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FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay

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Summary

A plasma metalloprotease, ADAMTS13, cleaves von Willebrand factor (VWF) multimers and downregulates their activity in platelet aggregation. Functional ADAMTS13 deficiency leads to the accumulation of hyperactive large VWF multimers, inducing a life-threatening disease, thrombotic thrombocytopenic purpura (TTP). Although measuring ADAMTS13 activity is important in TTP diagnosis, existing methods require time and skill. Here, we report a fluorescence resonance energy transfer (FRET) assay for ADAMTS13 activity. We developed a synthetic 73-amino-acid peptide, FRETS-VWF73. Cleavage of this substrate between two modified residues relieves the fluorescence quenching in the intact peptide. Incubation of FRETS-VWF73 with normal human plasma quantitatively increased fluorescence over time, while ADAMTS13-deficient plasma had no effect. Quantitative analysis could be achieved within a 1-h period using a 96-well format in commercial plate readers with common filters. The FRETS-VWF73 assay will be useful for the characterization of thrombotic microangiopathies like TTP and may clarify the importance of ADAMTS13 activity as a predictive marker for various thrombotic diseases.

Keywords: ADAMTS13, von Willebrand factor, platelet, thrombotic thrombocytopenic purpura, fluorescence resonance energy transfer.

Thrombotic thrombocytopenic purpura (TTP), a syndrome characterized by thrombocytopenia and microangiopathic haemolytic anaemia, is often associated with neurological dysfunction, renal failure and fever (Moschcowitz, 1924; Moake *et al*, 1982). Although most patients with TTP experience these crises idiopathically throughout adulthood, some patients present with neonatal onset and frequent relapses, also called Upshaw–Schulman syndrome (USS). Recent genetic studies have revealed that the majority of USS patients are homozygous or compound heterozygous for a critical mutation of the *ADAMTS13* gene (Levy *et al*, 2001; Kokame *et al*, 2002; Antoine *et al*, 2003; Assink *et al*, 2003; Savasan *et al*, 2003; Schneppenheim *et al*, 2003; Matsumoto *et al*, 2004; Pimanda *et al*, 2004). *ADAMTS13* encodes a plasma metalloprotease of the ADAMTS family (Levy *et al*, 2001; Soejima *et al*, 2001; Zheng *et al*, 2001; Plaimauer *et al*, 2002; Banno *et al*, 2004). Many patients with acquired TTP possess inhibitory auto-antibodies against ADAMTS13 (Furlan *et al*, 1998; Tsai & Lian, 1998). As the clinical characteristics of TTP are similar to those of other microangiopathic haemolytic anaemias, such as haemolytic uremic syndrome (HUS) and disseminated intravascular coagulation (DIC), an assay measuring ADAMTS13 activity would be an useful tool for appropriate diagnosis and treatment of TTP.

ADAMTS13 cleaves the peptidyl bond between Y1605 and M1606 in the A2 domain of von Willebrand factor (VWF) (Dent *et al*, 1990; Tsai *et al*, 1994; Furlan *et al*, 1996; Tsai, 1996), which circulates in plasma as large multimeric forms, ranging in size from 500 to 20 000 kDa. Functional ADAMTS13 deficiency can lead to the accumulation of large, hyperactive VWF multimers. A method to measure VWF-cleavage activity of ADAMTS13 was originally developed by Furlan *et al* (1996) and Tsai (1996), in which purified human VWF multimers were incubated with plasma in the presence of either urea or guanidine. The reaction products were separated by sodium dodecyl sulphate (SDS)-agarose (Furlan *et al*, 1996) or SDS-polyacrylamide (Tsai, 1996) gel electrophoresis, followed by Western blotting analysis with anti-VWF antibodies. Although these methods have significantly increased our understanding of the role of ADAMTS13 in TTP pathogenesis, they are not widely used at the clinical level because of technical complications.

Several groups have attempted to develop more simple and rapid diagnostic procedures for clinical use, including a

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collagen-binding assay (Gerritsen *et al*, 1999), an immunoradiometric assay using two site-directed VWF antibodies (Obert *et al*, 1999) and a ristocetin-cofactor assay (Böhm *et al*, 2002). Multicentre comparison studies of these different assays showed varied performance but supported the usefulness of the ADAMTS13 assay for TTP diagnosis (Studt *et al*, 2003; Tripodi *et al*, 2004). These assays, however, still demand complicated procedures and highly specialized materials. Therefore, a more rapid, reliable and convenient method of measuring VWF activity is eagerly awaited.

As chromogenic substrate assays are used in the clinical measurement of protease activities, initial studies were sought to identify a short oligopeptide that can be specifically cleaved by ADAMTS13 (Furlan & Lämmle, 2002). As these attempts have systematically failed, the cleavage at Y1605–M1606 of VWF probably depends on both the specific residues in the vicinity of the scissile bond and more remote sequences. Recently, we have succeeded in creating a recombinant substrate encompassing the shortest region of VWF that serves as a specific substrate for ADAMTS13 (Kokame *et al*, 2004). The peptide substrate, designated VWF73, contains 73-amino-acid residues of VWF from D1596 to R1668. In this study, we have chemically modified VWF73 to facilitate the quantitative measurement of ADAMTS13 activity in a single-step procedure.

Materials and methods

Materials

The fluorogenic substrate, FRETS-VWF73, was chemically synthesized by Thermo Electron GmbH (Sedanstrasse, Ulm, Germany) and the Peptide Institute, Inc. (Osaka, Japan). It was dissolved in 25% dimethyl sulphoxide/water to prepare the 100-μmol/l stock solution. Human plasma was obtained by centrifugation from whole blood that was treated with a 1/10 volume of 3·8% sodium citrate as an anti-coagulant. A protease inhibitor cocktail (Sigma, St Louis, MO, USA) used in the cleavage experiments contained 1 mmol/l 4-(2-aminoethyl) benzenesulphonyl fluoride, 15 μmol/l pepstatin A, 14 μmol/l *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, 36 μmol/l bestatin, 21 μmol/l leupeptin and 0·8 μmol/l aprotinin at a final concentration.

Fluorescent assay to measure the ADAMTS13 activity

Pooled human plasma (a range of 0–8 μl as a standard), or 4 μl of each test plasma, was diluted in 100 μl of assay buffer (5 mmol/l Bis–Tris, 25 mmol/l $CaCl_2$, 0·005% Tween-20, pH 6·0) in a 96-well white plate (Sumitomo Bakelite, Tokyo, Japan). Then, 100 μl of 4 μmol/l FRETS-VWF73 in the assay buffer was added to each well. Fluorescence was measured at 30°C in a Wallac 1420 ARVO multilabel counter (PerkinElmer Japan, Yokohama, Japan) equipped with a 340-nm excitation filter and a 450-nm emission filter. Fluorescence was measured every 5 min. The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 to 60 min using the PRISM software (GraphPad Software, San Diego, CA, USA).

Preparation of recombinant ADAMTS13 (rADAMTS13)

HeLa cells were cultured in Dulbecco's minimal essential medium (Invitrogen, Carlsbad, NM, USA) supplemented with 10% fetal bovine serum in humidified air with 5% CO_2 at 37°C. To produce rADAMTS13, the human ADAMTS13-expression plasmid was transfected into the subconfluent cells using FuGENE6 (Roche Diagnostics, Indianapolis, IN, USA), as described previously (Kokame *et al*, 2002; Matsumoto *et al*, 2004). Following a 4-h incubation, the culture medium was replaced with serum-free OPTI-MEM I medium (Invitrogen) and the culture was incubated for 44 h. The medium was collected and concentrated to one-eighth the original volume using Centricon YM-30 (Millipore, Billerica, MA, USA). As a negative control, a series of operations was performed in parallel as for the untransfected cells.

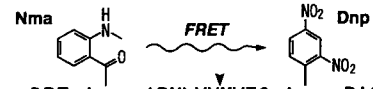
Subject population

The Suita study participants were arbitrarily selected from the municipality population registry of Suita city, stratified by gender and 10-year age groups. The basic sampling of the population started in 1989 with a cohort study base (Mannami *et al*, 1997). In the present study, 100 consecutive samples were selected from this population as a control group. This study was approved by the ethical committee on human research of the National Cardiovascular Centre. Written informed consent was obtained from all subjects prior to testing.

Results

Design of the fluorogenic substrate for ADAMTS13

To utilize fluorescence resonance energy transfer (FRET) to measure ADAMTS13 activity, we chemically synthesized a fluorogenic peptide, FRETS-VWF73 (Fig. 1), containing the 73-amino acids from D1596 to R1668 of VWF. Within this peptide, the Q1599 residue at the P7 position was converted to a 2,3-diaminopropionic residue (A2pr) modified with a 2-(*N*-



NH_2-DRE-A2pr-APNLVYMVTG-A2pr-PASDEIKRLPGDIQVVP

IGVGPNANVQELERIGWPNAPILIQDFETLPREAPDLVLQR-COOH

Fig 1. Structure of FRETS-VWF73. Within the 73-amino-acid peptide sequence, corresponding to the region from D1596 to R1668 of von Willebrand factor (VWF), Q1599 and N1610 were substituted with A2pr(Nma) and A2pr(Dnp) respectively. The arrowhead indicates the site cleaved by ADAMTS13.

methylamino)benzoyl group (Nma). The N1610 residue of the P5′ position was converted to A2pr modified with a 2,4-dinitrophenyl group (Dnp). When the Nma group is excited at 340 nm, fluorescence resonance energy is transferred to the neighbouring quencher, Dnp. If the bond between Y1605 and M1606 is cleaved, the energy transfer quenching the fluorescence does not occur, allowing the emission of fluorescence at 440 nm from Nma.

Cleavage of FRETS-VWF73 by plasma ADAMTS13

To explore the cleavage activity present in plasma, FRETS-VWF73 was incubated with normal human plasma in a fluorescent plate reader. Emission at 450 nm increased with time, indicating that FRETS-VWF73 was cleaved between the two A2pr residues by a plasma component (Fig. 2A). The increase of fluorescence was not inhibited by the addition of a protease inhibitor cocktail (mixed inhibitors effective against a broad range of serine proteases, cysteine proteases, aminopeptidases and acid proteases), but was completely inhibited by a divalent cation chelator (EDTA), suggesting that cleavage was mediated by the plasma metalloprotease, ADAMTS13, with minimal contribution of other plasma proteases. In fact, neither thrombin nor plasmin (5 μg/ml each, Sigma) increased fluorescence of FRETS-VWF73 (data not shown). The incubation of FRETS-VWF73 with plasma from an ADAMTS13-deficient patient showed no increase of fluorescence (Fig. 2A). The addition of ADAMTS13-deficient plasma to the normal plasma did not interfere with the cleavage of FRETS-VWF73 by the normal plasma (data not shown).

To verify further the cleavage by ADAMTS13, the substrate was incubated with the conditioned medium of cultured HeLa cells (Fig. 2B). Incubation with the medium of ADAMTS13-transfected cells showed the time-dependent increase of fluorescence, whereas the incubation with the medium of untransfected cells did not. All these data supported the conclusion that ADAMTS13 specifically cleaved FRETS-VWF73.

Plasma-dose dependency

FRETS-VWF73 cleavage was quantitatively dependent on plasma dosage (Fig. 3). We monitored fluorescence increase in the presence of variable volumes of normal plasma to the reaction mixture. The fluorescence over time increased with increasing plasma in a dose-dependent manner (Fig. 3A). To compensate for any differences in background fluorescence derived from plasma itself and to calculate the initial reaction rate, we estimated the slopes of the fluorescence over time using time points 0 and 60 min from a linear regression. These slopes (reaction rates) were then plotted against the plasma dosage (Fig. 3B). The data points fitted to a non-linear regression, indicating that ADAMTS13 activity in sample plasma could be estimated from the fluorescence reaction rate.

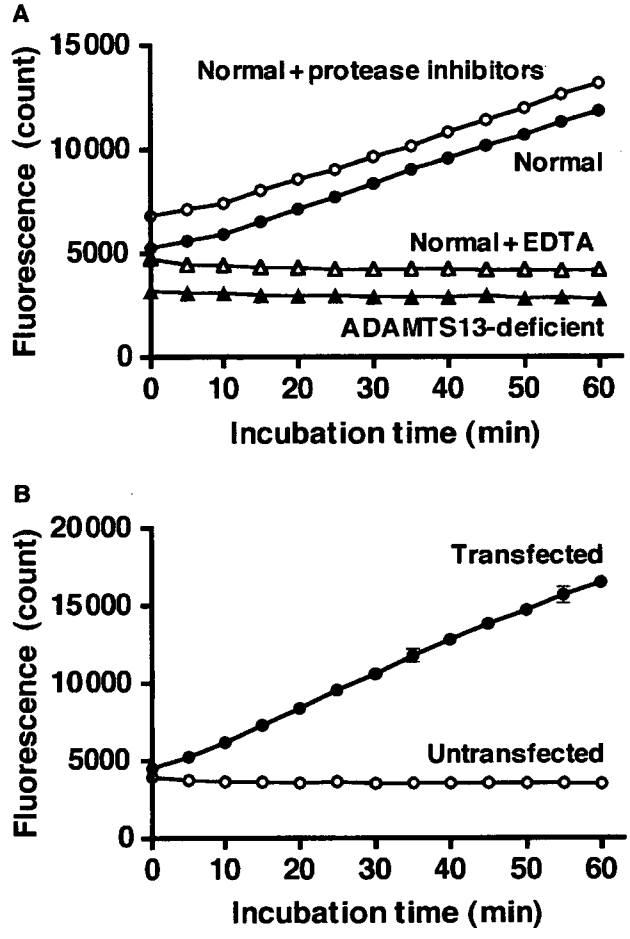


Fig 2. Cleavage of FRETS-VWF73 by ADAMTS13. (A) Fluorescence changes in FRETS-VWF73 during incubation with plasma. FRETS-VWF73 was incubated with normal plasma from a single donor in the absence (closed circles) or presence of either protease inhibitors (open circles) or EDTA (open triangles). The substrate was also incubated with ADAMTS13-deficient plasma from a congenital thrombotic thrombocytopenic purpura patient (closed triangles). Fluorescent emission at 450 nm was measured at the indicated times. A representative of three repetitive experiments is shown. (B) Fluorescence changes in FRETS-VWF73 during incubation with recombinant ADAMTS13 (rADAMTS13). FRETS-VWF73 was incubated with the conditioned medium of HeLa cells transfected with (closed circles) or without (open circles) ADAMTS13-expression plasmid DNA. Values shown are the mean and SD ($n = 3$).

Optimization of the FRETS-VWF73 assay

We next optimized reaction conditions to increase both the sensitivity and rapidity of measurement (Fig. 4). As ADAMTS13 requires divalent metal ions for proteolytic activity, we monitored the cleavage of FRETS-VWF73 by plasma in the presence of various metal ions (Fig. 4A). Ca^{2+} and Ba^{2+} ions were the most favourable for the reaction, although Mg^{2+} and Zn^{2+} also enhanced ADAMTS13 activity. In contrast, Mn^{2+} and Ni^{2+} could not activate the reaction, consistent with previous reports (Furlan *et al*, 1996; Tsai, 1996). Testing of various Ca^{2+} ion concentrations revealed that a range of

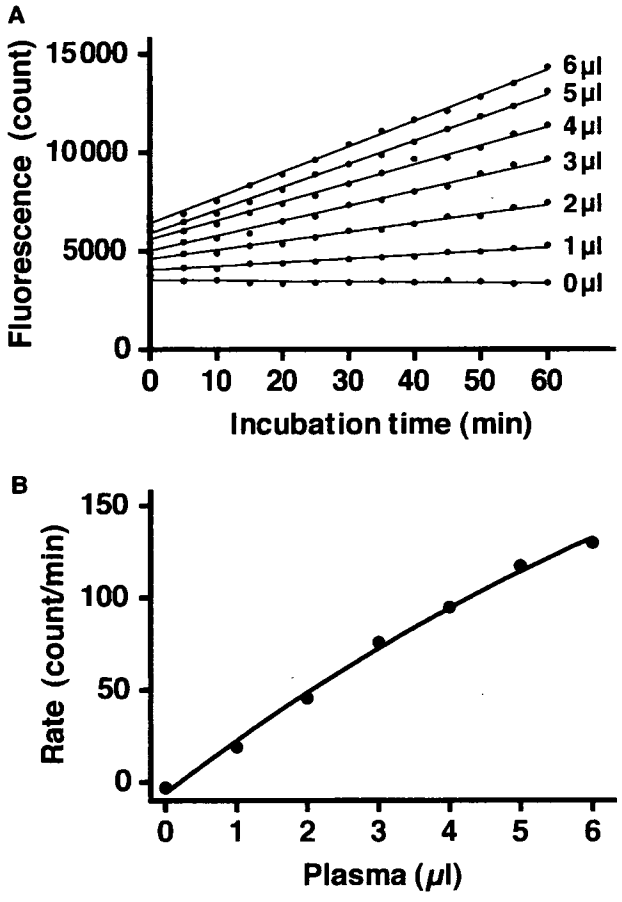


Fig 3. Plasma-dose dependency of FRETS-VWF73 cleavage. (A) Fluorescence was measured at 5-min intervals after the addition of FRETS-VWF73 to 0–6 μl normal plasma. (B) The reaction rates of time points 0 and 60 min were plotted against plasma dosage. Values were fit to a non-linear regression curve.

10–50 mmol/l Ca^{2+} was optimal for the reaction (Fig. 4B). We also examined the effect of differing NaCl concentrations, determining that lower concentrations provided more rapid cleavage (Fig. 4C), as seen in previous reports (Furlan *et al*, 1996; Kokame *et al*, 2004). The pH optimum for the FRETS-VWF73 assay was approximately 6·0 (Fig. 4D), which differed from previous studies reporting an optimal pH of 8·0–10·0 for the cleavage reaction (Furlan *et al*, 1996). This inconsistency may be a result of different reaction conditions, such as the presence or absence of denaturants. In addition, substitution of Q1599 and N1610 to A2pr(Nma) and A2pr(Dnp), respectively,

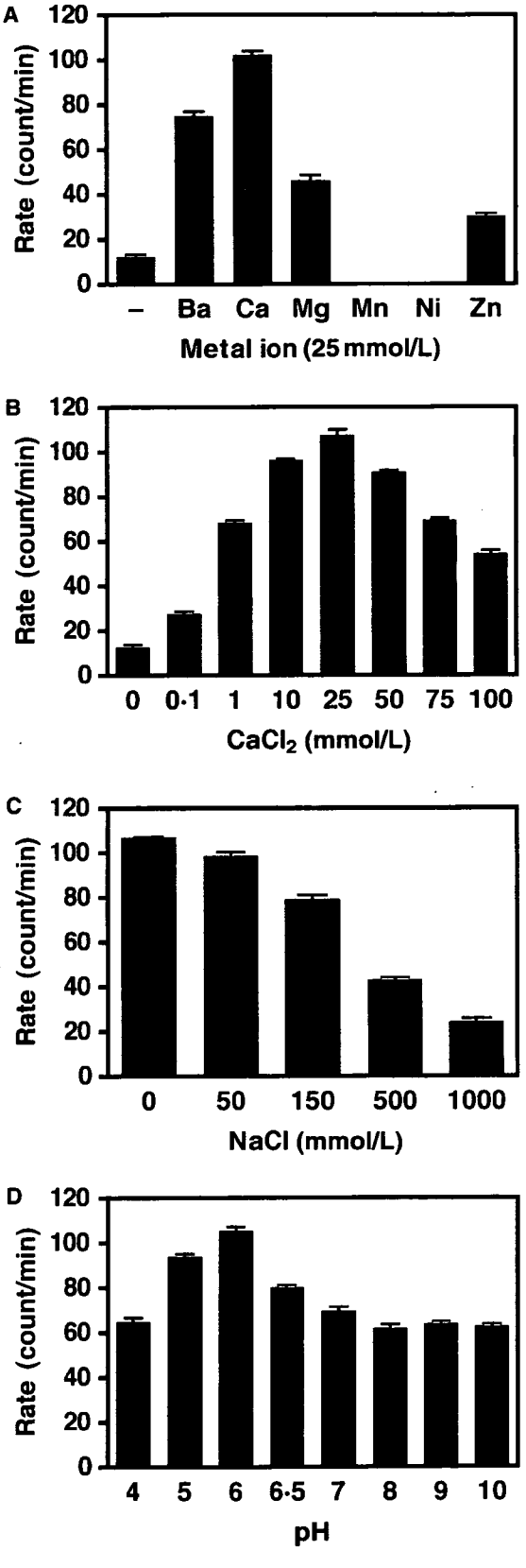


Fig 4. Optimization of the FRETS-VWF73 assay. (A) To determine metal ion dependency, FRETS-VWF73 was incubated with normal plasma from a single donor in the presence of the indicated divalent ions. (B) To measure Ca^{2+}-concentration dependency, FRETS-VWF73 was incubated with plasma in the presence of 0–100 mmol/l $CaCl_2$. (C) Ion-strength dependency was determined by incubating FRETS-VWF73 with plasma in the presence of 0–1000 mmol/l NaCl. (D) To measure pH dependency, FRETS-VWF73 was incubated with plasma in the indicated pH buffer. The reaction rates of time points 0 and 60 min are shown with the mean and SD (n = 3).

may affect the cleavage pH dependency. Alternatively, pH dependency of the assay might be affected not only by the cleavage efficiency, but also fluorescence emission, because most fluorescence reactions are highly pH dependent. Regardless, these data indicated that the FRETS-VWF73 assay was most efficient in the reaction buffer containing 5 mmol/l Bis–Tris, 25 mmol/l $CaCl_2$ and 0·005% Tween 20 at pH 6·0.

Reproducibility

We examined inter-run reproducibility of the FRETS-VWF73 assay. Plasma-dose dependency in the optimized condition was observed independently seven times. Each regression curve corresponded well with the other curves, indicating that the assay was obviously reproducible (data not shown). The relative ADAMTS13 activities of three different plasma samples were also measured independently seven times, where the activity of pooled plasma was normalized as 100%. The mean ± standard deviation (SD) values of the three samples were 113·9 ± 2·4, 62·5 ± 2·1 and 22·3 ± 1·4% (n = 7), respectively, indicating that the inter-assay variation was significantly small. The coefficients of variation of the three samples were 2·1, 3·4 and 6·3% (n = 7) respectively.

Plasma ADAMTS13 activity of patients and healthy individuals

To evaluate the FRETS-VWF73 assay for potential clinical use, we measured the relative ADAMTS13 activity in 78 plasma samples from various patients and 100 healthy individuals (Fig. 5A). The relative activities were estimated from the activity of pooled plasma prepared from all the 100 healthy individuals (66·0 ± 11·7 years old). Plasma samples from congenital TTP patients, homozygotes or compound heterozygotes of critical *ADAMTS13* mutations (Kokame *et al*, 2002; Matsumoto *et al*, 2004), all exhibited very low (<1%) or undetectable activities. The majority (33 samples) of plasma samples obtained from 41 patients with idiopathic TTP also showed low (<5%) or undetectable activities. The most possible explanation would be a deficiency of plasma ADAMTS13 level or generation of auto-antibodies against ADAMTS13, although there may be some other factor, such as auto-antibodies, that bind to the substrate and protect it from being cleaved. In contrast, plasma from parents or siblings of

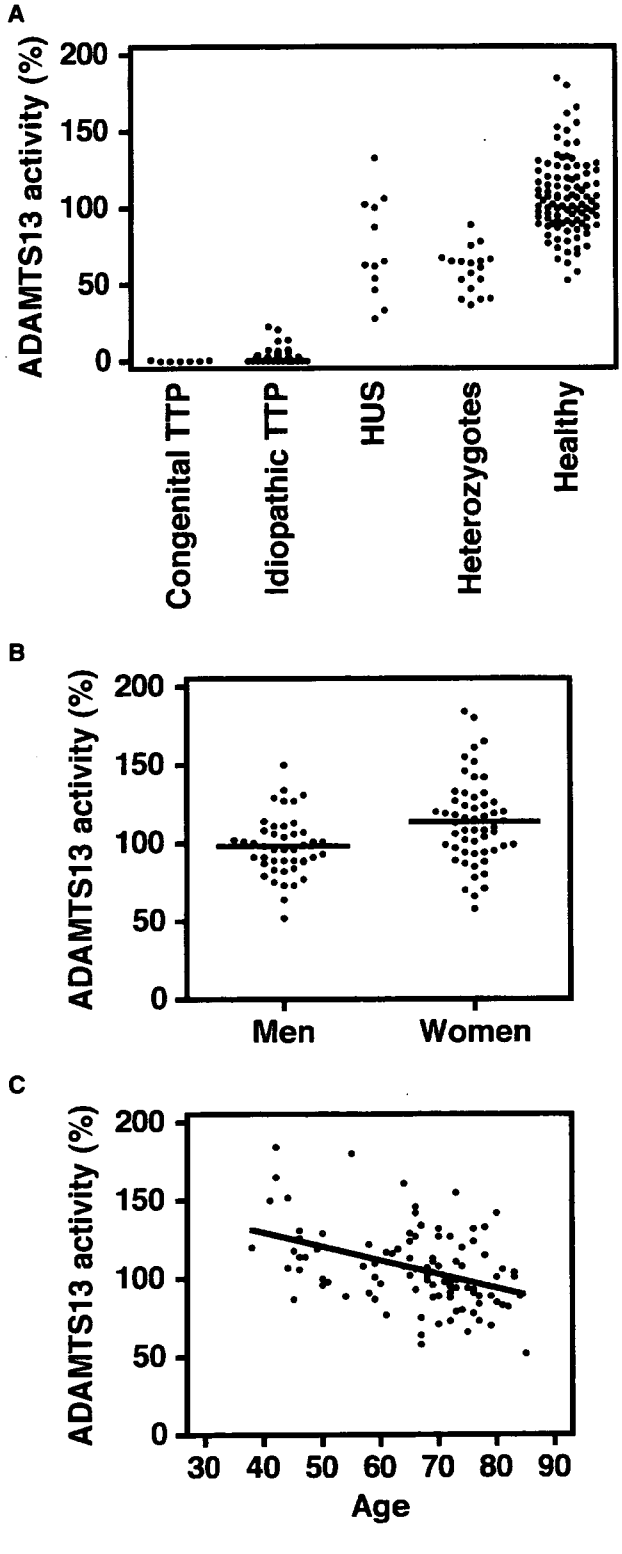


Fig 5. ADAMTS13 activity in plasma from patients and healthy individuals. (A) Relative ADAMTS13 activity was measured using the FRETS-VWF73 assay. FRETS-VWF73 was incubated with 4 μl of plasma from seven congenital thrombotic thrombocytopenic purpura (TTP) patients, 41 idiopathic TTP patients, 12 haemolytic uremic syndrome patients and 18 heterozygotes of ADAMTS13 with critical mutations. Plasma samples from randomly selected 100 healthy individuals were also examined. The relative ADAMTS13 activities were estimated from the standard curve, which was drawn up on the basis of the reaction rates of the pooled plasma prepared from all the 100 healthy individuals. (B) Association of ADAMTS13 activity with gender. Plasma ADAMTS13 activity in the healthy individuals was plotted by gender (45 men and 55 women). Bars indicate the means. Unpaired t-test identified significant differences between men and women (P = 0·0016). (C) Association of ADAMTS13 activity with age. The plasma ADAMTS13 activity correlated significantly with age (P < 0·0001). A line of best fit is indicated.

congenital TTP patients, heterozygotes of *ADAMTS13* mutations, exhibited on average approximately half the activity (59·0 ± 14·4%) of healthy individuals, while the plasma of patients with HUS showed substantial activity (73·2 ± 32·3%). Thus, the FRETS-VWF73 assay can be used to measure ADAMTS13 activity for TTP diagnosis in clinical samples.

Association of ADAMTS13 activity with gender and age

The measured ADAMTS13 activities of plasma samples from 100 healthy individuals (45 men aged 67·4 ± 11·5 years old and 55 women aged 64·9 ± 11·8 years old) were plotted according to gender (Fig. 5B). Comparison of the ADAMTS13 activities between men (97·9 ± 19·2%) and women (113·5 ± 27·1%) using the unpaired *t*-test demonstrated a significant difference between these groups ($P = 0{\cdot}0016$), suggesting that the ADAMTS13 activity of women should be significantly higher than that of men. Examination of the effect of age on ADAMTS13 activities using Spearman's rank correlation revealed a significant correlation ($r = -0{\cdot}396$, $P < 0{\cdot}0001$) (Fig. 5C). The slopes of best fit in linear regression analysis were −0·894 ± 0·196, with R^2 values of 0·175 ($P < 0{\cdot}0001$), suggesting that plasma ADAMTS13 activity should decrease with advancing age, at least after the early 40s.

Discussion

The Y1605–M1606 bond is inaccessible in native VWF and made sensitive to ADAMTS13 by denaturation and shear force. Structural modelling has suggested that the bond is buried in the core β-sheet of the VWF A2 domain (Jenkins *et al*, 1998; Sutherland *et al*, 2004). This partially explains the requirement for denaturants or shear force in the hydrolysis of the Y1605–M1606 bond by ADAMTS13. VWF73, corresponding to the C-terminal two-fifths of the A2 domain, can be efficiently cleaved by ADAMTS13 in the absence of denaturants and shear force (Kokame *et al*, 2004), suggesting that the N-terminal three-fifths of the A2 domain may prevent ADAMTS13 from accessing the cleavage site. A recent study indicated that the VWF A1 domain inhibits cleavage of the A2 domain by ADAMTS13; binding of platelet glycoprotein Ibα to the A1 domain appears to relieve the inhibition (Nishio *et al*, 2004). As VWF73 is a relatively small substrate, cleavage is less likely to be affected by other molecules. Therefore, VWF73 is an appropriate core for the convenient single-step fluorogenic assay for ADAMTS13 activity developed in this study.

Being a chemically modified version of VWF73 containing A2pr(Nma) and A2pr(Dnp), FRETS-VWF73 was a good substrate for ADAMTS13 cleavage, suggesting that Q1599 at the P7 position and N1610 at the P5′ position are not essential for the cleavage. We also examined the substitution of N1602 at the P4 position to A2pr(Nma). Although the peptide could be cleaved by plasma ADAMTS13, the efficiency was lower than that of the original FRETS-VWF73 (data not shown). The shorter distance of the modified residue from the cleavage site may interfere with efficient cleavage by ADAMTS13.

Enzymatic studies of ADAMTS13 will progress using FRETS-VWF73 as a model substrate in the future. The previously established substrate, purified plasma VWF, is comprised of non-uniform multimers with multiple cleavage sites. In contrast, FRETS-VWF73 is a monomeric molecule with a single cleavage site, facilitating the determination of cleavage kinetic parameters. No denaturants are required for the reaction, making this assay more closely reflect the physiological conditions. Although the optimal cleavage of FRETS-VWF73 still requires a hypotonic environment, isotonic solution gives approximately 80% of the activity observed in NaCl-free conditions (Fig. 3C) for kinetic analyses. VWF73, however, is not suitable for studying the functions of the other VWF domains, such as A1 and A3.

The greatest impact of the FRETS-VWF73 assay will be as a potential clinical diagnostic test. Unlike previous assays, the assay is a simple procedure, requiring no special reagents or equipment except a fluorescence spectrophotometer. These advantages may popularize ADAMTS13-activity measurement at the clinical level. The best possible application will be the appropriate diagnosis of TTP. The FRETS-VWF73 assay could be useful also for selecting curative plasma before administration to patients, as ADAMTS13 activity in the general population varies widely (Fig. 5). The selection of high-titre plasma may improve the responses of patients to plasma infusion or exchange treatment.

The relationship between ADAMTS13 deficiency and TTP is more complicated than originally thought (George & Vesely, 2004; Zheng *et al*, 2004); the problem may be because of symptomatical and pathological variety and diagnostic criteria of TTP. Not all patients with TTP present the classical five features of disease, thrombocytopenia, microangiopathic haemolytic anaemia, neurological dysfunction, renal failure and fever. Although severe ADAMTS13 deficiency is observed in most patients with idiopathic TTP without pre-existing medical conditions (Furlan *et al*, 1998; Tsai & Lian, 1998), the association between ADAMTS13 deficiency and TTP is unclear in less highly selected patient groups (Veyradier *et al*, 2001; Vesely *et al*, 2003). ADAMTS13 measurement cannot be used to predict exactly response to plasma exchange in patients that are clinically diagnosed with TTP (Vesely *et al*, 2003). An accurate ADAMTS13 assay may help to categorize TTP patients into subgroups and help establish objective diagnostic criteria.

What should be the cut-off value of ADAMTS13 activity for the diagnosis of TTP or ADAMTS13 deficiency? The present and previous studies (Mannucci *et al*, 2001; Veyradier *et al*, 2001; Böhm *et al*, 2002) showed a wide distribution of the ADAMTS13 activity in the healthy population. Further, we showed that the ADAMTS13 activity was associated with gender and age. As we used pooled plasma that was derived from relatively older individuals as a standard, the apparent ADAMTS13 activity of patient plasma may be over estimated

in the present study. To determine the universally applicable cut-off value, the definition of standard plasma will be of primary importance. The availability of purified or recombinant ADAMTS13 may help the standardization of ADAMTS13 assay. The gender- and age-oriented distribution of ADAMTS13 activity will need to be determined in the general population. Although the FRETS-VWF73 assay detected significant activity in some idiopathic TTP patients, the value was evidently lower than the lowest activity of 100 healthy individuals (Fig. 5A). Therefore, the cut-off value (for instance, the mean −2 SD % of normal activity) may have to be determined considering gender and age. The FRETS-VWF73 assay, suitable for high-throughput measurement, would accelerate such a population study.

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Giant platelet syndrome

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Introduction

Platelets are small, disk-shaped, anuclear cells with a mean diameter of 2 to 3 μm. They are derived from cytoplasmic fragmentation of megakaryocytes, released into the circulation and survived for 7–10 days. Megakaryocyte development and platelet formation are regulated by thrombopoietin and other cytokines. It is now generally assumed that platelets are released by extension and fragmentation of cytoplasmic processes (proplatelet) of megakaryocytes through sinus endothelial cells in the bone marrow. It is not known, however, how the number and the size of the platelet are controlled at this step [1].

Giant platelet syndrome is a group of unique disorders characterized by the presence of abnormally large platelets, and is usually accompanied by thrombocytopenia. Thus, it is also called macrothrombocytopenia. Giant platelets are occasionally observed as an incidental finding in routine blood smear examinations. Most of them are due to acquired disorders such as idiopathic thrombocytopenic purpura (ITP) and myelodysplastic syndrome (MDS) (Table I). In contrast, inherited giant platelet disorders are rare. The mechanisms of giant platelet formation and thrombocytopenia are not fully understood in both inherited and acquired disorders. It is important from a clinical standpoint that congenital disorders are distinguished from acquired disorders, especially ITP, to avoid unnecessary treatments. We will discuss the molecular basis, diagnosis, and management of some major inherited giant platelet syndromes.

Inherited giant platelet syndrome

In recent years we have seen remarkable progress in the molecular understanding of some giant platelet syndromes. The Table I lists some major inherited giant platelet syndromes according to the possible underlying cause: abnormalities in the platelet cytoskeleton, GPIb/IX/V, and transcription factors. Clinical and laboratory features as well as responsible genes and chromosomal localizations are also shown. There are still many inherited disorders in which the underlying genetic abnormality has not yet been elucidated.

Autosomal dominant macrothrombocytopenias with leukocyte inclusions (*MYH9* disorders)

May-Hegglin anomaly (MHA), first described a century ago, is the prototype of these disorders (Figure 1) [2]. Sebastian (SBS), Fechtner (FTNS), and Epstein (EPS) syndromes belong here. All four disorders have macrothrombocytopenia however, each of these disorders is distinguished from others by the presence or absence of granulocyte inclusion bodies and the presence or absence of a variable combination of Alport manifestations, including nephritis, deafness and cataracts (Table II). Although these four disorders were previously considered to be separate clinical entities, a recent positional cloning approach disclosed that these disorders are caused by mutations in the same gene, *MYH9*, which encodes the nonmuscle myosin heavy chain-A (NMMHCA) [3–5]. Thus, they appear to represent the same entity with different genetic penetrance and variable phenotypic expression. The bleeding tendency is usually mild.

The diagnosis of macrothrombocytopenia with leukocyte inclusions has been conventionally made on the basis of hematomorphological examinations. It is, however, not always easy to detect granulocyte inclusions on Wright stained smear. Immunofluorescence analysis of neutrophil NMMHCA localization has revolutionized the diagnosis of *MYH9* disorders [6]. Abnormal NMMHCA aggregates and accumulates in the neutrophil cytoplasm, and this abnormal

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Table I. Characteristics of Giant Platelet Syndromes

Disease	Inheritance	Gene	Chromosome	Clinical and laboratory features
Acquired				
ITP				almost normal RBC and WBC
MDS				anemia, abnormal WBC
Inherited				
Abnormalities in platelet cytoskeleton				
Autosomal dominant macrothrombocytopenia with leukocyte inclusions/ *MYH9* disorders*	AD	MYH9	22q12-13	Macrothrombocytopenia, granulocyte inclusions with/without Alport manifestations (Table II)
Abnormalities in GPIb/IX/V				
Bernard-Soulier syndrome	AR	GP1BA	17pter-p12	No ristocetin-induced platelet agglutination
		GP1BB	22q11	
		GP9	3q21	
Mediterranean macrothrombocytopenia/ Bernard-Soulier syndrome carrier	AD	GP1BA	17pter-p12	No bleeding tendency
		GP1BB	22q11	Mild thrombocytopenia with normal ristocetin-induced platelet agglutination
		GP9	3q21	
DiGeorge/Velocardiofacial syndrome	AD	GP1BB	22q11	Contiguous gene syndrome due to chromosome 22q11 microdeletion Parathyroid and thyroid hypoplasia, cardiac abnormalities, cleft palate, mental retardation
Abnormalities in transcription factors				
X-linked macrothrombocytopenia with dyserythropoiesis	XL	GATA1	Xp11	Dyserythropoiesis with/without β-thalassemia trait
Paris-Trousseau thrombocytopenia/ Jacobsen syndrome	AD	FLI1	11q23	Contiguous gene syndrome due to chromosome 11q23 microdeletion Growth and psychomotor retardation Dysmegakaryopoiesis associated with giant α-granules
Unknown cause				
Gray platelet syndrome	AR, AD	unknown		Gray or colorless platelets due to absent α-granules

*See Table II for details.
AD: autosomal dominant, AR: autosomal recessive, XL: X-linked.

subcellular localization of NMMHCA is present in every neutrophil from individuals with *MYH9* mutation. The localization pattern of neutrophil NMMHCA in *MYH9* disorders can be classified into three groups according to the number, size, and shape of the fluorescence-labeled NMMHCA granules: type I, II and III (Figure 2). In type I, NMMHCA forms one or two large and intensely stained cytoplasmic foci. Type II neutrophils consist of several cytoplasmic spots with circular to oval shape. Type III or speckled staining is detected in patients with EPS and isolated macrothrombocytopenia, in which Wright or May-Grünwald-Giemsa (MGG)-stained inclusion bodies have never been identified [6].

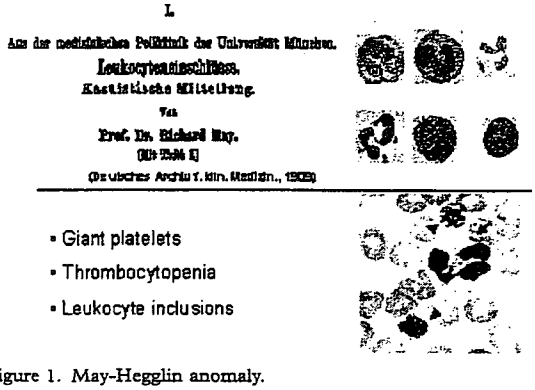

Figure 1. May-Hegglin anomaly.

An *MYH9* mutation is strictly associated with the hematological abnormalities. Although the molecular mechanism of the production of giant platelets has not been elucidated, it is suggested that abnormal NMMHCA, by interfering with the formation of myosin thick-filament, affects proper proplatelet formation in megakaryocytes. Genetic analysis of many kindred with *MYH9* disorders revealed that there is no clear relationship between clinical phenotypes and the sites of the *MYH9* mutations. It is likely that the *MYH9* mutation alone does not cause associated Alport manifestations and that unknown genetic and/or epigenetic factors might influence the phenotypic consequences of *MYH9* mutations [7,8].

We have recently generated and analyzed *MYH9* knock-out mice [9]. No homozygous mice were born, suggesting that *MYH9* expression is required for

Table II Macrothrombocytopenia with leukocyte inclusions/*MYH9* disorders

Phenotype	Macrothrombocytopenia	Leukocyte inclusions	Alport syndrome*
May-Hegglin anomaly (MHA)	+	+	−
Sebastian syndrome (SBS)	+	+	−
Fechtner syndrome (FTNS)	+	+	+
Epstein syndrome (EPS)	+	−	+

*Nephritis, deafness and cataracts.

embryonic development. By an antisense oligonucleotide-directed suppression of transcription, NMMHCA is involved in a rearrangement of the actin cytoskeleton and loss of cell adhesion [10]. *MYH9* −/− embryos, therefore, appeared to fail to develop a normal patterned embryo. Such fetal lethality may partly explain the absence of naturally occurring homozygous mutations in human subjects. In contrast, heterozygous mice (*MYH9* +/−) were viable and fertile without gross anatomical, hematological, and nephrological abnormalities. Immunofluorescence analysis showed the normal cytoplasmic distribution of NMMHCA. Interestingly, we found that some but not all mice have hearing loss. The distribution of *MYH9* expression in the inner ear has been studied in the developing fetal, neonate, adult mice [11], suggesting that *MYH9* may have important roles in the development and maintenance of auditory function. On the other hand, the unconventional myosins, Myosin VIIA is localized in the stereocilia and cell body of hair cells and is critical in differentiation, formation, and/or maintenance of sensory hair cell structure [12–14]. Myosin VIIA have been linked to nonsyndromic and syndromic hearing loss. Therefore, the requirement of NMMHCA in the mammalian auditory system might be limited and could be compensated by other conventional myosins. Thus heterozygous loss of NMMHCA might result in the phenotype that does not appear uniformly, or the severity of phenotype might be unrecognizable.

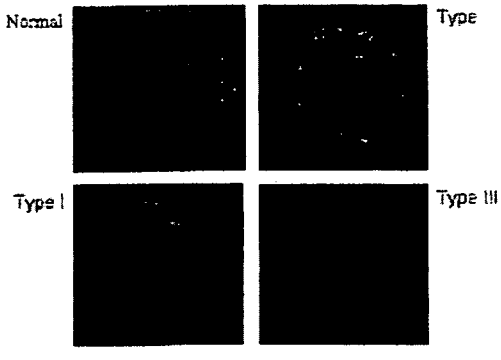

Figure 2. Subcellular localization of neutrophil NMMHCA.

Bernard-Soulier syndrome

Bernard-Soulier syndrome (BSS) is an autosomal recessive bleeding disorder characterized by giant platelets, thrombocytopenia and prolonged bleeding time (Figure 3), originally described by Bernard and Soulier in 1948 [15]. BSS is caused by quantitative or qualitative abnormalities in the glycoprotein (GP) Ib/IX/V complex, the platelet receptor for von Willebrand factor [16,17]. As a result of the absence of GPIb/IX/V complexes on the platelet membrane, platelets cannot stick to the damaged blood vessel walls, and consequently patients bleed. In the central cytoplasmic domain, GPIb associates with the actin cross-linking protein, filamin A. Thus, the defective linkage between GPIb/IX/V and cytoskeleton is the proposed molecular cause of the giant platelets.

The classical diagnostic features are a prolonged bleeding time, moderate to severe thrombocytopenia, and giant platelets. Especially, giant platelets and the absence of ristocetin-induced platelet agglutination are the laboratory hallmarks of BSS (Table I). Flow cytometric determination of platelet GPIb/IX expression is a convenient method for diagnosis of BSS in a clinical laboratory.

Thus far, 39 mutations in the genes for GPIbα, GPIbβ, and GPIX have been described. Approximately half of the mutations were found in the GPIbα gene (17 mutations), and the remaining were found in the GPIbβ (12 mutations) and GPIX genes (10 mutations). A majority of the mutations correspond to single-base substitutions or small base deletion and insertion mutations. Recent studies have shown that the phenotypes caused by mutations in the subunits of GPIb/IX span a wide spectrum, from

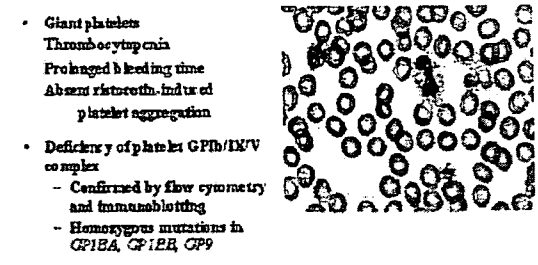

Figure 3. Bernard-Soulier syndrome.

the normal phenotype to isolated macrothrombocytopenia with normal platelet function, to full-blown BSS and platelet-type von Willebrand disease. It is important to note that although heterozygous BSS carriers are generally asymptomatic with subnormal platelet number and function, they have giant platelets. Several individuals with heterozygous GPIb/IX mutation have been initially identified as having undifferentiated thrombocytopenia or refractory ITP. In Italy, a mutation in GPIbα (Ala156Val) was found to be a founder mutation responsible for the autosomal dominant macrothrombocytopenia previously known as a Mediterranean macrothrombocytopenia [18]. In addition, patients with DiGeorge/velo-cardio-facial syndrome due to a heterozygous chromosome 22q11.2 microdeletion, which includes the GPIbβgene, have macrothrombocytopenia [19] (Table I).

X-linked macrothrombocytopenia with dyserythropoiesis

Recently, several unrelated families with X-linked macrothrombocytopenia with mild to moderate dyserythropoiesis have been found to have mutations in the GATA-1 gene. GATA-1 is a megakaryocyte- and erythroid-specific transcription factor required for normal growth and differentiation of both lineages. Defective GATA-1 function due to missense mutations causes reduced transcription and thus protein expression of its target genes, including GPIbα, GPIbβ, GPIX, and GPV. The concomitant decrease in the expression of GPIb/IX/V and other platelet-specific gene products is related to the macrothrombocytopenia and bleeding tendency in this disorder [20].

Paris-Trousseau syndrome/Jacobsen syndrome

Paris-Trousseau syndrome/Jacobsen syndrome is a contiguous gene syndrome characterized by mental retardation, and facial and cardiac abnormalities due to a heterozygous 11q23 deletion. The platelets contain giant α granules on peripheral blood smears, and in the bone marrow megakaryocytes are increased with many micro megakaryocytes. Hemizygous deletion of the transcription factor Fli1 contributes to the hematopoietic defects in this disorder [21].

Inherited macrothrombocytopenias of unknown cause

Gray platelet syndrome

Gray platelet syndrome (GPS) is characterized by thrombocytopenia and abnormal giant platelets with absent platelet α-granules. Patients with GPS have a bleeding tendency of variable severity. The gene(s) responsible for the disease are currently not known. The most characteristic feature and thus the laboratory hallmark of the syndrome is agranular platelets. On Wright- or MGG- stained peripheral blood smears, platelets appear gray or colorless due to the absence of platelet α-granules and their constituents. Because platelet α-granule proteins such as platelet-derived growth factor are synthesized but not properly stored in the granules and released from megakaryocytes into the bone marrow, myelofibrosis is present in most cases [22].

Type 2B von Willebrand disease

Patients have a prolonged bleeding time, decidedly low vWF activity measured as ristocetin cofactor activity, a mild deficiency of vWF antigen level, and enhanced ristocetin-induced platelet aggregation at low concentrations of ristocetin. Although the molecular mechanisms remain to be elucidated, some patients with type 2B vWD have been reported to have giant platelets [23].

Approach to patients with macrothrombocytopenia [24]

First of all, acquired causes of macrothrombocytopenia, including ITP and myelodysplastic syndromes, should be ruled out. Complete history and physical examination should be carefully performed. In syndromic forms, patients show complications of physical abnormalities such as facial, cardiac, skeletal anomalies and/or mental retardation. If the patient previously had normal platelet counts, acquired rather than congenital conditions are more likely to be the underlying cause. In inherited macrothrombocytopenias, platelet counts are constantly decreased, ranging from as low as 10×10^9/l to near normal 150×10^9/l. On a peripheral blood smear, the majority of platelets are large, being similar to or larger than red blood cells or small lymphocytes. In contrast, in patients with the much more common ITP, large platelets are present but the majority are of normal size. Because routine automated blood cell counting systems differentiate blood cells by their size and do not recognize giant platelets as platelets, these instruments underestimate platelet counts in patients with macrothrombocytopenia. The mean platelet volume, usually calculated as a parameter of the complete blood count, also does not reflect actual platelet size in the case of giant platelets. Platelet count should therefore be determined manually in a calculating chamber or on peripheral blood smears. Careful examination of a smear also allows morphological assessment of leukocytes and erythrocytes. If granulocyte inclusion bodies are obscure or absent, immunofluorescence analysis for neutrophil NMMHCA localization is helpful to make a clear distinction. Flow cytometric analysis of

platelet GPIb/IX expression can differentiate BSS heterozygotes from patients with "true" isolated macrothrombocytopenia.

Patients with congenital macrothrombocytopenia generally do not respond to standard ITP treatments, including corticosteroids, intravenous immunoglobulin, and splenectomy. If treatment for bleeding is clinically indicated, the administration of antifibrinolytic agents such as ε-aminocaproic acid or tranexamic acid and recombinant activated factor VII may transiently improve the episodes [25]. Transfusion of platelets is effective for serious bleeding and as prophylaxis prior to major surgery, but may be complicated by the development of alloantibodies. In certain instances, hematopoietic stem cell transplantation (SCT) may be a curative therapeutic option [26]. It is important to make a proper diagnosis to avoid unnecessary treatment. Affected families should be educated about their diagnosis to avoid unnecessary medications and potentially dangerous treatments for presumed ITP. When evaluating patients with refractory ITP or undifferentiated thrombocytopenia, congenital macrothrombocytopenias should be included in the differential diagnosis.

Conclusions

Inherited giant platelet syndromes are rare conditions, yet the study of them has been instrumental in elucidating the structure and function of normal platelets as well as the mechanisms of thrombopoiesis. Further research is needed to understand the pathogenesis of many congenital disorders with unknown causes.

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Concise Report

Two types of autoantibody-mediated thrombocytopenia in patients with systemic lupus erythematosus

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Objectives. **To determine whether autoantibodies to two platelet-specific antigens, glycoprotein IIb/IIIa (GPIIb/IIIa) and thrombopoietin receptor (TPOR), contribute to thrombocytopenia in patients with systemic lupus erythematosus (SLE).**
Methods. **Circulating B cells producing anti-GPIIb/IIIa antibodies and serum anti-TPOR antibodies were measured in 32 SLE patients with thrombocytopenia, 30 SLE patients without thrombocytopenia, 92 patients with idiopathic thrombocytopenia and 60 healthy controls. The megakaryocyte density in bone-marrow smears from all the patients with thrombocytopenia was evaluated.**
Results. **Anti-GPIIb/IIIa and anti-TPOR antibody responses were more frequent in SLE patients with thrombocytopenia than in those without thrombocytopenia (88 *vs* 17%, $P<0.0001$; and 22% *vs* 0%, $P=0.01$, respectively). The frequencies of these platelet-related antibodies were comparable between SLE patients with thrombocytopenia and patients with idiopathic thrombocytopenia. Twenty-nine (91%) SLE patients with thrombocytopenia had either anti-GPIIb/IIIa or anti-TPOR antibody, and six had both. In SLE patients with thrombocytopenia, the anti-TPOR-positive patients had significantly higher frequencies of megakaryocytic hypoplasia and poorer therapeutic responses to corticosteroids and intravenous immunoglobulin than did the anti-TPOR-negative patients, most of whom had the anti-GPIIb/IIIa antibody alone.**
Conclusions. **Anti-GPIIb/IIIa and anti-TPOR antibodies are major factors contributing to SLE-associated thrombocytopenia, but the clinical presentations associated with these autoantibodies are different.**

KEY WORDS: Autoantibodies, Glycoprotein IIb/IIIa, Systemic lupus erythematosus, Thrombocytopenia, Thrombopoietin receptor.

Thrombocytopenia is a major haematological complication in patients with systemic lupus erythematosus (SLE) [1]. The pathogenesis of thrombocytopenia in SLE patients is heterogeneous, but the most common mechanism is believed to be increased platelet clearance mediated by anti-platelet autoantibodies, which is analogous to the mechanism seen in patients with idiopathic thrombocytopenic purpura (ITP) [1]. Other potential mechanisms include thrombotic thrombocytopenic purpura, disseminated intravascular coagulation, haemophagocytic syndrome, antiphospholipid syndrome and impaired thrombopoiesis. Anti-platelet autoantibodies in ITP patients preferentially recognize platelet surface glycoproteins (GP), and the most common target is GPIIb/IIIa [2]. A recent study by Michel *et al.* [3] showed that anti-GPIIb/IIIa antibodies also play a primary role in SLE-associated thrombocytopenia. On the other hand, we recently identified autoantibodies to thrombopoietin receptor (TPOR), also called c-Mpl, which is clinically associated with thrombocytopenia in SLE patients and inhibits thrombopoietin (TPO)-dependent megakaryogenesis *in vitro* [4]. In this study, the roles of these two types of autoantibody responses in SLE-associated thrombocytopenia were evaluated.

Materials and methods

Patients and controls

We studied 32 patients with SLE who had thrombocytopenia (mean platelet count 23×10^9/l, range $5–57\times10^9$/l) and were followed at Keio University Hospital between 1997 and 2004. The inclusion criteria were as follows: (i) requirement for treatment because of a significant bleeding tendency; (ii) pretreatment bone marrow films were available; and (iii) exclusion of clinically apparent conditions that can cause thrombocytopenia, i.e. disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, haemophagocytic syndrome and drug-induced thrombocytopenia. The control subjects were 30 SLE patients who had never been thrombocytopenic and 60 healthy individuals. All the SLE patients satisfied the American College of Rheumatology preliminary criteria [5], and three with thrombocytopenia and four without thrombocytopenia additionally satisfied the Sapporo criteria for antiphospholipid syndrome [6]. We also examined 92 patients with idiopathic thrombocytopenia, defined as thrombocytopenia ($<100\times10^9$/l) that is not accompanied by morphological evidence of dysplasia in the bone marrow and cannot be attributed to other

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primary diseases or conditions. Blood samples were obtained after the patients and control subjects had given their written informed consent, as approved by the Keio University Institutional Review Board.

Clinical findings

The demographic and clinical features of each SLE patient were evaluated at the time of blood collection. Thirty-seven clinical and laboratory findings were recorded; these were the individual items included in the American College of Rheumatology preliminary classification criteria [5] and the SLE Disease Activity Index (SLEDAI) [7]. All SLE patients with thrombocytopenia received moderate- to high-dose oral corticosteroids (>40 mg/day; $n=26$) or methylprednisolone pulse therapy (1 g/day for 3 days; $n=6$), and eight of them simultaneously received intravenous immunoglobulin (IVIG; 0.4 g/day for 3–5 days) and/or platelet transfusion. During the course of the disease, 19 patients who required surgical or invasive procedure received IVIG without increase in the corticosteroid dosage or the initiation of immunosuppressant. A therapeutic response was defined as a platelet count $>100\times10^9$/l in association with these therapies. The efficacy of the corticosteroid treatment was assessed 3 months afterwards, when the potential influence of IVIG or platelet transfusion could be ignored; the efficacy of IVIG was assessed at 1 week.

Autoantibody analysis

Anti-double-stranded DNA antibody was measured by the Farr assay, and anti-Sm and anti-SSA antibodies were identified using an RNA immunoprecipitation assay [8]. IgG anti-cardiolipin antibodies were measured with an enzyme-linked immunosorbent assay (ELISA) kit (MBL, Nagano, Japan).

Anti-GPIIb/IIIa antibody-producing B cells

The anti-GPIIb/IIIa antibody response was evaluated by detecting peripheral blood B cells secreting IgG anti-GPIIb/IIIa antibodies. For this, we used an enzyme-linked immunospot assay, which is a sensitive and specific method for evaluating the presence or absence of autoantibody-mediated thrombocytopenia [9]. Briefly, peripheral blood mononuclear cells (10^5/well) were cultured in pentaplicate on GPIIb/IIIa-coated 96-well microplates at 37°C for 4 h, and subsequently incubated with alkaline phosphatase-conjugated goat anti-human IgG. Finally, the anti-GPIIb/IIIa antibodies that bound to the membrane were visualized as spots by incubation with a substrate. The frequency of circulating anti-GPIIb/IIIa antibody-producing B cells was calculated as the number per 10^5 peripheral blood mononuclear cells. The cut-off value was defined as 2.0 [9].

Anti-TPOR antibody

Serum anti-TPOR antibody was detected by ELISA using a recombinant protein encoding the entire extracellular domain of human TPOR as the antigen, as described before [4]. Antibody units were calculated from the optical density at 450 nm, using a standard curve obtained from serial concentrations of rabbit anti-human TPOR polyclonal antibodies (Kirin Brewery, Takasaki, Japan), and the cut-off value was defined as 18.0 units [4].

Evaluation of bone-marrow megakaryocyte density

Bone-marrow films from all the patients with thrombocytopenia were available. The proportion of megakaryocytes to the total number of nucleated cells was evaluated from Wright–Giemsa-stained bone-marrow smears. At least 1000 nucleated cells were counted for each sample. A proportion of megakaryocytes that was $\leq 0.2\%$ was regarded as a decrease and one of $>1.0\%$ as an increase.

Statistical analysis

All continuous results were expressed as the mean ± S.D. Comparisons to determine statistical significance between two groups were performed using Fisher's exact test or unpaired Student's *t*-test, as appropriate.

Results

Anti-GPIIb/IIIa and anti-TPOR antibody responses in SLE patients with thrombocytopenia

Anti-GPIIb/IIIa antibody-producing B cells and anti-TPOR antibody levels in SLE patients with thrombocytopenia were significantly higher than in SLE patients without thrombocytopenia or healthy controls, but were comparable to those in patients with idiopathic thrombocytopenia (Fig. 1). When all the subjects were stratified above or below the cut-off, an anti-GPIIb/IIIa antibody response was detected in 28 (88%) SLE patients with thrombocytopenia, but in five (17%) without thrombocytopenia ($P<0.0001$). Anti-TPOR antibody was detected exclusively in SLE patients with thrombocytopenia and in those with idiopathic thrombocytopenia, and its frequency was significantly higher in SLE patients with thrombocytopenia than in SLE patients without it (22 *vs* 0%, $P=0.01$). The respective frequencies of anti-GPIIb/IIIa and anti-TPOR antibodies in SLE patients with thrombocytopenia were comparable to those in patients with idiopathic thrombocytopenia (86 and 10%). Finally, 29 (91%) of the SLE patients with thrombocytopenia produced either anti-GPIIb/IIIa or anti-TPOR antibody, and six of these patients produced both.

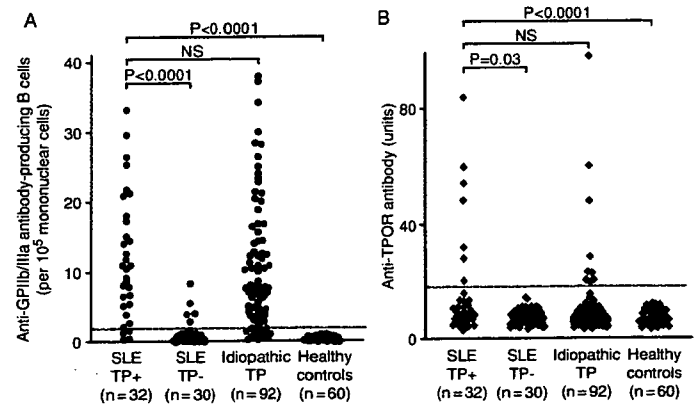

FIG. 1. Circulating anti-GPIIb/IIIa antibody-producing B cells (A) and serum anti-TPOR antibody (B) in 32 SLE patients with thrombocytopenia (TP), 30 SLE patients without thrombocytopenia, 92 patients with idiopathic thrombocytopenia and 60 healthy controls. A broken line indicates a cut-off level (2.0 for anti-GPIIb/IIIa antibody-producing B cells and 18.0 for anti-TPOR antibody). Levels were compared between SLE patients with thrombocytopenia and other groups using the unpaired *t*-test. NS, not significant ($P>0.05$).

Megakaryocyte density in association with autoantibody status

Examination of the bone marrow from SLE patients with thrombocytopenia revealed that eight (25%), 17 (53%) and seven (22%) patients had increased, normal and decreased bone megakaryocytes, respectively. A similar distribution was observed in the 92 patients with idiopathic thrombocytopenia; i.e. 20, 66 and 14% had increased, normal and decreased bone megakaryocytes, respectively. The status of anti-GPIIb/IIIa and anti-TPOR antibodies was compared with the bone marrow megakaryocyte density of patients with SLE and thrombocytopenia and those with idiopathic thrombocytopenia. Seven SLE patients who had anti-TPOR antibody had a significantly higher frequency of megakaryocytic hypoplasia than 25 patients who did not (86 *vs* 4%, $P<0.0001$; Table 1), and this association appeared to be independent of anti-GPIIb/IIIa antibody production. In contrast, none of the SLE patients who produced anti-GPIIb/IIIa antibody but not anti-TPOR antibody had megakaryocytic hypoplasia. Similarly, in patients with idiopathic thrombocytopenia, megakaryocytic hypoplasia was significantly more frequent in the nine patients with anti-TPOR antibody than in the 83 without this antibody (79 *vs* 7%, $P<0.0001$).

Clinical associations with anti-TPOR antibody

Additional clinical and laboratory findings for SLE patients with thrombocytopenia were compared based on the presence or absence of anti-TPOR antibody (Table 1). There was no significant difference in sex, age at examination, SLE-related clinical findings, lowest platelet count, autoantibody status, including anti-GPIIb/IIIa antibody-producing B cells, or SLEDAI between these two groups. A poor therapeutic response to corticosteroids was more prevalent in patients with anti-TPOR antibody than in those without, most of whom had anti-GPIIb/IIIa antibody alone ($P=0.0006$). Thus, the immunosuppressant use for thrombocytopenia was significantly more frequent in anti-TPOR-positive patients than in anti-TPOR-negative patients (71 *vs* 8%, $P=0.002$). Furthermore, all five anti-TPOR-positive patients who received IVIG were non-responders, while only one patient (10%) without this antibody showed a poor response to IVIG ($P=0.002$).

TABLE 1. Clinical and laboratory findings for SLE patients with thrombocytopenia who did or did not produce anti-TPOR antibody

Clinical and laboratory findings	Anti-TPOR-positive ($n=7$)	Anti-TPOR-negative ($n=25$)	P
Sex (% female)	86	88	NS
Age at examination (yr, mean ± S.D.)	44.0 ± 5.6	37.3 ± 15.0	NS
Malar rash (%)	43	36	NS
Discoid rash (%)	14	8	NS
Photosensitivity (%)	28	20	NS
Oral ulcers (%)	14	16	NS
Arthritis (%)	14	12	NS
Serositis (%)	14	8	NS
Renal disorder (%)	14	24	NS
Neurological disorder (%)	14	4	NS
Haemolytic anaemia (%)	0	4	NS
Leucopenia (%)	57	60	NS
Lowest platelet count ($\times 10^9$/l; mean ± S.D.)	20.7 ± 17.9	24.1 ± 12.1	NS
Anti-dsDNA antibody (%)	71	76	NS
Anti-Sm antibody (%)	14	4	NS
Anti-SSA antibody (%)	57	44	NS
Anti-cardiolipin antibody (%)	28	48	NS
Anti-GPIIb/IIIa antibody-producing B cells ($/10^6$ mononuclear cells, mean ± S.D.)	8.4 ± 8.1	12.8 ± 8.8	NS
Megakaryocytic hypoplasia (%)	86	4	<0.0001
Poor response to corticosteroids (%)	86	12	0.0006
Poor response to IVIG (n/n,%)	5/5 (100)	1/10 (10)	0.002
SLEDAI (mean ± S.D.)	9.0 ± 5.8	7.5 ± 7.3	NS

NS, not significant ($P \geq 0.05$).
dsDNA, double-stranded DNA; IVIG, intravenous immunoglobulin; SLEDAI, SLE disease activity index.

Discussion

Our findings demonstrate that both anti-GPIIb/IIIa and anti-TPOR antibodies are associated with thrombocytopenia in SLE patients, although the tests used were not necessarily comparable: antibody-secreting peripheral blood B cells were measured to detect the anti-GPIIb/IIIa antibody response while serum samples were used to detect the anti-TPOR antibody response. More than 90% of SLE patients with thrombocytopenia had at least one of these platelet-related autoantibodies, indicating that thrombocytopenia mediated by these two types of autoantibody is a dominant mechanism for SLE-associated thrombocytopenia, as for idiopathic thrombocytopenia.

Interestingly, anti-GPIIb/IIIa and anti-TPOR antibodies were associated with different phenotypes of thrombocytopenia, in terms of bone-marrow megakaryocyte density and therapeutic responses to standard treatment regimens for immune thrombocytopenia. All the SLE patients with anti-GPIIb/IIIa antibody alone had normal or increased megakaryocyte density, whereas the anti-TPOR antibody was strongly associated with megakaryocytic hypoplasia. This different phenotype can be explained by the distinct biological effects of these antibodies: anti-GPIIb/IIIa antibody binds circulating platelets and facilitates Fcγ receptor-mediated clearance of opsonized platelets by reticuloendothelial phagocytes [2], whereas anti-TPOR antibody blocks TPO signalling, resulting in inhibition of megakaryogenesis in the bone marrow [4]. This different mode of action may also account for the lack of therapeutic response to IVIG of patients with anti-TPOR antibody. Since interactions between the Fc portion of the infused immunoglobulins and the Fc receptors on target cells are thought to be a primary action of IVIG [10], it is likely that IVIG has little effect on the TPO signal blockade through the variable region of the antibodies.

In summary, measurement of anti-GPIIb/IIIa anti-TPOR antibody responses is useful in distinguishing between subsets of patients with SLE and thrombocytopenia and predicting their therapeutic response.

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Key messages

- Anti-GPIIb/IIIa and anti-TPOR antibodies are major contributory factors to SLE-associated thrombocytopenia.
- Anti-TPOR antibody is associated with megakaryocytic hypoplasia and poor therapeutic responses to corticosteroids and intravenous immunoglobulin.

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ORIGINAL ARTICLE

Preliminary laboratory based diagnostic criteria for immune thrombocytopenic purpura: evaluation by multi-center prospective study

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Summary. *Background:* We proposed diagnostic criteria for immune thrombocytopenic purpura (ITP) by modifying the existing guidelines for diagnosis of ITP and by incorporating laboratory tests found useful for predicting its diagnosis, for example erythrocyte count, leukocyte count, anti-GPIIb/IIIa antibody-producing B cells, platelet-associated anti-GPIIb/IIIa antibodies, percentage of reticulated platelets, and plasma thrombopoietin. *Objective and methods:* To validate our criteria, we conducted a multi-center prospective study involving 112 patients with thrombocytopenia and a morphologically normal peripheral blood film at the first visit. Each patient underwent a physical examination, routine laboratory tests, and specialized tests for the anti-GPIIb/IIIa antibody response and platelet turnover. *Results:* Ninety-one patients (81%) satisfied the proposed criteria at first visit. Clinical diagnosis was made by skilled hematologists > 6 months after the first visit; ITP was diagnosed in 88 patients and non-ITP disorders in 24. The proposed criteria had 98% sensitivity, 79% specificity, a 95% positive predictive value, and a 90% negative predictive value. A relatively low specificity appears to be attributed to a few patients who had both ITP and aplastic anemia or myelodysplastic syndrome. *Conclusions:* Our preliminary diagnostic criteria based on ITP-associated laboratory findings were useful for the differential diagnosis of ITP, but additional evaluations and modifications will be necessary to develop criteria that can be used routinely.

Keywords: clinical studies, immune thrombocytopenic purpura, platelet antibodies.

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Introduction

Immune thrombocytopenic purpura (ITP) is an acquired hemorrhagic condition of accelerated platelet consumption caused by antiplatelet autoantibodies [1]. This condition is seen in patients with various diseases, such as systemic lupus erythematosus (SLE) and human immunodeficiency virus (HIV) infection, and can also occur without an underlying disease, in which case it is known as idiopathic or primary form of ITP. Currently, the diagnosis of ITP is principally based on the exclusion of other possible concurrent causes of thrombocytopenia [1,2]. According to the guidelines proposed by the American Society of Hematology (ASH), the diagnosis of ITP should be made in patients with thrombocytopenia who lack findings that are atypical or suggest another diagnosis in their history, physical examination, complete blood count, and peripheral blood film, and no further laboratory tests are considered necessary [3]. A similar guideline has been proposed in the UK [4]. However, it is difficult for physicians who are not experts in ITP to exploit the guidelines' recommendations, because these guidelines are principally aimed at detecting alternative causes of thrombocytopenia and require comprehensive expertise and experience in platelet disorders. One potential solution to this problem is development of consistent and reproducible criteria, which do not rely on the skill of the clinicians. For this purpose, it is potentially useful to