Fig. 3. Detection and analysis of platelet-associated anti-glycoprotein (GP)VI antibodies in the case 1 patient. (A) Elevated platelet-associated IgG (PAIgG) in the patient (case 1). Platelets obtained from the patient in November 2006 showed elevated PAIgG levels. The number in the histogram indicates mean fluorescence intensity (MFI) of the gated region. (B) Upper panel: the binding of anti-β_3 monoclonal antibody, VI-PL2 (shaded) or MOPC (open) to $\alpha_{IIb}\beta_3$-expressing 293 cells is shown. Lower panel: platelet-associated antibodies were eluted with diethyl ether, and IgG binding in the eluates was examined using $\alpha_{IIb}\beta_3$-expressing 293 cells. (C) Detection of platelet-associated anti-GPVI antibodies in the patient (case 1). Left panels: platelet eluates obtained from a normal control, an immune thrombocytopenic purpura (ITP) patient who had elevated PAIgG (MFI 43.87) or case 1 were incubated with GPVI–FcRγ Chinese hamster ovary (CHO) cells, followed by Alexa488-conjugated anti-human IgG. GPVI expression was monitored by incubation with 204-11, followed by phycoerythrin-conjugated anti-mouse IgG. Right panel: binding of human IgG to the cells expressing high levels of GPVI (gated area in the dot blots) was examined and expressed as a histogram (open dotted line for control subject, open solid line for ITP patient, and shaded histogram for case 1). (D) One hundred nanograms of recombinant human GPVI (rhGPVI) was dotted onto a nitrocellulose membrane in duplicate and incubated with platelet eluate from the patient. IgG binding to rhGPVI was detected with biotinylated anti-human IgG, followed by horseradish peroxidase (HRP)-conjugated streptoavidin. The left panel shows the binding of biotinylated convulxin as a positive control. (E) Two hundred and fifty nanograms of rhGPVI was coated on each well of a microtiter plate. Fifty microliters of eluate or buffer was added to each well and incubated for 1 h. After washing, 1 μg mL^{-1} 204-11 or MOPC was added to each well and incubated for another 1 h, and this was followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG. Alkaline phosphatase activity was measured using disodium phenylphosphate as a substrate. Relative optical density (OD) against buffer is indicated as mean + standard deviation of three independent experiments. (F) *N*-ethylmaleimide (NEM) treatment of platelets. Platelets were treated with 2 mM NEM for 15 min at room temperature. GPVI expression of the NEM-treated platelets was analyzed by fluorescence-activated cell sorting analysis with 204-11 (right upper panel), and immunoblotting with anti-GPVI cytoplasmic tail antibody (left panel) or anti-FcRγ polyclonal antibody (right lower panel). ◀, full-length GPVI; *, ~ 10-kDa platelet-associated remnant fragment of GPVI. Note that the NEM treatment led to complete loss of the ectodomain of GPVI in platelets, whereas significant amounts of the remnant fragment of GPVI and FcRγ remained platelet-associated. (G) Platelet eluates were preincubated with NEM-treated platelets (NEM: shaded) or control platelets (control: open, dotted line), or diluted with the same amount of buffer (buffer: open, solid line), and IgG binding to CHO cells expressing high levels of GPVI was then analyzed. In contrast to the preincubation with control platelets, preincubation with NEM-treated platelets failed to significantly inhibit IgG binding to CHO cells expressing high levels of GPVI.

~ 10-kDa platelet-associated remnant GPVI fragment and FcRγ remained on NEM-treated platelets (Fig. 3F). Absorption of the eluate with the NEM-treated platelets did not show a significant decrease in the binding of anti-GPVI antibodies to GPVI–FcRγ CHO cells (Fig. 3G). These results indicate that the anti-GPVI antibodies in case 1 did not recognize epitope(s) on the remnant 10-kDa GPVI fragment or FcRγ chain, but mainly recognized epitope(s) on the extracellular domain of GPVI, which is (are) apparently different from the 204-11 epitope.

Detection of anti-GPVI antibodies in plasma

We next tried to detect anti-GPVI antibodies in the plasma from the case 1 patient. We first examined whether the patient's plasma could induce platelet aggregation. However, addition of 20% of the patient's plasma to normal platelet-rich plasma did not induce platelet aggregation or inhibition of collagen-induced aggregation (Fig. 4A). We also failed to detect plasma anti-GPVI antibodies with the use of GPVI–FcRγ CHO cells (Fig. 4B). Although this experiment suggested that collagen-induced platelet aggregation may be increased in the presence of the patient's plasma as compared with control and the mother's plasma, this characteristic was not reproducible in additional experiments (data not shown). These results indicate that there were no detectable anti-GPVI antibodies in the patient's plasma.

Detection of platelet-associated anti-GPVI autoantibodies in a second GPVI-deficient patient (case 2)

We also searched for the presence of platelet-associated anti-GPVI autoantibodies in the case 2 GPVI-deficient patient. Like the case 1 patient, she had suffered ITP, but no plasma anti-GPVI autoantibodies were detected, despite intensive analysis [11]. We confirmed that GPVI expression on the case 2 patient's

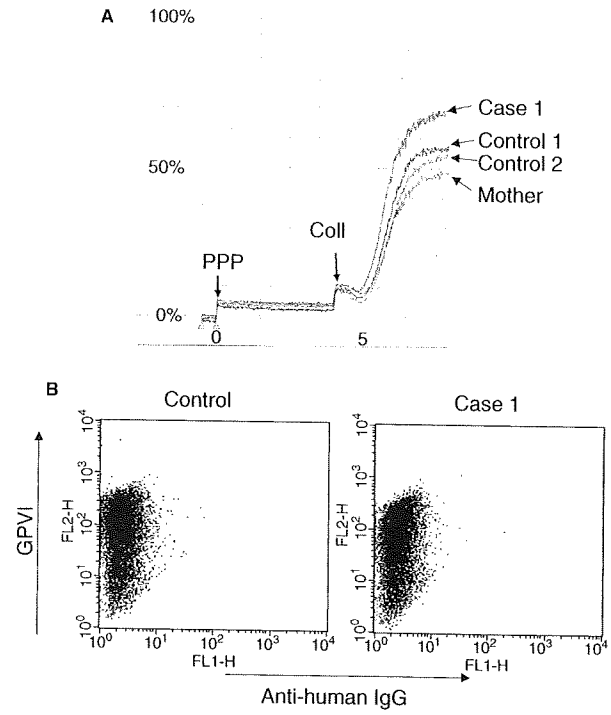

Fig. 4. Detection of anti-glycoprotein (GP)VI antibodies in plasma from the case 1 patient. (A) Effects of the patient's (case 1) plasma on control platelet function. Twenty-four microliters of platelet-poor plasma (PPP) obtained from case 1, the mother and two normal controls was added to 96 μL of platelet-rich plasma obtained from a control. After 4 min of incubation, 1 μg mL^{-1} collagen (Coll) was added. (B) Platelet-poor plasma from the patient was preincubated with wild-type Chinese hamster ovary (CHO) cells for 1 h to remove non-specific binding, and then incubated with GPVI–FcRγ CHO cells. Binding of human IgG to CHO cells expressing high levels of GPVI was analyzed as described in Fig. 3C.

platelets was still markedly impaired over a 5-year follow-up after the initial diagnosis in 2003 (Fig. 5A,B). The expression level of GPVI was much lower than that of the case 1 patient.

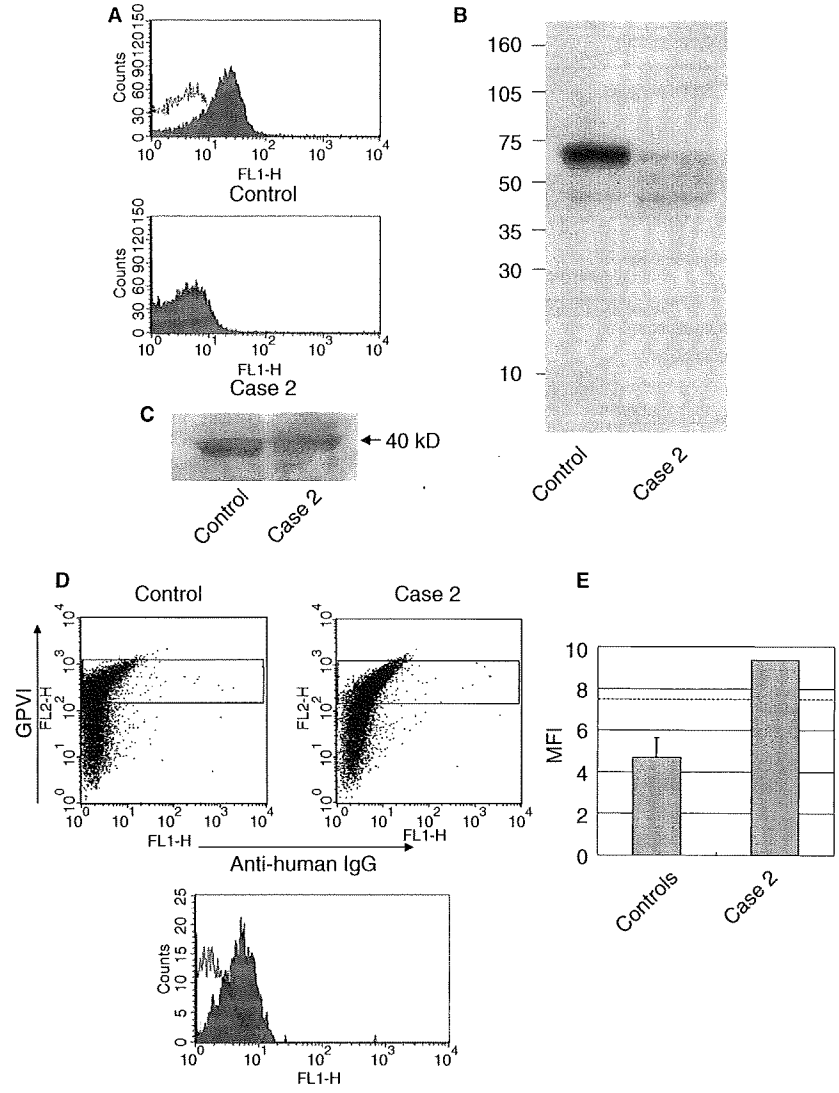

Fig. 5. Detection of platelet-associated anti-glycoprotein (GP)VI antibodies in the case 2 patient. (A) Flow cytometric analysis of platelets obtained from a control or the patient (case 2) with 204-11 (shaded) or MOPC (open). (B) Immunoblotting of platelet lysate obtained from a control or the patient (case 2) with polyclonal anti-GPVI cytoplasmic tail antibody. (C) FcγRIIA expression in platelets obtained from a control and the patient (case 2). (D) Detection of platelet-associated anti-GPVI antibodies in the patient (case 2). Binding of human IgG to the cells expressing high levels of GPVI (the gated area in the dot blots) was examined and expressed as a histogram (open histogram for control subject, shaded histogram for the patient, case 2). (E) Mean fluorescence intensity (MFI) for five control subjects (4.70 ± 0.94; mean ± standard deviation) from Fig. 5D and MFI for the patient (case 2) (9.35) are shown. The dotted line represents mean + 3 standard deviations (7.53) of control subjects.

Again, we could not detect any ~ 10-kDa remnant fragment of GPVI in the case 2 platelet lysate (Fig. 5B), but detected normal FcγRIIA expression (Fig. 5C). PAIgG was slightly elevated (data not shown). In the eluate obtained from the patient's platelets, we detected low but significant antibody binding to GPVI–FcRγ CHO cells (Fig. 5D,E).

Restoration of collagen-induced platelet aggregation and GPVI expression in the case 1 patient

In February 2008, the platelet count of the case 1 patient increased to $200 \times 10^3\ \mu L^{-1}$ and the bleeding tendency improved (Fig. 1A). As shown in Fig. 6A, the platelet response to collagen was still impaired, but was clearly improved as compared with that in November 2006 (see Fig. 1B). Flow cytometric analysis with 204-11 revealed that surface expression levels of GPVI on the patient's platelets were restored to 9% and 40% of control in February 2008 and September 2008, respectively (Fig. 6B). Comparable results were obtained by western blotting using anti-GPVI tail antibodies (12% and 38% in February and September 2008, respectively; Fig. 6C). Anti-GPVI autoantibodies were still detectable in eluates obtained from patient's platelets in February and September 2008, but the amounts of the antibodies relative to GPVI expression levels were markedly decreased in September 2008 (Fig. 6D). The amounts of anti-$\alpha_{IIb}\beta_3$ autoantibodies in the eluates of February and September 2008 were also markedly decreased (Fig. 6E).

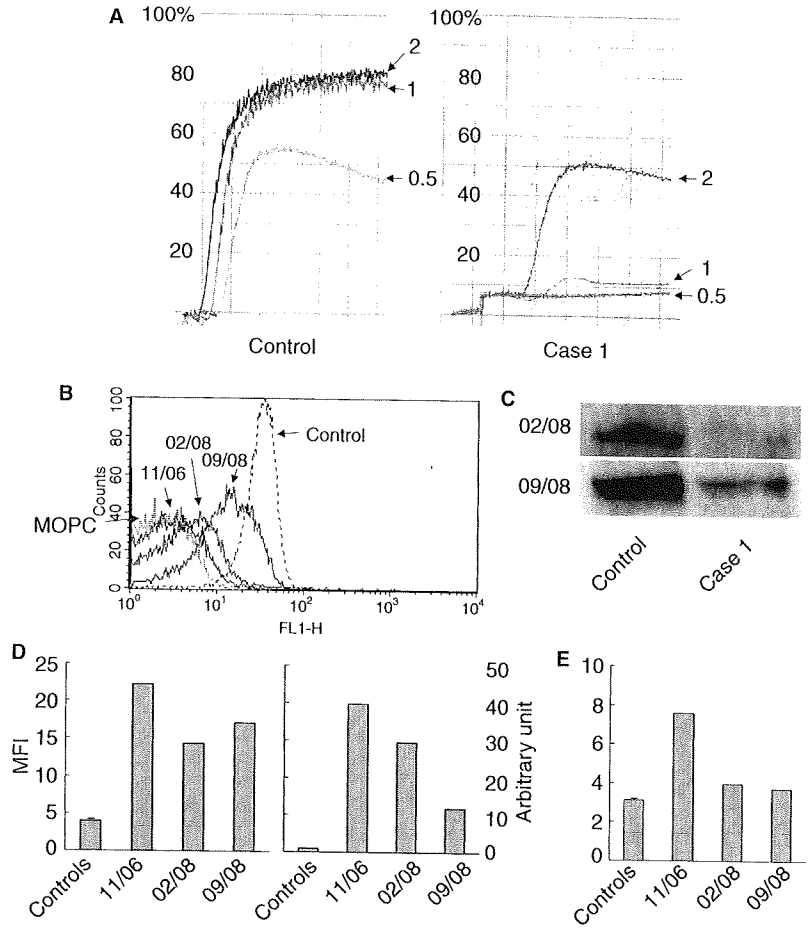

Fig. 6. Transition of platelet aggregation, glycoprotein (GP)VI expression and anti-GPVI antibodies in the case 1 patient. (A) Collagen-induced platelet aggregation in the patient (case 1) in February 2008. Platelet aggregation of a control and of case 1 with the indicated concentration of collagen are shown. Note that the patient's platelets showed clear aggregation induced by 2 μg mL^{-1} collagen (compare with Fig. 1B). (B) Surface GPVI expression in case 1 platelets obtained in November 2006 (06/11), February (08/02) and September 2008 (08/09) was analyzed by flow cytometry with 204-11. (C) GPVI expression in platelet lysates obtained in February and September 2008 was analyzed by western blotting with the anti-GPVI cytoplasmic tail antibody. (D) Anti-GPVI antibodies in eluates obtained on 06/11, 08/02 and 08/09 were analyzed using GPVI–FcRγ Chinese hamster ovary (CHO) cells as described in Fig. 3C. Left panel: mean fluorescence intensity (MFI) for antibody binding with CHO cells expressing high levels of GPVI is shown. Right panel: the amounts of anti-GPVI antibodies relative to GPVI expression levels. The MFI for the GPVI antibodies was divided by the GPVI expression levels estimated by immunoblotting at each indicated date. Data are expressed as the ratio to a normal control, which is defined as one arbitrary unit. (E) Anti-$\alpha_{IIb}\beta_3$ antibodies in eluates were analyzed as described in Fig. 3B, and the MFI is shown.

Discussion

In this article, we have demonstrated for the first time the presence of platelet-associated anti-GPVI autoantibodies in two GPVI-deficient patients with no detectable plasma anti-GPVI antibodies. Furthermore, we have demonstrated spontaneous restoration of platelet GPVI expression in one of these two patients. Thus, GPVI deficiency is an acquired abnormality in these two patients. Although molecular abnormalities in the GPVI gene have been identified in at least two patients with congenital GPVI deficiency [13,14], an immunologic mechanism has been suggested for the pathogenesis of acquired GPVI deficiency. Except for the congenital cases, nine of 11 GPVI-deficient patients, including our new case, have mild or severe thrombocytopenia, which improved with prednisolone treatment [4,6–12]. Indeed, plasma anti-GPVI antibodies have been demonstrated in five cases [4,8,10,12,15]. Nonetheless, no detectable plasma anti-GPVI antibodies were detected in the remaining cases. It has been well established that platelet-associated rather than plasma autoantibodies play a critical role in the pathogenesis of ITP [26,34]. However, the presence of platelet-associated anti-GPVI antibodies is unexpected in patients with GPVI deficiency. We propose that the high affinity of the antibodies in the two patients in this study and/or the high sensitivity of our method using GPVI–FcRγ CHO cells enabled the detection of the anti-GPVI antibodies bound to the residual surface GPVI. Another possible explanation for the successful detection of platelet-associated anti-GPVI antibodies may be that we are detecting internalized anti-GPVI antibodies as discussed below.

Recent reports have demonstrated that there are at least two distinct mechanisms for GPVI downregulation induced by the binding of anti-GPVI antibodies: shedding by activated metalloprotease, and internalization/degradation [18,19]. GPVI is

shed near the transmembrane domain by a metalloprotease after binding of ligands or anti-GPVI antibodies, resulting in the production of an ~ 55-kDa soluble ectodomain fragment and an ~ 10-kDa platelet-associated remnant fragment [31]. In this regard, GPVI shedding has been clearly demonstrated in a GPVI-deficient patient with plasma anti-GPVI antibodies capable of inducing platelet aggregation [12]. In contrast, we failed to detect the membrane-associated remnant GPVI fragment in platelet lysates in our cases (Figs 2D and 5B). GPVI ligation causes not only GPVI–FcRγ shedding, but also calpain-dependent cleavage of FcγRIIA, another ITAM-containing receptor in platelets [12,35]. Again, we failed to detect FcγRIIA cleavage (Figs 2F and 5C). These results suggest that GPVI shedding was not the main cause of GPVI depletion, at least in our cases. GPVI expression in case 1 platelets in November 2006 was estimated to be < 5% by flow cytometry using 204-11 and 5% or less by western blotting using biotinylated convulxin or 204-11, whereas it was estimated to be more than 10% by western blotting using the anti-GPVI cytoplasmic tail antibody. Convulxin and 204-11 binding are tertiary structure-dependent, whereas anti-GPVI cytoplasmic tail antibody binding is not. Thus, this discrepancy may reflect GPVI internalization, as anti-GPVI cytoplasmic tail antibody would detect internalized GPVI complexed with anti-GPVI antibodies. This may explain why we could detect platelet-associated anti-GPVI antibodies despite a marked reduction in the expression of GPVI on the platelet surface, as ether treatment of platelets for antibody elution lyses the plasma membrane. In this context, it has been demonstrated that internalized platelet JAQ1 and GPVI were detectable as long as 48 h after JAQ1 treatment in mice [18]. Moreover, this property of human antibodies is similar to that of the recently reported mouse monoclonal anti-GPVI antibody mF1232, which induces downregulation of GPVI in monkeys without inducing GPVI shedding [19].

Restoration of GPVI expression appeared to be associated with remission of the ITP and the decrease in platelet-associated anti-GPVI autoantibodies relative to GPVI expression. In addition to this case, a meeting abstract has reported partial restoration of GPVI in a GPVI-deficient patient with systemic lupus erythematosus and plasma anti-GPVI antibodies after immunosuppressive therapy [15] These data strongly support an immunologic etiology of GPVI deficiency. However, we could still observe anti-GPVI autoantibodies in eluates obtained in September 2008, despite a marked increase in GPVI expression. Like anti-$\alpha_{IIb}\beta_3$ or anti-GPIb autoantibodies in thrombocytopenia [26,36], anti-GPVI autoantibodies may be heterogeneous in terms of GPVI internalization. In this context, it is noteworthy that *in vivo* administration of some monoclonal anti-GPVI antibodies, such as OM2, OM4, and 9O12.2, does not induce GPVI depletion [37–39]. Alternatively, factors other than antibody binding might be necessary for GPVI deficiency. Further investigation is necessary for a complete understanding of the immunologic mechanisms resulting in GPVI depletion.

Our results also demonstrate that collagen-induced platelet aggregation was dramatically improved in case 1 in February 2008, with only a slight increase of surface GPVI expression (9% of normal). In a mouse model, expression of ~ 20% of control GPVI is sufficient for normal thrombus formation on a collagen-coated surface [40]. These results suggest that even a marked decrease in GPVI expression in platelets might be overlooked in many ITP patients. This possibility is under investigation in our laboratory.

In summary, we have shown for the first time the presence of platelet-associated anti-GPVI antibodies in GPVI-deficient patients. We also demonstrate the restoration of GPVI expression associated with the remission of ITP and a decrease in platelet-associated anti-GPVI autoantibodies. Our present findings thus provide strong evidence for an autoimmune mechanism in the pathophysiology of acquired GPVI deficiency. Moreover, our findings may not only serve to clarify the mechanism of GPVI depletion, but may also lead to the development of new anti-GPVI reagents for the prevention of atherothrombosis.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Platelet count response to *H. pylori* treatment in patients with immune thrombocytopenic purpura with and without *H. pylori* infection: a systematic review

Donald M. Arnold,[1] Ashley Bernotas,[1] Ishac Nazi,[2] Roberto Stasi,[3] Masataka Kuwana,[4] Yang Liu,[1] John G. Kelton,[1,2] and Mark A. Crowther[1]

[1]Michael G. DeGroote School of Medicine, Department of Medicine, McMaster University, Hamilton, Ontario, Canada; [2]Michael G. DeGroote School of Medicine, Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada; [3]Department of Medical Sciences, Ospedale "Regina Apostolorum", Albano Laziale, Italy, and [4]Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

Eradication of *H. pylori* improves thrombocytopenia in some patients with immune thrombocytopenic purpura by mechanisms that remain obscure. Platelet count responses may occur independently of *H. pylori* infection as a result of the immune modulating effects of macrolide antimicrobials or the removal of other commensal bacteria. We performed a systematic review of the literature to determine the effect of *H. pylori* eradication therapy in patients with immune thrombocytopenic purpura by comparing the platelet response in patients who were, and who were not infected with *H. pylori*. MEDLINE, EMBASE, Cochrane central registry and abstracts from the American Society of Hematology (from 2003) were searched in duplicate and independently without language or age restrictions. Eleven studies, 8 from Japan, were included enrolling 282 patients with immune thrombocytopenic purpura who received eradication therapy; 205 were *H. pylori*-positive and 77 were *H. pylori*-negative. The odds of achieving a platelet count response following eradication therapy were 14.5 higher (95% confidence interval 4.2 to 83.0) in patients with *H. pylori* infection (51.2% *vs.* 8.8%). No study reported bleeding or quality of life. Adverse events were reported in 12 patients. *H. pylori* eradication therapy was of little benefit for *H. pylori*-negative patients. These findings strengthen the causal association between *H. pylori* infection and immune thrombocytopenia in some patients. Randomized trials are needed to determine the applicability of *H. pylori* eradication therapy across diverse geographical regions.

Key words: helicobacter, autoimmunity, purpura, platelets.

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Introduction

A causal link between *H. pylori* infection and immune thrombocytopenic purpura (ITP) is suggested by clinical studies demonstrating a platelet count response in approximately 50% of patients following *H. pylori* eradication.[1-3] However, eradication therapy may result in the improvement of thrombocytopenia by mechanisms independent of *H. pylori* including the immune modulating effects of the treatment itself[4] or the removal of other commensal bacteria. In support of these hypotheses is a recent meta-analysis showing an increase in platelet count following treatment in some patients with ITP regardless of the outcome of eradication therapy.[2] Furthermore, in certain geographical regions, the prevalence of *H. pylori* infection among patients with ITP is no different than the general population[5] suggesting that the association between *H. pylori* and ITP is not certain. Understanding the mechanism of the platelet count response following *H. pylori* treatment may help explain pathophysiology and determine whether or not uninfected patients stand to benefit from treatment. To determine the independent effect of *H. pylori* eradication therapy on platelet count response, we performed a systematic review of all studies reporting treatment responses compared to *H. pylori*-negative controls.

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Correspondence: Donald M. Arnold MD, MSc, FRCP(C), HSC 3V-48, 1200 Main Street West, Hamilton, Ontario, Canada. E-mail: arnold@mcmaster.ca

Design and Methods

Literature Search

The electronic databases of MEDLINE, EMBASE and the Cochrane central registry were searched in duplicate and independently using the subject headings *Purpura, Thrombocytopenic, Idiopathic, Purpura, Thrombocytopenic,* and the keywords *idiopathic thrombocytopenic purpura, thrombocytopenic purpura, immune thrombocytopenia, and ITP,* combined with the subject headings *Helicobacter pylori, Helicobacter Infections* and the keywords *Helicobacter pylori, helicobacter infections* and *H. pylori*. Abstracts from the American Society of Hematology (2003 to 2007) were searched in duplicate electronically using the term *pylori*. Experts were canvassed for additional references and relevant bibliographies were searched by hand. No age or language restrictions were applied. Articles were included if they enrolled patients with ITP and described the platelet count response following *H. pylori* eradication for both infected and uninfected patients. Eradication therapy had to include at least one antibiotic and a proton pump inhibitor. Studies that selected only *H. pylori* -positive patients and case reports of fewer than 5 patients were excluded.

Study quality

The quality of included studies was assessed independently by two reviewers with expertise in methodology. Reviewers assessed each study for the presence or absence of 4 key design features derived from the specifications for reporting meta-analyses of observational studies[6] and from a quality assessment tool for non-randomized studies[7]: (i) prospective data collection; (ii) enrolment of consecutive patients unselected for *H. pylori* infection status; (iii) a clearly stated duration of follow-up; and (iv) a description of losses to follow-up. Disagreements were resolved by consensus. Funnel plots relating sample size to treatment effect expressed as the log of the odds ratio (OR) were examined for evidence of publication bias.

Analysis

Platelet count response, bleeding and quality of life were captured. We used two definitions of overall response – the definitions from primary reports (which varied) and a standardized definition of a platelet count rise to at least 30×10^9/L within six months (reported in most studies). Group level proportions for overall, partial and complete response following eradication therapy were derived for *H. pylori*-positive, eradicated, non-eradicated and negative patients. Age, duration of ITP, time to response and duration of response were derived by weighted means adjusted for sample size; when means and standard deviations were not reported for these variables, they were imputed from quartiles assuming a normal distribution. The comparison of proportion of responders between the *H. pylori*-positive and negative groups was performed using an exact test (two-tailed) for stratified data,[8] and the common OR was reported with exact 95% confidence intervals (CI) to account for small sample sizes. Unweighted chance-corrected kappa was used to assess agreement between reviewers for study selection.[9] This systematic review had no external source of funding. The authors had complete access to all study data, and all have seen and approved this manuscript.

Results

Article selection

The initial literature search yielded 266 citations. Titles were screened for relevance and duplicates leaving 165 citations. Of those, 76 did not meet eligibility criteria leaving 90 articles which underwent detailed review. Finally, 11 articles were included (Figure 1). Agreement on article selection was moderate (k=0.48) and discrepancies, which were often due to uncertainty about the inclusion of *H. pylori*-negative patients, were resolved by consensus.

Study characteristics

Of 11 studies, 8 were from Japan and one was from each of Italy, USA and Canada (Table 1). Median prevalence of *H. pylori* infection from studies from Japan was 73% (first, third quartile 70%, 81%) compared with 22%[11] and 18%[15] as reported in the 2 studies from North America, and 51%[10] in the study from Italy. Most studies defined ITP as thrombocytopenia in the absence of any other cause and consistent with the American Society of Hematology guidelines;[21] 8 of 11 studies required a bone marrow examination showing normal or increased megakaryocytes. The platelet count values for inclusion were heterogenous from study to study, ranging from $\leq50\times10^9$/L[12] to $\leq120\times10^9$/L.,[11,16,19] and with an overall mean (± SD) baseline platelet count of $43\pm28\times10^9$/L. The definitions of platelet count responses were variable.

Patient characteristics

In total, 355 patients with ITP (68.2% female) with a

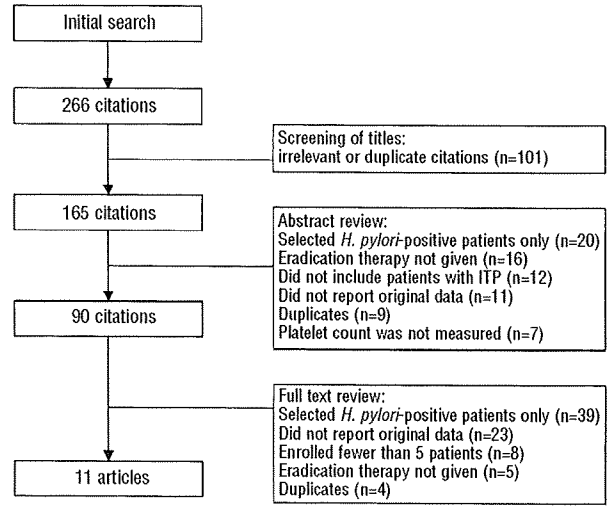


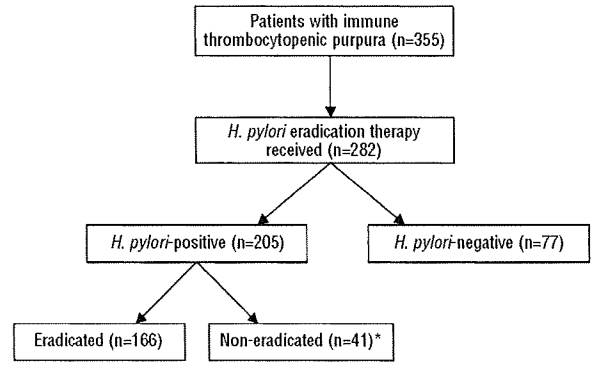
Figure 1. Results of study search and selection.

Table 1. Characteristics of *H. pylori* eradication studies that met inclusion criteria (n=355 patients) and the criteria used to define platelet count responses.

Emilia 2007[10]	Italy	75	PLT <100 for 6 months	51%	PLT >150	PLT 40-149, and doubling from baseline	PLT >40 and doubling	3 months
Michel 2004[11]	USA	74	PLT < 60	22%	PLT >150	PLT >50, and doubling from baseline	PLT >50, and doubling	3 months
Asahi 2006[12]	Japan	37	PLT < 50 for 3 months	70%	NR	NR	PLT >100	6 months
Inaba 2005[13]	Japan	35	PLT < 100 for 6 months	71%	NR	NR	PLT >100, and increase by 30	6 months
Hino 2003[14]	Japan	30	PLT < 100	70%	NR	NR	Significant increase	3 months
Jackson 2008[15]	Canada	22	PLT < 120	18%	PLT >150	PLT >30	PLT >30	2 months
Takahashi 2004[16]	Japan	20	PLT <120	75%	PLT >120	PLT Increase by 20	Increase by 20	4 months
Ando 2004[17]	Japan	20	PLT < 100	85%	PLT >120	PLT 90-120	PLT >90	1 month
Kato 2004[18]	Japan	20	PLT < 100	85%	PLT >150 or increase by 100	Increase by 70-100	Increase by 70-100	
Morimoto 2007[19]	Japan	15	PLT < 120	80%	PLT >120	PLT increase by 20	Increase by 20	4 months
Ohguchi 2005[20]	Japan	7	PLT < 100	32%	NR	NR	Significant increase	6 months

PLT: platelet count ($\times 10^9$/L); NR: not reported.

mean age of 51.6±17.1 years were enrolled. None were children. Mean prior duration of ITP was 61.5 months and 47 (13.2%) patients had undergone splenectomy. Mean duration of follow-up was 34.5±23.9 months. Of 355 patients, 282 received eradication therapy as part of the study design; 205 (72.7%) were *H. pylori*-positive and 77 (27.3%) were *H. pylori*-negative. *H. pylori* was successfully eradicated in 166 (81.0%) infected patients (Figure 2).



* Data on platelet count response are reported for 12 non-eradicated patients

Figure 2. Patients who received *H. pylori* eradication therapy.

H. pylori diagnosis and treatment

The urea breath test was used to diagnose *H. pylori* in all studies except one[15] in which fecal antigen testing was used. Additional tests for *H. pylori* included serology (n=5 studies) and tissue culture (n=1). Treatment consisted of amoxicillin 750 to 1500 mg twice daily, clarithromycin 200 to 400 mg twice daily, and a proton pump inhibitor for seven days in all studies except one[11] in which the duration of therapy was 14 days.

Response

The overall platelet count response (as variably defined in primary reports) for *H. pylori*-positive (n=205), eradicated (n=166) and negative (n=77) patients was 53.1%, 53.6% and 6.5% respectively (Table 2). The OR (OR; positive *vs.* negative) was 24.0, exact 95% CI 7.1 to 125.1. Using the homogeneous definition of a platelet count increase to 30×10^9/L or higher by six months,[10-13,15,18] overall platelet count response was 51.2%, 52.5% and 8.8% for positive (n=125), eradicated (n=115) and negative (n=57), respectively; OR (positive *vs.* negative) 14.5, 95% CI 4.2 to 83.0 (Figure 3). Complete platelet count response (platelet count greater than 150×10^9/L) was not significantly different between *H. pylori*-positive and negative patients (OR 3.4, 95% CI 0.7 to 22.4), although only 4 studies reported this outcome. A comparison of partial response was not possible since the definition varied too widely between studies. Relapses were reported in 3 *H. pylori*-positive responders, and one *H. pylori*-negative responder.

We performed a descriptive sensitivity analysis using only those studies that enrolled consecutive patients whose infection status was not known until after the effect of treatment had been assessed (n=4 studies). Three of those studies, one from Canada[15] and 2 from Japan,[12,13] showed a positive effect of treatment in infected compared with uninfected patients [3/4 (75%) *vs.* 3/18 (17%); 16/26 (62%) *vs.* 0/11 (0%); and 11/25 (44%) *vs.* 0/10 (0%)]. In contrast, the American study by Michel[11] showed no significant difference between groups [1/15 (6.7%) *vs.* 0/10 (0%)].

Among responders, the mean time to response was 2.7 weeks (range 1-6),[10-12,15-17] and the mean duration of response was 19.9 months (range 7-60) measured to the end of follow-up.[10-13,15,17-18]

Of 12 patients whose infection could not be eradicated with treatment, 3 (25.0%, 95% CI 5% to 57%) achieved a platelet count response.[10,15-16] The median platelet count increase by group could not be derived because of insufficient data from primary reports, and an analysis by severity of thrombocytopenia was not possible with the study-level data available for this review. None of the studies reported bleeding or quality of life measures. A response to eradication therapy correlated with a reduction in platelet associated IgG levels in 3 studies,[13,16,19] and in one study[12] suppression of B cells secreting anti-glycoprotein IIb-IIIa antibody was demonstrated in responders by 24 weeks.

Adverse events

Diarrhea (n=6 patients), taste disturbance (n=3), constipation (n=1), vomiting (n=1) and skin rash (n=1) were reported among treated patients.[11,13-14] There were 2 deaths reported;[10] neither were related to ITP or to *H. pylori* treatment.

Study quality

Data collection was prospective for all studies, however only one study[13] met all methodological quality criteria (Table 3). Duration of follow-up was often not explicitly stated and reporting of losses to follow-up was inadequate; however, the data accounted for virtually all patients enrolled. Four studies[11-13,15] entered consecutive patients with ITP without knowledge of *H. pylori* status; whereas 7 studies identified patients' infection status prior to treatment.[10,14,16-20] Funnel plots relating sample size to OR were asymmetric suggesting that publication bias may have been present; however small studies with large effect sizes were not overrepresented.

Discussion

H. pylori is a commensal gram-negative microaerophilic bacterium that colonizes the human stomach. It is recognized as the causative agent of active chronic gastritis and peptic ulcer disease[22] and is associated with the development of adenocarcinoma and mucosa-associated lymphoma.[23] The association with ITP was first noted by Gasbarrini who described a platelet count improvement in 8 of 11 patients following eradication of *H. pylori* with disappearance of platelet autoantibodies in 6 of these 8

Table 2. Platelet count responses among patients with immune thrombocytopenic purpura given *H. pylori* eradication therapy.

All studies				
Overall response (n=11)	109/205 (53.2%)	5/77 (6.5%)	<0.001	24.0 (7.1, 125.1)
Complete response (n=6)	41/106 (38.7%)	4/43 (9.3%)	0.012	5.7 (1.4, 30.1)
Partial response (n=6)	14/106 (13.2%)	1/43 (2.4%)	0.071	21.0 (0.9, 588.0)
Homogeneous outcome criteria[2]				
Overall (n=6)	64/125 (51.2%)	5/57 (8.8%)	0.001	<14.5 (4.2, 83.0)
Complete (n=4)	26/74 (35.1%)	4/36 (11.1%)	0.148	3.4 (0.7, 22.4)
Partial (n=4)	11/74 (14.9%)	1/36 (2.8%)	0.135	16.5 (0.7, 554.9)

[1]Exact test (two-tailed) for stratified data; [2]Overall response: achievement of a platelet count of 30x10⁹/L or higher by 6 months; complete response: platelet count 150x10⁹/L or higher; partial response was variably defined.

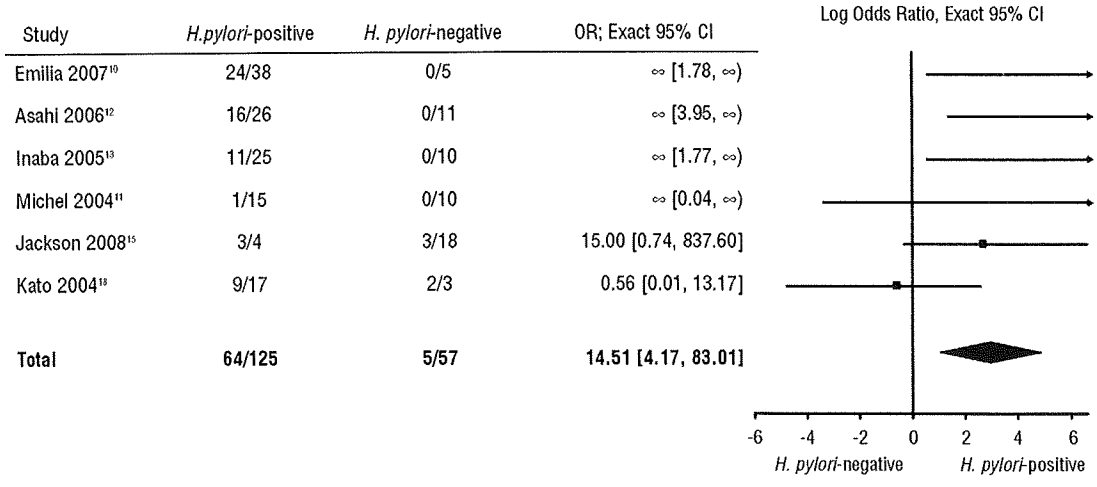


Figure 3. Pooled odds ratio (OR) of *H. pylori* eradication therapy on overall platelet count response (platelet count greater than 30x10⁹/L by 6 months) in *H. pylori*-positive and *H. pylori*-negative patients with immune thrombocytopenic purpura.

patients.[24] The association between platelet count improvement and *H. pylori* eradication has since been corroborated in other studies.[1-3] However, an independent effect of *H. pylori* treatment has been hypothesized, since 1) macrolide antibiotics including clarithromycin (universally included in eradication regimens) possess anti-inflammatory properties which may improve platelet autoreactivity in ITP by blocking the production of proinflammatory cytokines;[4] and 2) antimicrobials used for *H. pylori* treatment may eradicate other commensal bacteria that stimulate cross reactive platelet antibodies. Using treated, *H. pylori* -negative patients as controls, this systematic review confirms that the effect of *H. pylori* treatment is indeed due to eradication of the bacteria and not to the treatment itself. Platelet responses were only rarely observed in uninfected patients and in patients whose infection could not be eradicated, at a rate similar to that of spontaneous remissions expected in this patient population.[25,26]

The methods that we adopted and the results that we produced in this systematic review are different from other reviews on this topic (Table 4). For one, we used treated, *H. pylori*-negative patients as controls. This design allowed us to assess the effect of eradication therapy, independent of *H. pylori* infection, and to determine whether or not uninfected patients might also

Table 3. Key features of study design.

Jackson 2008[15]	Prospective cohort	Yes	No	None described
Emilia 2007[10]	Prospective cohort	No	Yes	None described
Morimoto 2007[19]	Prospective cohort	No	No	None described
Asahi 2006[12]	Prospective cohort	Yes	No	None described
Takahashi 2004[16]	Prospective cohort	No	No	None described
Inaba 2005[13]	Prospective cohort	Yes	Yes	One patient withdrawn
Ohguchi 2005[20]	Unable to assess	No	No	None described
Michel 2004[11]	Prospective cohort	Yes	Yes	None described
Ando 2004[17]	Prospective cohort	No	Yes	None described
Kato 2004[18]	Prospective cohort	No	Unable to assess	None described
Hino 2003[14]	Prospective cohort	No	No	None described

**H. pylori infection status was not known until after eradication therapy was administered.[2]*

Table 4. Comparison of reviews of *H. pylori* eradication in patients with immune thrombocytopenic purpura.

Arnold*	282	Treated, HP-negative	Platelet count response (>30×10⁹/L lasting 6 months or more)	Exact odd ratio (HP-positive *vs.* HP-negative) = 14.5 (95% CI 4.2 to 83.0)	The odds of responding to HP eradication therapy was 14.5 times higher in patients with ITP who were HP-positive, compared with those who were HP-negative.
Stasi[1]	866	None	Platelet count response [doubling of baseline and >100×10⁹/L (complete) or >30×10⁹/L (overall)]	Overall response = 50.3% (95% CI, 41.6% to 59.0%)	Of HP-positive ITP patients who received eradiation therapy, 50% achieved a platelet count response.
Franchini[2]	409	Untreated; HP-positive, eradicated; HP-positive, non-eradicated; and HP-negative†	Difference in platelet count level	Weighted mean difference (treated *vs.* untreated) = 34×10⁹/L (95% CI, 20-47)	HP-positive ITP patients who received eradication therapy had a platelet count level that was 34×10⁹/L higher than those who did not receive eradication therapy.
Jackson[3]	234	None	Platelet count response (variably defined)	Response = 52%	Of HP-positive ITP patients who received eradication therapy, 52% achieved a platelet count response.

**N = treated patients; †H. pylori-negative patients did not receive eradication therapy in the review by Franchini; *Arnold DM, Kelton JG. Current options for the treatment of idiopathic thrombocytopenic purpura. Semin Hematol. 2007 Oct;44(4 Suppl 5):S12-23.*

benefit from treatment. The review by Franchini used as controls, untreated *H. pylori*-positive patients, eradicated and non-eradicated *H. pylori*-positive patients, and untreated *H. pylori*-negative patients;[2] whereas other reviews did not include a control group.[1,3] Second, our review used comparative statistical methods (exact OR) to determine the effect of treatment on the proportion of responders; whereas Franchini reported the difference in platelet count levels between groups,[2] and other reviews described the number of responders among treated *H. pylori*-positive patients.[1,3] The review by Stasi did describe 41 treated, *H. pylori*-negative patients, none of whom showed a platelet count recovery.[1]

Several mechanisms have been proposed to explain the association between *H. pylori* and ITP. For one, antibodies against *H. pylori*, specifically against CagA protein have been shown to cross react with platelet antigens[16,27] causing accelerated platelet clearance. This hypothesis may explain the observed variability in treatment effect by country since CagA-positive strains of *H. pylori* are more prevalent in Japan than in North America.[28] Other proposed mechanisms are modulation of host immunity following colonization by *H. pylori* to favor the emergence of autoreactive B-1 cells[29] and the enhancement of phagocytic capacity of monocytes together with low levels of the inhibitory Fcγ receptor IIB following *H. pylori* infection.[30]

Our findings raise questions that merit further research. For instance, we found that the rate of complete response (platelet count greater 150×10^9/L) was low in both *H. pylori*-positive and negative patients following eradication therapy (35.1% *vs.* 11.1%, respectively) suggesting that while treatment may improve platelet counts, it may not reverse the autoimmune process. Also, extended follow-up is needed to address the durability of the platelet count response. Finally, we, like others, observed regional differences in the proportion of *H. pylori*-positive responders with the highest rates from Japan[12] and Italy[10] [16/26 (62%) and 24/38 (63%), respectively], and the lowest rate from North America[11] [1/15 (7%)]. While differences in the prevalence of CagA-positive strains may explain some of this variability,[31] additional prospective studies with sufficient power and geographical representation are needed.

Strengths of this review were the inclusion of controlled studies only, allowing for the determination of the treatment effect with respect to controls; the use of standardized outcome criteria for defining platelet count response, limiting ascertainment bias; and the use of exact statistical tests to account for small sample sizes and large variability. Exact statistical methods involve synthesizing the evidence over studies based on the available data without requiring point estimates to be computed for each study. As opposed to standard meta-analytic methods, exact methods are most suitable when data are sparse and are particularly useful when estimates of effect cannot be derived from individual studies because of small sample sizes.

None of the primary reports were randomized trials, thus measured and unmeasured bias including concomitant treatments and variable platelet count inclusion thresholds confound our results. Pooling results of such non-randomized trials is subject to bias; however we felt that studies using the same definition of platelet count response were sufficiently homogeneous. Furthermore a sensitivity analysis based only on studies of consecutive patients, where investigators were effectively blinded to *H. pylori* infections status, showed similar results. Our results are also limited by the lack of individual patient data and the lack of detailed reporting of prior ITP treatments, demographic data and outcome responses in some studies. The data from primary reports did not support an analysis by severity of thrombocytopenia (mild *vs.* severe), as these groups were generally not reported separately. While in practice, patients with mild thrombocytopenia often do not require any treatment, the goal of this review was to show a biological effect compared with controls; thus even patients with mild thrombocytopenia (baseline platelet count for the cohort was 43×10^9/L) contribute meaningfully to the data.

Although empiric *H. pylori* eradication therapy in ITP is appealing because of its simplicity and low toxicity, our data suggest that treatment should be reserved for *H. pylori*-positive patients only. High response rates and high prevalence of the infection in certain countries like Japan support routine screening for *H. pylori* in those regions. Even in countries like the US and Canada where prevalence is low, screening may be justified since some (albeit few) patients may respond to treatment, which is brief and well tolerated. Nevertheless, the efficacy and cost effectiveness of screening in North America remains uncertain. Randomized trials in unselected patients from diverse geographical regions are needed to confirm our findings and to determine the durability of the platelet count response.

Authorship and Disclosures

DMA: conception and design of the study, acquisition of data from original reports, interpretation of the data, drafting the manuscript and final approval; AB: acquisition of data from original reports, interpretation of the data, revision of the manuscript and final approval; IN: acquisition of data, interpretation of the data, revision of the manuscript and final approval; RS: conception of the study, acquisition of data from original reports, interpretation of the data, revision of the manuscript and final approval; MK: acquisition of data, interpretation of the data, revision of the manuscript and final approval; YL: data analysis and interpretation, revision of the manuscript and final approval; JGK: conception of the study, acquisition of data, interpretation of the data, revision of the manuscript and final approval; MAC: conception and design of the study, interpretation of the data, drafting the manuscript and final approval.

The authors reported no potential conflicts of interest.

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Heterogeneous pathogenic processes of thrombotic microangiopathies in patients with connective tissue diseases

Tomomi Matsuyama[1]; Masataka Kuwana[2]; Masanori Matsumoto[1]; Ayami Isonishi[1]; Shigeko Inokuma[3]; Yoshihiro Fujimura [1]

[1]Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Nara, Japan; [2]Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan; [3]Rheumatology Center, Japanese Red Cross Medical Center, Tokyo, Japan

Summary

To clarify the pathogenic processes of thrombotic microangiopathies (TMAs) in patients with connective tissue disease (CTD), we analysed clinical characteristics and plasma ADAMTS13 levels in 127 patients with CTD-TMAs, including patients with systemic lupus erythematosus (SLE), systemic sclerosis, polymyositis/dermatomyositis, and rheumatoid arthritis (RA), and 64 patients with acquired idiopathic thrombotic thrombocytopenic purpura (ai-TTP). Plasma levels of ADAMTS13 activity, antigen, and inhibitors were determined by enzyme immunoassays. IgG type anti-ADAMTS13 antibodies were also detected by immunoblots using purified ADAMTS13. ADAMTS13 activity was significantly decreased in CTD-TMAs, regardless of the underlying disease, but the frequency of severe deficiency (defined as <0.5% of normal) was lower in CTD-TMA patients than in ai-TTP patients (16.5% vs. 70.3%, $p<0.01$). Severe deficiency of ADAMTS13 activity was predominantly detected in patients with RA- and SLE-TMAs, and was closely associated with the presence of anti-ADAMTS13 IgG antibodies. CTD-TMA patients with severe deficiency of ADAMTS13 activity appeared to have lower platelet counts and better therapeutic outcomes. At least two phenotypic TMAs occur in patients with CTDs: a minor population with deficient ADAMTS13 activity caused by neutralising autoantibodies, and a major population with normal or moderately reduced activity. Classifying CTD-TMAs by ADAMTS13 activity may be useful in predicting the clinical course and therapeutic outcomes, as patients with moderately reduced activity are likely to have more prominent renal impairment and poor prognoses.

Keywords

Connective tissue disease, TMA, ADAMTS13, autoantibody

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Introduction

Thrombotic thrombocytopenic purpura (TTP) and haemolytic uraemic syndrome (HUS) are life-threatening diseases, characterised pathologically by thrombotic microangiopathies (TMAs), microangiopathic haemolytic anaemia, destructive thrombocytopenia, and organ dysfunction caused by platelet-thombi (1). The discovery of ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motif 13) (2–7), which specifically cleaves the Tyr1605-Met1606 bond in the von Willebrand factor (VWF)-A2 domain, facilitated the recognition that enzymatic deficiency due to genetic mutation or acquired autoantibodies was a more specific feature of TTP (8, 9). In the absence of ADAMTS13, unusually large VWF multimers (UL-VWFMs) are not appropriately cleaved in circulation; as a result, platelets aggregate excessively under high shear stress (10, 11).

In 1939, Gitlow and Goldmark (12) first reported a close relationship between TTP and systemic lupus erythematosus (SLE). Since then, this concept has become well accepted (13–16), but investigations of the underlying pathogenic processes are still lacking. More recently, several investigators (17–21) have observed deficient ADAMSTS13 activity caused by inhibitory IgG-autoantibodies in some patients with SLE-related TTP; however, many other patients have slightly reduced or almost normal activity (18). Thus, the pathogenesis of SLE-related TTP is still controversial.

During 1997–2006, we identified a total of 783 patients with TMAs by analysing their clinical and laboratory findings, including the levels of ADAMTS13 activity (ADAMTS13:AC)

Correspondence to:
Yoshihiro Fujimura, MD
Department of Blood Transfusion Medicine, Nara Medical University
Shijyo-cho 840, Kashihara city, Nara, 634–8522, Japan
Tel.: +81 744 22 3051(3289), Fax: +81 744 29 0771
E-mail: malon@naramed-u.ac.jp

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and ADAMTS13 inhibitor (ADAMTS13:INH), at Nara Medical University (22). In this database, we found 33 patients with congenital ADAMTS13:AC deficiency (termed Upshaw-Schulman syndrome), a rare thrombo-haemorrhagic disease, and elucidated their ADAMTS13 genetic status (23, 24). Using the same database, we identified 127 patients who developed TMAs in association with connective tissue diseases (CTDs), including SLE, systemic sclerosis (SSc), polymyositis/dermatomyositis (PM/DM), and rheumatoid arthritis (RA).

In this study, to further characterise the clinical expression and underlying pathogenesis of CTD-TMAs, we have extensively evaluated ADAMTS13 profiles, antibodies to ADAMTS13, and clinical features in patients with CTD-TMAs in comparison with 64 patients with acquired idiopathic (ai)-TTP.

Patients, materials and methods

Study subjects

One hundred twenty-seven patients with CTD-TMAs in a Nara Medical University database (22) included 64 SLE patients, 42 SSc patients, 11 PM/DM patients, and 10 RA patients. All patients fulfilled the corresponding classification criteria (25–28). SSc patients with complicating classical hypertensive renal crisis were excluded from the analysis. Blood samples and detailed clinical information were provided by referring physicians in area hospitals across Japan, and only patients who were confirmed to have TMAs were included in the database.

Based on the previous reports (29–31), TMAs were defined as having all of the followings: (i) microangiopathic haemolytic anemia (haemoglobin ≤12 g/dl), Coombs test negative, undetectable serum haptoglobin (<10 mg/dl), more than two fragmented red cells (schistocytes) in a microscopic field with a magnification of 100, and concurrent increased serum lactate dehydrogenase (LDH) above institutional baseline; (ii) thrombocytopenia (platelet count ≤100 x 10^9/l); and (iii) a variety of organ dysfunction (renal or neurological involvement) devoid of the stigmata of disseminated intravascular coagulation.

Sixty-four ai-TTP patients in a Nara Medical University database generated during the past two years (March 2006-April 2008) were used in this study for comparison. A diagnosis of ai-TTP was made for patients (i) without apparent underlying disease, and (ii) with the aforementioned clinical and laboratory features of TMAs.

Normal plasma samples were prepared from 20 healthy individuals (10 male, 10 female) between 20 and 40 years of age for use as a control. The study protocol conformed to the ethical principles of the World Medical Association Declaration of Helsinki as reflected in a *priori* approval from the Ethics Committee of Nara Medical University, and written informed consent was obtained from all patients at each referral hospital.

Therapeutic regimens and outcomes

Steroid pulse therapy is administration of intravenous methylprednisolone 1 g/day for 3 days, followed by a moderate or high dose of steroid therapy (0.5–1.0 mg of oral prednisolone/kg body weight/day). Therapeutic plasma exchange was conducted according to the following regimen: daily plasma exchange (PE) was performed at 1.5-fold body plasma volume with fresh frozen plasma (FFP) for the first 3 days, and PE was then performed at one body plasma volume daily for up to 14 days, until normal platelet counts (>150 x 10^9/l) were achieved. Response to therapy for TMAs was evaluated in two ways: (i) remission, defined as normalisation of platelet count (>150 x 10^9/l) and clinical manifestations without requiring PE, or (ii) death due to TMA.

Blood sampling

Before therapeutic plasma exchange or plasma infusions, blood samples (4.5 ml) were taken from each patient into plastic tubes containing 0.5 ml of 3.8% sodium citrate. Plasma was isolated by centrifugation at 3,000 x g for 15 minutes (min) at 4°C. Plasma samples were kept in aliquots at –80°C until testing, and sent to our institution.

Plasma ADAMTS13:AC, ADAMTS13:INH, ADAMTS13:AG, and VWF:Ag

Plasma levels of ADAMTS13:AC were determined using a commercially available chromogenic act-ELISA (Kainos Inc., Tokyo, Japan) (32). The detection limit of ADAMTS13:AC by this method was 0.5% of the control. A good correlation in plasma levels of ADAMTS13:AC between the classic VWFM assay and the chromogenic ADAMTS13-act-ELISA has previously been shown in normal individuals (R^2=0.72, $p<0.01$) (32, 33).

In terms of plasma levels of ADAMTS13:AC, since SSC-ISTH (Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis) has not defined "severe" deficiency of ADAMTS13:AC, we have here tentatively categorized into three types of plasma ADAMTS13:AC: less than 0.5%, 0.5% to less than 25%, and 25% or higher of the normal control as severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal, respectively.

ADAMTS13:INH titers were evaluated by act-ELISA using plasma that was heat-inactivated at 56°C for 30 min. Inhibitor titers are expressed as Bethesda units (BU) (34). One BU is defined as the amount necessary to reduce ADAMTS13:AC to 50% of control levels. Titers greater than 0.5 Bethesda U/ml were classified as inhibitor-positive.

Plasma ADAMTS13 antigen (ADAMTS13:AG) levels were analysed by sandwich antigen (ag)-ELISA, using two murine anti-ADAMTS13 mAbs, A10 and C7. The A10 antibody recognizes an epitope in the disintegrin-like domain, completely inhibiting enzyme activity at a final concentration of 10 μg/ml (35). The C7 antibody recognizes the 7th and 8th thrombospondin-1 domains without affecting activity. The detection limit of the ag-ELISA for plasma ADAMTS13:AG was 0.1% of the normal control (35).

Plasma levels of VWF:Ag were assayed by sandwich ELISA using rabbit anti-human VWF polyclonal antibodies (DAKO, Denmark). The detection limit of this assay was 0.3% of the normal control.

Detection of IgG autoantibodies specific to ADAMTS13

Plasma-derived ADAMTS13 was purified using A10-agarose immunoaffinity chromatography followed by size-exclusion chromatography. Purified ADAMTS13 had a specific activity of 302 units/mg. SDS-5% polyacrylamide gel electrophoresis (PAGE) analysis revealed a 170kD-band before and a 190 kD-band after reduction (36).

To detect IgG-type autoantibodies specific for ADAMTS13, 0.15 μg purified ADAMTS13 per lane was separated by SDS-5% PAGE under non-reducing conditions, and electrophoretically transferred to polyvinylidine fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After blocking nonspecific binding with 5% skim milk, PVDF membranes were cut longitudinally into small pieces (3 x 800 mm). Each strip was incubated overnight at 4°C with 3 ml 5% skim milk containing 50 μl heat-treated plasma from each CTD-TMA patient. The heat-treated plasma was prepared by incubation at 56°C for 30 min. After centrifugation, the supernatant was used in assays. Human IgG bound to the purified ADAMTS13 on PVDF membranes was detected using a horseradish peroxidase (HRP)-conjugated anti-human IgG polyclonal antibody (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA). Binding was visualised using the Western Lightning Chemiluminescence reagent (Perkin-Elmer Life Science Inc., Boston, MA, USA) and imaged by X-ray autoradiography (Eastman Kodak, Rochester, NY, USA) (37, 38). Heated plasma from a patient with acquired idiopathic TTP with IgG inhibitors against ADAMTS13 was used as a positive control, while that from a normal individual without ADAMTS13:INH was used as a negative control.

Statistical analysis

All continuous values are shown as median values (25, 75 percentile). All comparisons among three (severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal ADAMTS13 activity in both CTD-TMAs and ai-TTP) or five (SLE, SSc, PM/DM, RA, and ai-TTP) patient groups were tested for statistical significance using the Kruskal-Wallis H test or chi-square tests with Yates' correction for 2 x3 or 2 x 5 tables. Significant differences between three or five groups (overall $p < 0.05$) were further analysed by the Mann-Whiteney U-test or chi-square test. A two-tailed p-value less than 0.05 was considered to be significant.

Results

Clinical and laboratory features of CTD-TMAs

The clinical features and therapeutic outcomes of 127 patients with CTD-TMAs in comparison to 64 patients with ai-TTP are summarised in Table 1. SLE patients were younger at age at onset than patients with PM/DM or RA. There was a gender disparity, with female predominance, for patients with CTD-TMAs relative to ai-TTP patients. Platelet counts in SSc-TMA patients appeared to be higher than in patients with other CTD-TMAs or

Table 1: Clinical features and therapeutic outcomes of patients with CTD-TMAs and ai-TTP.

	CTD-TMAs (n=127)				ai-TTP (n=64)	Overall *P* *
	SLE (n=64)	SSc (n=42)	PM/DM (n=11)	RA (n=10)		
Clinical features						
Median age at onset of TMAs, years (25, 75 percentile)	44 (30, 54)	59 (54, 70)	57 (49, 63)	62 (56, 73)	54 (40, 69)	<0.01[a]
Female (%)	84	95	82	90	64	<0.01[b]
Renal involvement (%)	91	95	100	100	83	NS
CNS involvement (%)	69	48	64	80	70	NS
Laboratory findings at TMA diagnosis						
Median platelet count, 10^9/l (25, 75 percentile)	29 (9, 40)	50 (31, 74)	32 (9, 46)	23 (14, 28)	9 (9, 20)	NS
Median haemoglobin, g/dl (25, 75 percentile)	7.5 (6.1, 8.8)	8.3 (7.3, 9.3)	7.4 (6.6, 9.0)	7.2 (6.9, 8.1)	8.1 (6.4, 9.2)	NS
Median serum creatinine, mg/dl (25, 75 percentile)	1.6 (0.7, 2.6)	2.8 (1.9, 3.3)	1.5 (1.2, 2.3)	3.1 (1.1, 4.4)	2.1 (0.7, 2.1)	<0.01[c]
Median VWF:Ag , % (25, 75 percentile)	207 (147, 325)	256 (191, 370)	339 (225, 461)	302 (245, 454)	147 (114, 202)	<0.01[d]
Therapies						
Plasma exchange (%)	70	79	81	60	77	NS
Plasma infusion without plasma exchange (%)	27	21	18	40	25	NS
Steroid therapy without pulse therapy (%)	53	40	82	60	39	NS
Steroid pulse therapy (%)	38	26	0	20	30	NS
Rituximab (%)	0	0	0	0	9	NS
Immunosuppressants (%)	31	14	9	20	10	NS
Therapeutic response	(n=50)	(n=26)	(n=7)	(n=9)	(n=61)	
Remission of TMAs (%)	74	42	57	33	79	<0.01[e]
Death due to TMAs (%)	26	58	43	67	21	<0.01[f]

NS: not significant differences ($P \geq 0.05$). Overall *P* values were calculated using the Kruskal-Wallis H test or chi-square tests with Yates' correction for 2 x 5 tables. Significant differences between 5 groups (overall $P < 0.5$) were further analyzed by Mann-Whiteney U-test or chi-square test. [a] $P<0.01$ between SLE and PM/DM, RA. [b] $P<0.01$ between SLE, SSc, PM/DM, RA and ai-TTP. [c] $P<0.05$ between SLE and RA. [d] $P<0.01$ between SLE, and PM/DM. $P<0.01$ between PM/DM, RA and ai-TTP. [e] $P<0.01$ between SLE and SSc. $P<0.01$ between SSc, RA and ai-TTP. $P<0.05$ between SLE and RA. [f] $P<0.01$ between SLE and SSc. $P<0.01$ between SSc, RA and ai-TTP. $P<0.05$ between SLE and RA.

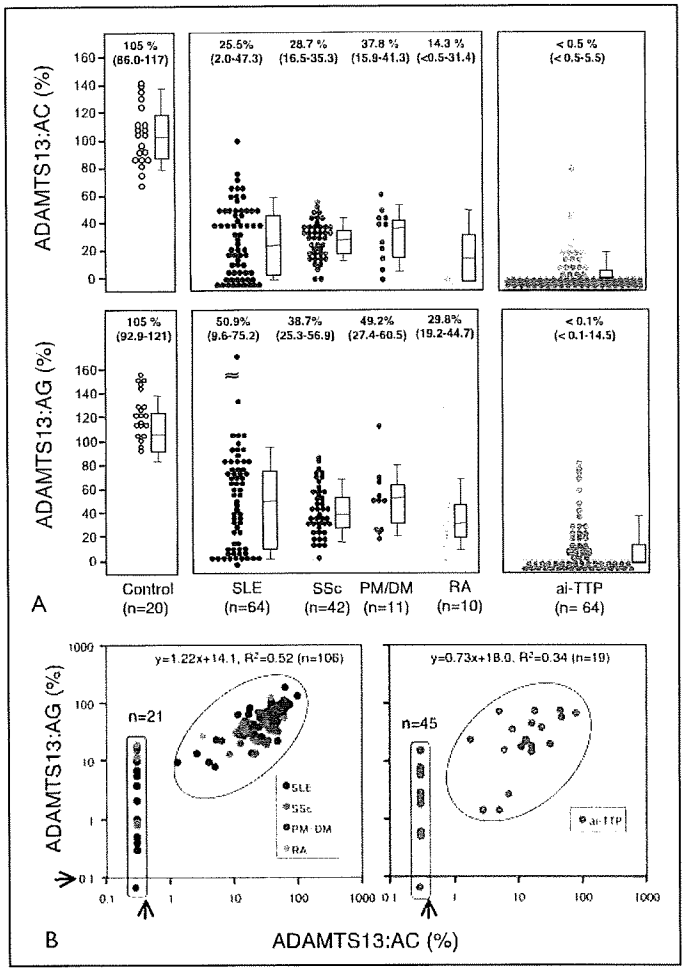

Figure 1: Plasma levels of ADAMTS13:AC and ADAMTS13:AG in patients with CTD-TMA or ai-TTP. A; top) Plasma levels of ADAMTS13:AC measured by chromogenic ADAMTS13-act-ELISA in patients with CTD-TMA or ai-TTP. The median values (25, 75 percentiles) of ADAMTS13:AC in these patients are shown at the top of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

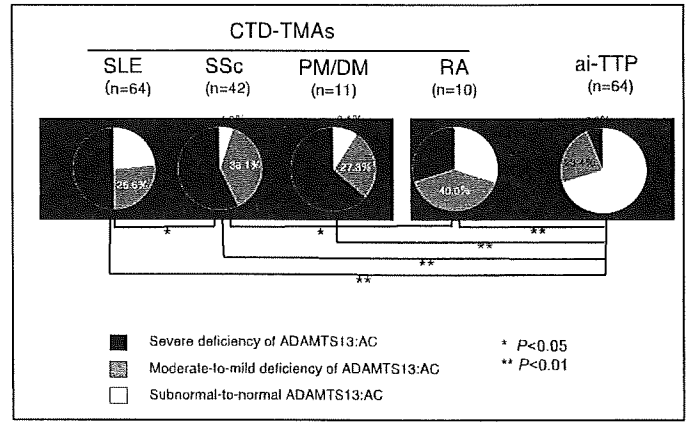

Figure 2: The proportion of CTD-TMA or ai-TTP patients grouped by three levels of plasma ADAMTS13:AC. The proportion of severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal plasma ADAMTS13:AC is illustrated in this figure. Comparison among five patient groups was tested for statistical 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 ($p=0.053$).

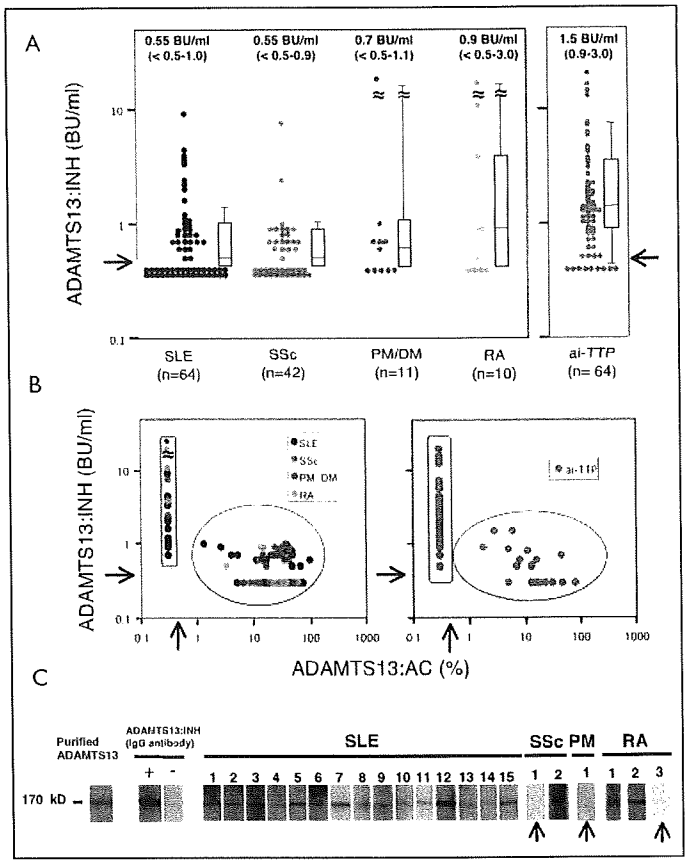

Figure 3: Plasma levels of ADAMTS13:INH and immunoblot analysis of IgG-autoantibodies to ADAMTS13. A) Plasma levels of ADAMTS13:INH in both the CTD-TMA and ai-TTP patient groups. The median values (25, 75 percentiles) of ADAMTS13:INH in these patients are shown at the top of the figure. B) Relationship 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.

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Conflict-of interest disclosure

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

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