} in November 2006, suggesting that functional abnormalities may exist in the patient's platelets. Collagen-induced platelet aggregation was markedly impaired in the patient (Fig. 1B). Although epinephrine-induced aggregation was impaired, impairment is observed in ~ 16% of Japanese healthy controls and is not associated with a bleeding diathesis [32]. The impaired collagen-induced platelet aggregation was not corrected even at 10 μg mL^{-1} collagen, although modest concentration-dependent aggregation was observed (Fig. 1B). These data suggest that the patient possessed a defect in a platelet collagen receptor.

Expression of GPVI and FcRγ

GPVI and $\alpha_2\beta_1$ are known to be the two major collagen receptors on platelets [1–3]. Flow cytometric analysis using an anti-GPVI monoclonal antibody, 204-11, indicated that surface GPVI expression was markedly reduced (3% of control) on the patient's platelets, whereas the expression levels of $\alpha_2\beta_1$, GPIb, $\alpha_{IIb}\beta_3$ and CD36 (data not shown) were normal (Fig. 2A). No reduction in GPVI expression was observed on platelets obtained from the patient's mother or the elder sister. Next, we examined binding of convulxin, 204-11 and a polyclonal anti-GPVI cytoplasmic tail antibody to platelet lysates in an immunoblot assay. Biotinylated convulxin binding was markedly reduced, reflecting the result obtained for GPVI expression by flow cytometry (< 5% of control) (Fig. 2B). Binding of 204-11 was also greatly reduced (~ 5% of normal control Fig. 2C). In contrast, anti-GPVI cytoplasmic tail antibody revealed that the patient platelet lysate contained a significant amount (14% of control) of normal-sized GPVI (Fig. 2D,E). It is known that GPVI is shed near the transmembrane domain by a metalloprotease after binding its ligands such as collagen or by anti-GPVI antibodies, resulting in production of an ~ 55-kDa soluble ectodomain fragment and an ~ 10-kDa platelet-associated remnant fragment [31]. Treatment of platelets with a metalloprotease activator, NEM, also induces the same effects (Fig. 3F) [31]. However, we could not detect any remnant GPVI fragment in the patient platelet lysate (Fig. 2D). In addition, we confirmed that there were no abnormalities, at least in the coding region of GPVI cDNA obtained from the patient's platelets, except for the common polymorphism

Gln317 → Leu as homozygote by direct sequencing analysis (data not shown) [33].

FcRγ expression in platelets from the case 1 patient was markedly reduced, whereas FcRγ expression was normal in the patient's mononuclear cells (Fig. 2F). We confirmed that there were no sequence abnormalities in the coding region of FcRγ cDNA obtained from the patient's platelets (data not shown). In addition, we detected normal expression of FcγRIIA in platelets from the case 1 patient (Fig. 2F).

Detection and analysis of platelet-associated anti-GPVI antibodies

As the case 1 patient's platelets possessed an elevated level of platelet-associated IgG (PAIgG) (MFI 42.44; Fig. 3A), we eluted antibodies from the platelets using diethyl ether. We confirmed that the eluted antibodies had the ability to bind to control platelets (data not shown). We detected anti-$\alpha_{IIb}\beta_3$ antibodies in the eluate by employing $\alpha_{IIb}\beta_3$-transfected 293 cells (Fig. 3B). We then used GPVI–FcRγ CHO cells to search for the presence of anti-GPVI antibodies. We monitored the expression of GPVI with 204-11, and two-color flow cytometric analysis was performed because of heterogeneity in the expression of GPVI. We used eluates obtained from normal controls and an immune thrombocytopenic purpura (ITP) patient with elevated PAIgG (MFI 43.87) as negative controls. As shown in Fig. 3C, antibody binding depending on the expression of GPVI was clearly observed in the eluate of case 1. To exclude the possibility of cross-reactivity of the anti-$\alpha_{IIb}\beta_3$ antibodies with the GPVI–FcRγ CHO cells, anti-$\alpha_{IIb}\beta_3$ antibodies in the eluate were precleared by incubation with $\alpha_{IIb}\beta_3$-transfected 293 cells, and then IgG antibody binding was reanalyzed. Antibody binding to the GPVI–FcRγ CHO cells was not affected by the depletion of the $\alpha_{IIb}\beta_3$ antibodies (data not shown). These data clearly demonstrate the presence of platelet-associated autoantibodies specific for GPVI in case 1. Moreover, preclearing the eluate with $\alpha_{IIb}\beta_3$-transfected 293 cells and with $\alpha_{IIb}\beta_3$-transfected 293 cells plus GPVI–FcRγ CHO cells decreased IgG binding to control platelets by 45% and by 87%, respectively. These results indicate that the PAIgG of the patient is mainly composed of anti-GPIIb–IIIa and anti-GPVI antibodies (data not shown).

To localize the epitope(s) for the anti-GPVI antibodies, we next examined the binding of the anti-GPVI antibodies to

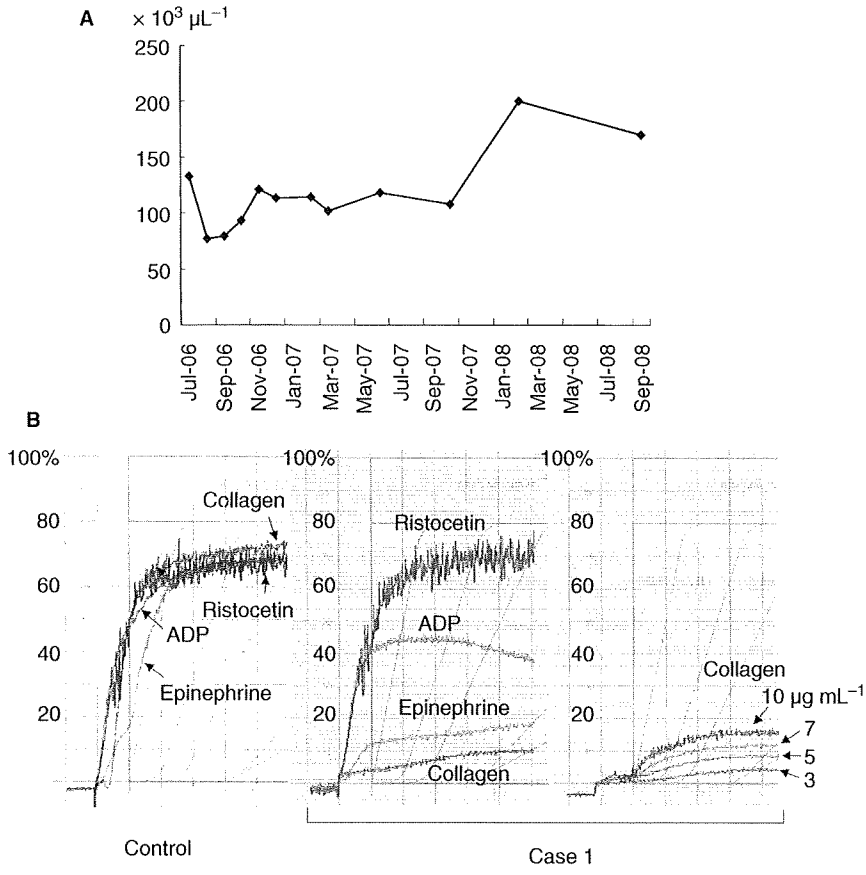

Fig. 1. Platelet counts and platelet aggregation study of the case 1 patient. (A) Time course of the platelet count for the case 1 patient from July 2006 to September 2008. (B) Platelet-rich plasma obtained from a normal control (left panel) or the patient (case 1) in November 2006 (middle panel) was stimulated with ADP (2.0 μM), ristocetin (1.5 mg mL^{-1}), epinephrine (2.0 μM), or collagen (3.0 μg mL^{-1}). Platelet-rich plasma from case 1 was stimulated with collagen at the indicated concentrations (3.0–10.0 μg mL^{-1}) (right panel).

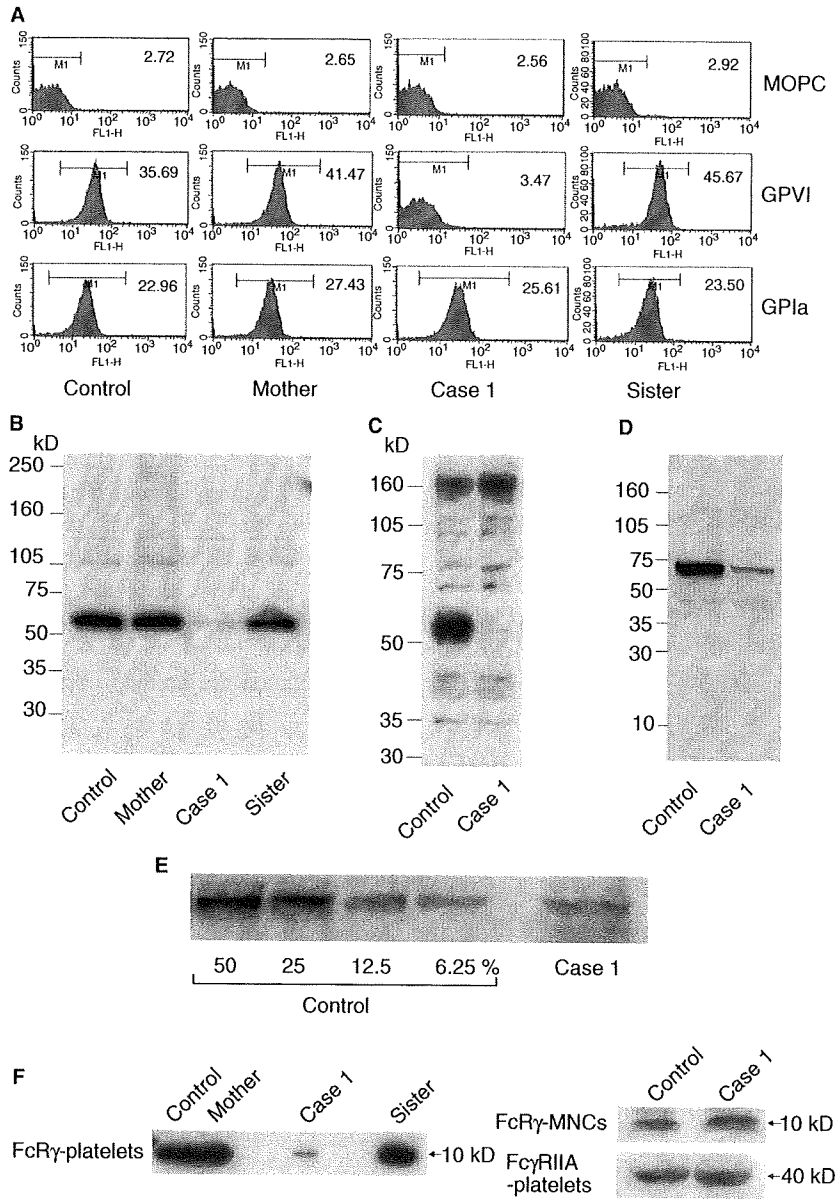

Fig. 2. Expression of glycoprotein (GP)VI and FcRγ in the case 1 patient. (A) Flow cytometric analysis of patient platelets (case 1). Platelets obtained in November 2006 from a control, the patient's mother, the patient (case 1) and an elder sister were incubated with either a monoclonal anti-GPVI antibody, 204-11, a monoclonal anti-α_2 antibody, Gi9, or a control IgG, MOPC, and analyzed by flow cytometry. Numbers in each histogram represent the mean fluorescence intensity of the gated cells. (B) Platelet lysates (20 μg) obtained from the control, the mother, the patient (case 1) and the sister were electrophoresed on a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel under non-reducing conditions. After transfer to poly(vinylidene difluoride) membranes, the membranes were incubated with biotinylated convulxin, followed by horseradish peroxidase-conjugated avidin. (C) Platelet lysates (20 μg) were electrophoresed on an SDS-PAGE gel under non-reducing conditions, and this was followed by blotting with 204-11. (D) Platelet lysates (20 μg) were electrophoresed on an SDS-PAGE gel under reducing conditions, and this was followed by blotting with polyclonal anti-GPVI cytoplasmic tail antibody. (E) Quantification of GPVI in platelet lysates of case 1. Serially diluted control lysates and the patient's lysate were electrophoresed on an SDS-PAGE gel and detected by polyclonal anti-GPVI cytoplasmic tail antibody. (F) Left panel: platelet lysates were electrophoresed under reducing conditions, and FcRγ expression was analyzed using an anti-FcRγ polyclonal antibody. Right panel: mononuclear cell (MNC) lysates (upper) or platelet lysates (lower) were electrophoresed under reducing conditions, and FcRγ expression in mononuclear cells and FcγRIIA expression in platelets were examined using anti-FcRγ and anti-FcγRIIA polyclonal antibodies, respectively.

rhGPVI (amino acids 24–219). With dot blot analysis, antibody binding to rhGPVI was clearly detected in the patient's platelet eluate (Fig. 3D). We then investigated whether the epitope for 204-11 and the anti-GPVI autoantibodies might be close to each other. As shown in Fig. 3E, the eluate obtained from case 1 did not inhibit 204-11 binding to immobilized rhGPVI. Preincubation of GPVI–FcRγ CHO cells with 204-11 also did not inhibit anti-GPVI autoantibody binding to the cells (data not shown). NEM treatment led to complete loss of the ectodomain of GPVI, whereas significant amounts of an