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ORIGINAL ARTICLE

Association of the antagonism of von Willebrand factor but not fibrinogen by platelet $\alpha_{IIb}\beta_3$ antagonists with prolongation of bleeding time

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Summary. *Background:* The $\alpha_{IIb}\beta_3$ antagonists inhibit platelet aggregation and are used as antithrombotic agents for cardiothrombotic disease. The present study investigates the correlation of inhibition of fibrinogen and von Willebrand factor (VWF) binding by $\alpha_{IIb}\beta_3$ antagonists with the inhibition of platelet aggregation and prolongation of bleeding time (BT). *Methods:* Inhibition of fibrinogen and VWF binding were assessed in a purified $\alpha_{IIb}\beta_3$-binding assay. As an *in vitro* cell-based assay, platelet aggregation and VWF-mediated adhesion studies were performed using human platelets. *In vivo* e ects on BT were measured using a template device in dogs at the same time as an *ex vivo* aggregation study was performed. *Results: In vitro* studies demonstrated that the antiaggregatory e ects of $\alpha_{IIb}\beta_3$ antagonists correlate with their inhibition of fibrinogen binding, but not VWF. Interestingly, the e ects of $\alpha_{IIb}\beta_3$ antagonists on BT could be di erentiated from the inhibition of platelet aggregation. Furthermore, this di erentiation was strongly correlated with the di erent inhibitory potencies between fibrinogen and VWF binding, as well as that between VWF-mediated adhesion and aggregation. *Conclusions:* Our study provides novel evidence showing that the inhibitory e ect of $\alpha_{IIb}\beta_3$ antagonists on VWF, but not fibrinogen binding, correlates with their ability to prolong BT.

Keywords: bleeding, fibrinogen, platelet $\alpha_{IIb}\beta_3$ antagonists, von Willebrand factor.

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Introduction

Platelet aggregation and adhesion are key mechanisms for normal hemostasis and limiting blood loss from injured tissue [1,2]. $\alpha_{IIb}\beta_3$, the major integrin on the surface of the platelet membrane, is a physiologic receptor for fibrinogen and von Willebrand factor (VWF). The binding of these two ligands plays a crucial role in platelet aggregation and adhesion, in the initial process of thrombus formation. Antagonism of $\alpha_{IIb}\beta_3$ is an attractive strategy for antiplatelet therapy and achieves complete inhibition of aggregation [3,4]. Several $\alpha_{IIb}\beta_3$ antagonists have been used as antithrombotic drugs, and have demonstrated favorable efficacy in reducing the incidence of ischemic events in unstable angina and in preventing vascular events after percutaneous transluminal coronary angioplasty (PTCA) [5–7], although they appeared to increase the risk of bleeding [8,9]. Thus, careful management and monitoring of patients, such as weight adjustment of heparin dose and assessment of hemostatic markers [10–11], is required to avoid bleeding complications. Mechanistic studies on the hemorrhagic properties of $\alpha_{IIb}\beta_3$ antagonists would provide more detailed information about controlling bleeding complications.

Current mechanistic studies on platelet-thrombus formation under flow conditions suggest that the $\alpha_{IIb}\beta_3$ ligands, VWF and fibrinogen, play different functions in the process of thrombus formation. In the initial process of hemostasis, VWF binding to $\alpha_{IIb}\beta_3$ is a key step for formation of platelet adhesion, causing the initial surface coverage of injured vascular lumen. In contrast, fibrinogen binding plays a major role in the later phase of thrombus formation on the covered surface by recruiting platelet aggregates [12–14], and leads to formation of a mature thrombus to plug the injured vessels. Therefore, we hypothesized, if inhibition of the VWF-dependent surface coverage by platelets is the critical hemorrhagic determinant for $\alpha_{IIb}\beta_3$ antagonists, rather than the later fibrinogen-dominant phase, $\alpha_{IIb}\beta_3$ antagonists with minimal inhibition of VWF

binding should show reduced tendency for prolonged bleeding time (BT) but retain potent antiaggregation effects.

In this study, we assessed the inhibitory effects of synthetic $\alpha_{IIb}\beta_3$ antagonists on VWF and fibrinogen binding and examined their association with *ex vivo* inhibition of platelet aggregation and an *in vivo* hemorrhagic effect, prolongation of BT.

Materials and methods

Materials

FK419 [15], FK633 [16], FR184764, FR233229, FR231643, lamifiban [17], xemilofiban [18], and tirofiban [19] (Fig. 1) were synthesized in Fujisawa Pharmaceutical Co., Ltd (Osaka, Japan), with purity >98% by HPLC and mass spectrum analysis. All antagonists were dissolved in isotonic sodium chloride solution or buffer prior to use.

Proteins and antibodies

Fibrinogen and VWF were purchased from American Diagnostica Inc. (Stamford, CT, USA). Purified human $\alpha_{IIb}\beta_3$ was purchased from Enzyme Research Laboratories Inc. (South Bend, IN, USA). Anti-$\alpha_{IIb}\beta_3$ (PT25-2) was purchased from Takara (Ohtsu, Japan). HPR-F(ab')$_2$ fragments of goat antimouse IgG (H + L) antibody was purchased from Zymed Laboratories Inc. (San Francisco, CA, USA) . Bovine serum albumin (BSA) was purchased from Nakarai (Kyoto, Japan).

Inhibition assay for the interaction of purified $\alpha_{IIb}\beta_3$ with fibrinogen and von Willebrand factor

Fibrinogen (1 μg per well) was immobilized on a 96-well microtiter plate overnight at 4 C. The residual exposed plastic was blocked with 1% BSA. After the plate was washed twice with buffer, 50 μL of purified $\alpha_{IIb}\beta_3$ at 20 μg mL^{-1} in the presence or absence of 50 μL of drug were added to each well and incubated for 3 h at room temperature. Bound $\alpha_{IIb}\beta_3$ was measured by ELISA by using primary (antihuman $\alpha_{IIb}\beta_3$) and secondary (goat antimouse IgG coupled to HPR) antibodies. In the case of VWF-binding assay, $\alpha_{IIb}\beta_3$ was immobilized on the plate. VWF was added and binding was measured by ELISA. IC_{50} values were calculated by using the curve fitting and statistics software, PRISM version 4, and are expressed as the mean ± SEM of five experiments. All experiments were performed with the same lot of the proteins. $\alpha_{IIb}\beta_3$ antagonists were compared under the same assay conditions. We also measured K_d values for fibrinogen and VWF binding in our assay formats. The K_d values (Fg: K_d = 87 nM, VWF: K_d = 11 nM) were comparable with those reported in the literature [20,21].

Platelet adhesion to von Willebrand factor coated plate

Venous blood from healthy male volunteers was collected onto sodium citrate. Platelet-rich plasma (PRP) was prepared by rapid centrifugation of whole blood. Platelets were washed with modified HEPES-Tyrode's buffer containing 1 μM PGE_1. After washing, platelets were suspended in modified HEPES-Tyrode's buffer containing 1.0 mM $CaCl_2$ without PGE_1, and platelet count was adjusted. Adhesion assay protocol was performed as described previously [22]. Briefly, 96-well microtiter plates were coated with 1 μg per well of VWF. The plates were then blocked with 1% BSA. After washing the plates with buffer, ADP-activated washed platelets were added to each well in presence of drugs or buffer and incubated for 30 min at 37 C. The plates were then washed three times with buffer. The number of adhered cells was determined by the acid

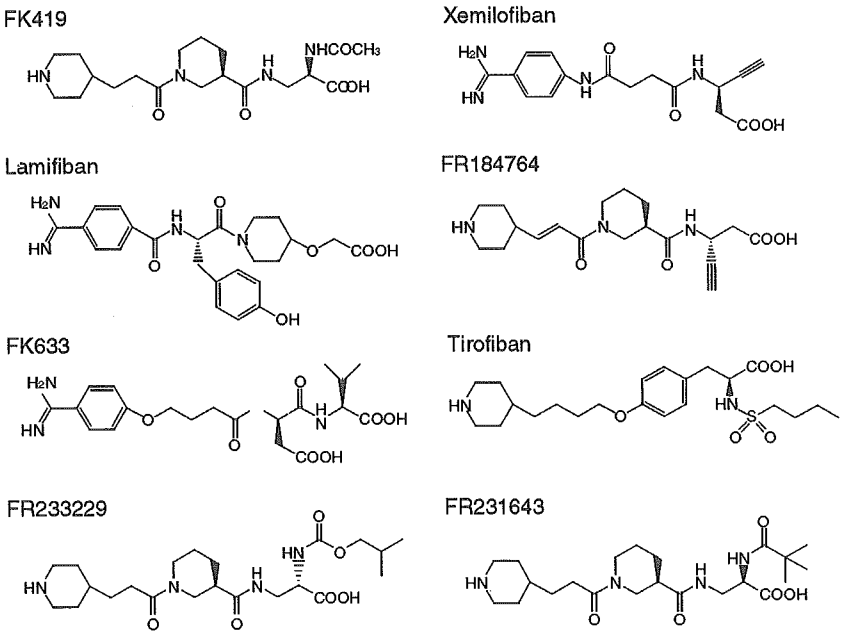

Fig. 1. Chemical structures of synthetic $\alpha_{IIb}\beta_3$ antagonists.

phosphatase activity of cells at 410 nm using a microplate reader.

Platelet aggregation study

Platelet aggregation assays were performed using NBS HEMA TRACER 801, an eight-channel aggregometer (Nikobioscience, Tokyo, Japan). Light transmittance of platelet poor plasma (PPP) was calibrated as 100%. PRP was incubated for 2 min in the aggregometer at 37 C. ADP (2.5 μM for humans and 20–40 μM for dogs) was added as an agonist at which full response of platelet aggregation was obtained, and the change in light transmittance was monitored by a PL500 recorder (Yokogawa, Tokyo, Japan). Percent inhibition of aggregation in the drug-treated samples was calculated by comparison with the aggregation in the absence of drug or in the pretreatment period.

Measurement of template bleeding time

All experiments were carried out in accordance with the guidelines provided by the Ethical Committee of Fujisawa Pharmaceutical Co., Ltd. Beagle dogs (Kashou Co., Ltd or Japan Laboratory Animals Co., Ltd, Tokyo, Japan) were anesthetized with sodium pentobarbital at 30 mg kg^{-1} intraperitoneally. $\alpha_{IIb}\beta_3$ antagonists were administered by a bolus injection (0.2 mL kg^{-1}), followed by continuous infusion (0.1 mL kg^{-1} h^{-1}) for 3 h, from a catheter inserted into the vein on the surface of the hind limb. Blood samples were taken six time-points from the cephalic vein for the platelet aggregation study and for measurement of plasma drug concentrations at 30-min intervals after dosing. At the same time as blood sample collection, BT was assessed with an automated spring-loaded device (Simplete R, Organon Teknika, Durham, NC, USA) designed to produce a standardized incision applied to the inner side of the upper jowl. Blood from the incision was blotted with Whatman No.2 filter paper every 30 s until all bleeding had stopped. In order to eliminate the experimental validity, we have excluded animals with the basal BT of > 5 or < 3 min. Our laboratory standard of basal BT was 3.9 ± 0.3 min. When bleeding did not stop within 30 min, BT was recorded as 30 min. Prolongation of BT was determined by comparison with the time in the pretreatment period.

Determination of plasma concentration of $\alpha_{IIb}\beta_3$ antagonists

Plasma samples were mixed with water, and purified by solid-phase extraction. The eluate was evaporated under a nitrogen stream, dissolved in water, and analyzed by LC-MS-MS. HPLC separation was achieved using a mobile phase consisting of formate buffer/acetonitrile/methanol with flow rate 0.2 mL min^{-1}. The lower limit of quantification was 10 ng mL^{-1} using 50 μL plasma, and intra-day precision measurements of the assay as indicated by coefficients of variation were $< 10\%$.

Calculation of mean plasma concentrations for 50% inhibition of platelet aggregation and for 2.5-fold prolongation of bleeding time

Data for inhibition of ADP-induced *ex vivo* platelet aggregation (Yi%) and prolongation of BT (Zi-fold) at all doses and time points were sorted in order of increasing plasma concentrations (Xi, ng mL^{-1}) of an antagonist. Mean concentrations for 50% inhibition of platelet aggregation and 2.5-fold prolongation of BT were calculated using curve fitting and statistics software, PRISM version 4. The values are expressed as mean ± SEM.

Results

Inhibition of fibrinogen and von Willebrand factor binding to purified $\alpha_{IIb}\beta_3$

Inhibitory effects of synthetic $\alpha_{IIb}\beta_3$ antagonists on fibrinogen and VWF binding were determined by ELISA assays of purified $\alpha_{IIb}\beta_3$. In this binding assay, all antagonists effectively inhibited both fibrinogen and VWF binding to purified $\alpha_{IIb}\beta_3$ in a concentration-dependent manner (Fig. 2). Interestingly, the rank orders of antagonists for the inhibition were different between fibrinogen and VWF binding (fibrinogen inhibition; tirofiban < FK419 = xemilofiban < FK633, VWF inhibition; tirofiban ≤ xemilofiban < FK419 = FK633). For instance, FK419 inhibited VWF binding with similar potency as FK633; however, its potency for fibrinogen was fourfold higher than FK633. Thus, FK419 is a relatively selective inhibitor of fibrinogen binding compared with FK633. To validate the relative selectivity of $\alpha_{IIb}\beta_3$ antagonists, we calculate ratios of IC_{50}s for fibrinogen to VWF binding as a selectivity index, VWF/Fg (Table 1). The results of this study suggest that the selectivity index varied for each $\alpha_{IIb}\beta_3$ antagonist.

Inhibition of human platelet aggregation and von Willebrand factor-mediated adhesion

To further assess the cell-based antagonism of $\alpha_{IIb}\beta_3$ antagonists against fibrinogen and VWF, we assessed their inhibitory effects of the antagonists on ADP-induced aggregation and adhesion onto immobilized VWF *in vitro*. As summarized in Table 2, all antagonists were effective in inhibiting ADP-induced platelet aggregation (AG1) with IC_{50} values ranging from 21 to 103 nM, with comparable potency to their inhibition of fibrinogen binding to purified $\alpha_{IIb}\beta_3$. Furthermore, in the platelet adhesion assay, they also effectively inhibited ADP-activated platelet adhesion to VWF-coated plates. However IC_{50} values for VWF-mediated adhesion (AD) were not parallel to that for aggregation. The $\alpha_{IIb}\beta_3$ antagonists, xemilofiban, lamfiban, and tirofiban, showed similar or greater potency for inhibiting VWF-mediated adhesion compared with their inhibitory effects on platelet aggregation, and had a lower ratio (AD/AG1). In other words, these antagonists had lower ratios of AD/AG1 than those of FK419 and FR233229. The

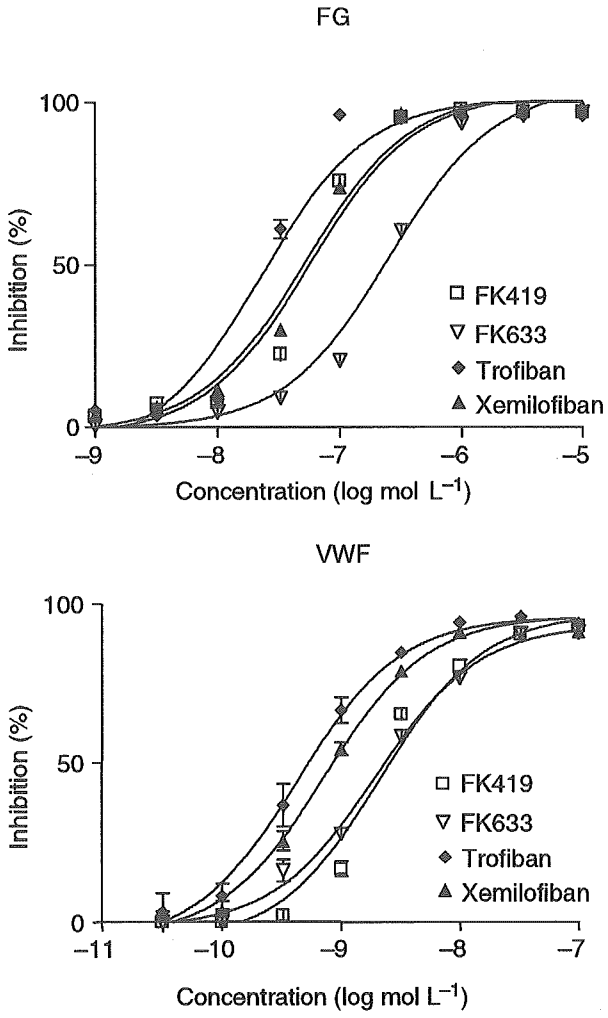

Fig. 2. E ects of synthetic $\alpha_{IIb}\beta_3$ antagonists, FK419, FK633, tirofiban, and lamifiban, on fibrinogen and von Willebrand factor binding to purified $\alpha_{IIb}\beta_3$ receptors. All experiments were performed by using the same protein preparation and assay plates. Data are presented as the mean ± SEM of five experiments.

Table 1 Inhibitory e ects of $\alpha_{IIb}\beta_3$ antagonists for purified $\alpha_{IIb}\beta_3$-ligand binding

	Fibrinogen (Fg) IC_{50} (nM)	von Willebrand factor (VWF), IC_{50} (nM)	Ratio (VWF)/(Fg)
FK419	53 ± 2.7	3.2 ± 0.34	0.060
FK633	200 ± 14	2.8 ± 0.49	0.014
Xemilofiban	45 ± 0.2	0.87 ± 0.15	0.019
Lamifiban	20 ± 4.9	0.76 ± 0.06	0.038
Tirofiban	26 ± 0.2	0.73 ± 0.30	0.028
FR184764	34 ± 3.6	0.68 ± 0.14	0.020
FR233229	9.5 ± 0.6	0.47 ± 0.07	0.049
FR231643	24 ± 2.8	1.2 ± 1.3	0.050

results of cell-based studies suggest that each $\alpha_{IIb}\beta_3$ antagonists have different inhibitory effects between platelet aggregation and VWF-mediated platelet adhesion.

Table 2 Inhibitory e ects of $\alpha_{IIb}\beta_3$ antagonists on platelet aggregation and von Willebrand factor-mediated adhesion

	ADP-induced aggregation (AG1), IC_{50} (nM)	VWF-mediated adhesion (AD) IC_{50} (nM)	Ratio (AD)/(AG1)
FK419	53 ± 8.3	219 ± 12	5.5
FK633	103 ± 5.3	190 ± 23	1.8
Xemilofiban	26 ± 7.8	29 ± 6.1	1.1
Lamifiban	45 ± 3.0	20 ± 1.6	0.44
Tirofiban	46 ± 7.6	6.7 ± 2.2	0.15
FR184764	43 ± 5.3	157 ± 10	3.7
FR233229	21 ± 1.1	92 ± 14	4.3
FR231643	37 ± 2.5	94 ± 6.2	2.5

Ex vivo *inhibition of ADP-induced platelet aggregation and template bleeding time*

The effects of dose escalation of $\alpha_{IIb}\beta_3$ antagonists, FK419, xemilofiban and lamifiban on ADP-induced *ex vivo* platelet aggregation and template BT are represented in Fig. 3. These antagonists effectively inhibited ADP-induced aggregation in a dose-dependent manner, with time-courses of inhibition that paralleled their pharmacokinetics (data not shown). In template BT measurements, all the antagonists prolonged BT in a dose dependent manner; however, their potency was not dependent on the inhibition of platelet aggregation. It is noteworthy that the i.v. administration of FK419 at doses of 200 + 70 μg kg^{-1} h^{-1} completely inhibited ADP-induced aggregation without any prolongation of BT. Even at 500 + 150 μg kg^{-1} h^{-1}, FK419 induced only modest prolongation of BT. In sharp contrast, xemilofiban and lamifiban markedly prolonged BT even at doses causing more than 70% (100 + 92 μg kg^{-1} h^{-1}) and 85% (200 + 70 μg kg^{-1} h^{-1}) inhibition of platelet aggregation, respectively.

To clarify the separation of the effects on BT and inhibition of aggregation, data points were plotted as a function of plasma concentrations attained during infusion of the four $\alpha_{IIb}\beta_3$ antagonists, FK419, FK633, xemilofiban, and lamifiban, at all time points and doses given (Fig. 4). In FK419-treated animals, plasma concentration-dependent inhibition of platelet aggregation and prolongation of BT were observed at different concentration ranges. Although FK419 completely inhibited platelet aggregation at plasma levels of 300 ng mL^{-1}, it started to prolong BT at the levels over 700 ng mL^{-1}. However, in FK633, xemilofiban, or lamifiban-treated animals, prolongation of BT was observed in accordance with their inhibition of platelet aggregation. Table 3 summarizes the mean plasma concentrations of $\alpha_{IIb}\beta_3$ antagonists for inducing 50% inhibition of platelet aggregation (AG2), and 2.5-fold prolongation of BT compared with pretreatment value (BT). Antagonists such as xemilofiban and FK633 showed narrow differences between inhibition of aggregation and prolongation of BT, with BT/AG2 ratios of 2.7 and 2.2, respectively. On the contrary, FK419 and FR233229 had a wide separation of these

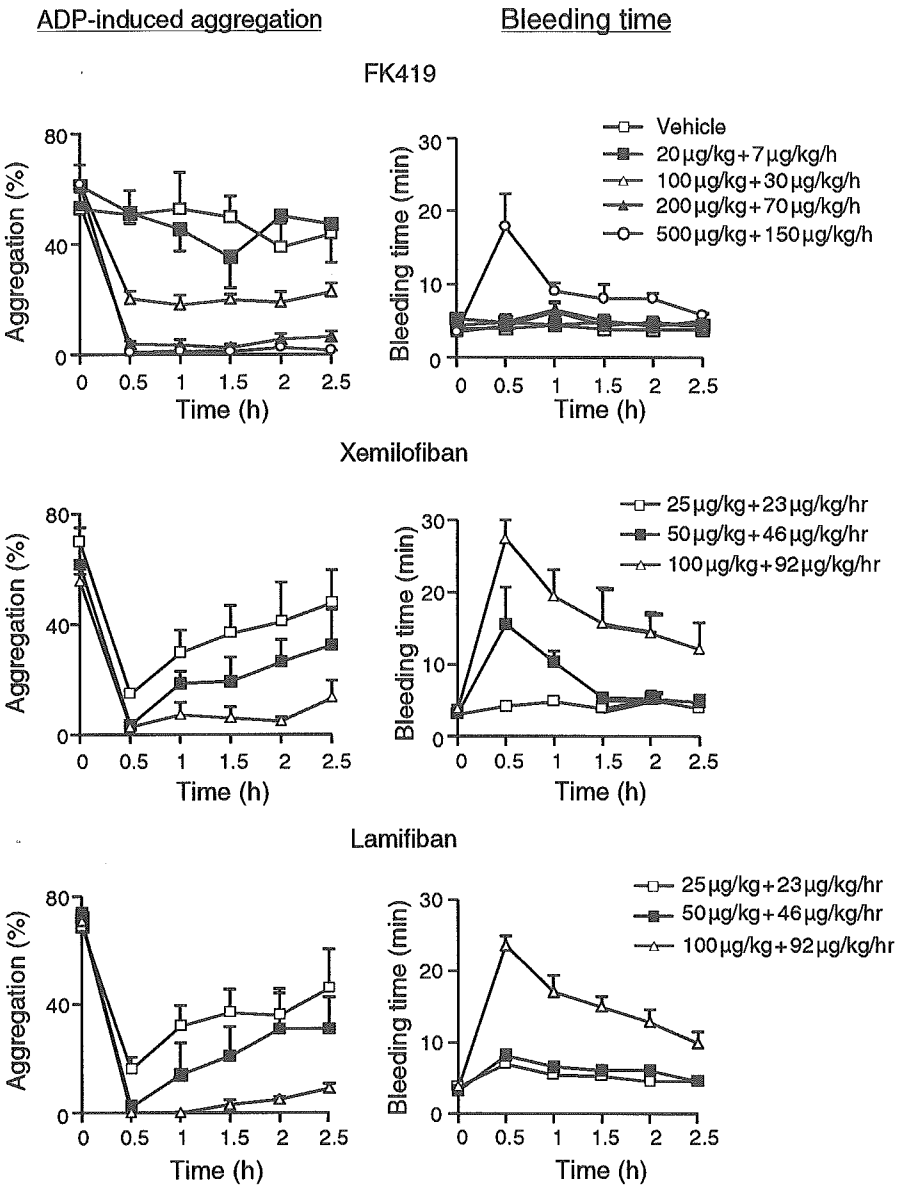

Fig. 3. E ects of synthetic $\alpha_{IIb}\beta_3$ antagonists on the platelet aggregation in response to ADP and template bleeding time during bolus plus continuous infusion in dogs. Data are presented as the mean ± SEM of four experiments.

parameters with a ratio of 17. These results suggest that the inhibitory effect of $\alpha_{IIb}\beta_3$ antagonists on platelet aggregation could be discriminated from their effect on BT.

Correlation between in vivo *BT/AG2 ratio and ligand selectivity, or* in vitro *AD/AG1*

To further investigate the mechanism of *in vivo* separation between inhibition of fibrinogen-mediated platelet aggregation and prolongation of BT (BT/AG2), we assessed the correlation between BT/AG2 and selectivity of the inhibition of ligand binding (VWF/Fg), or AD/AG1 obtained from *in vitro* platelet adhesion and aggregation studies. In Fig. 5a, BT/AG2 ratios for antagonists were plotted against ligand selectivity (VWF/Fg ratios). There was a good correlation between BT/AG2 ratios and VWF/Fg ($r = 0.81$, $n = 8$). These results suggest that the selectivity of antagonists for inhibition of two-ligand binding associate with separation between inhibition of aggregation and prolongation of BT. Moreover, as shown in Fig. 5b, there was also a strong correlation between BT/AG2 and AD/AG1 ratios ($r = 0.89$, $n = 8$). Together with the results of fibrinogen dependence of platelet aggregation, the present study strongly imply that inhibition of the interaction between $\alpha_{IIb}\beta_3$ and VWF is responsible for their hemorrhagic profile, prolongation of BT.

Discussion

The antagonism of platelet $\alpha_{IIb}\beta_3$ represents an attractive antithrombotic approach for the treatment of vaso-occlusive

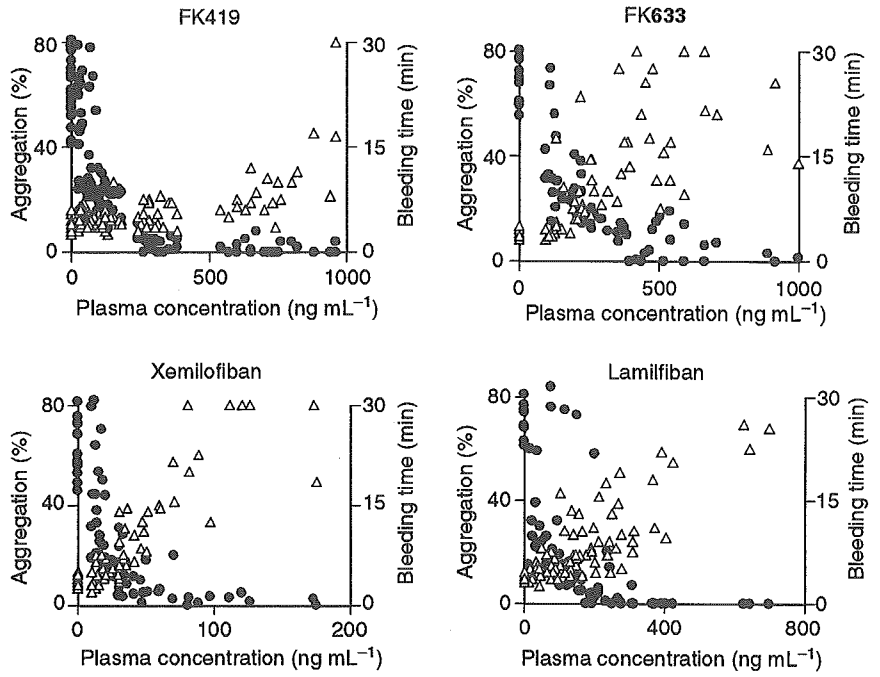

Fig. 4. E ects of synthetic $\alpha_{IIb}\beta_3$ antagonists on ADP-induced platelet aggregation (•) and template bleeding time (△) in dogs as a function of plasma concentrations of synthetic $\alpha_{IIb}\beta_3$ antagonists at all doses and time points.

Table 3 Inhibitory e ects of antagonists on *ex vivo* platelet aggregation and bleeding time in dogs

	ADP-induced aggregation (AG2), IC_{50} (ng mL^{-1})	Bleeding time (BT), BT 2.5 (ng mL^{-1})	Ratio (BT)/(AG2)
FK419	46 ± 4.0	770 ± 20	17
FK633	108 ± 23	238 ± 16	2.2
Xemilofiban	16 ± 0.2	43 ± 1.6	2.7
Lamifiban	50 ± 5.4	212 ± 5.7	4.2
Tirofiban	57 ± 1.0	184 ± 5.8	3.2
FR184764	33 ± 1.0	260 ± 8.0	7.9
FR233229	2.8 ± 0.51	47 ± 3.0	17
FR231643	31 ± 3.0	244 ± 12	7.8

disorders. Intravenous $\alpha_{IIb}\beta_3$ antagonists such as abciximab, tirofiban, and integrelin, have been demonstrated to exert favorable efficacy in reducing the incidence of ischemic events in unstable angina as well as in preventing adverse events after PTCA [5–7]. However, a major concern of treatment with $\alpha_{IIb}\beta_3$ antagonists is the risk of critical bleeding. Early clinical studies with $\alpha_{IIb}\beta_3$ antagonists found substantial increases in the rate of major bleeding events and requirements for transfusion [8]. Therapeutic doses of these antagonists in unstable angina patients increase bleeding complications as well as BT by six to eightfold over baseline, when platelet aggregation is inhibited by > 80% [23]. Careful management and monitoring of patients are required to avoid bleeding

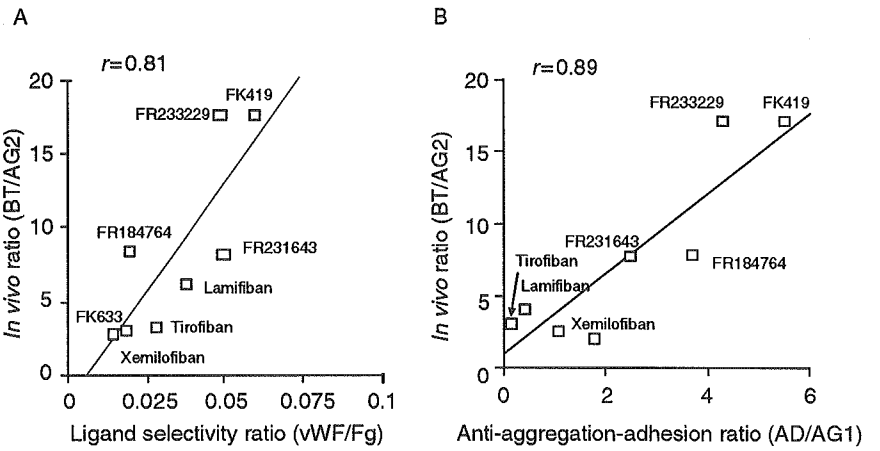

Fig. 5. Association of ligand selectivity or antiaggregation–adhesion e ects of synthetic $\alpha_{IIb}\beta_3$ antagonists with the hemorrhagic profile. (A) Comparison of *in vitro* ligand selectivity (VWF/Fg) with *in vivo* safe separation between inhibition of platelet aggregation and prolongation of bleeding time (BT/AG2). (B) Comparison of *in vitro* antiaggregation–adhesion e ects (AD/AG1) with *in vivo* safe separation between inhibition of platelet aggregation and prolongation of bleeding time (BT/AG2).

complications, including weight-adjustment of heparin dose and assessment of hemostatic markers.

In the present *in vivo* and *ex vivo* studies, we have demonstrated the prolongation of BT caused by $\alpha_{IIb}\beta_3$ antagonists can be dissociated from their inhibition of *ex vivo* platelet aggregation. $\alpha_{IIb}\beta_3$ antagonists such as FK419 and FR233229 did not markedly prolong BT even though they completely inhibited platelet aggregation (high BT/AG2 ratios). In contrast, other antagonists such as xemilofiban and lamifiban showed poor dissociation between these two effects (low BT/AG2 ratios). This discrepancy cannot be explained by pharmacokinetic differences (e.g. rapid decline of plasma concentration of antagonists might underestimate the ability to prolong BT during measurement period). For example, FK419 showed less tendency to promote bleeding even with a slower decline in plasma concentration, compared with xemilofiban and lamifiban, which showed a relatively rapid decline. These results suggest that distinct mechanisms underlie the inhibition of platelet aggregation and prolongation of BT by $\alpha_{IIb}\beta_3$ antagonists.

In terms of the inhibitory effect of $\alpha_{IIb}\beta_3$ antagonists on platelet aggregation, it is well documented that fibrinogen binding to $\alpha_{IIb}\beta_3$ is crucial for platelet aggregation. It has been shown that genetic mutation of the fibrinogen γ-chain abolished platelet aggregation induced by similar condition as the present study [24]. Thus, we hypothesized that blockade of VWF binding by $\alpha_{IIb}\beta_3$ antagonist is responsible for the prolongation of BT. To prove this hypothesis, we have assessed the inhibitory effects of $\alpha_{IIb}\beta_3$ antagonists on fibrinogen and VWF binding using a purified receptor-binding assay. To classify the relative VWF selectivity of $\alpha_{IIb}\beta_3$ antagonists against fibrinogen, we determined the ratio of IC_{50} values for VWF to fibrinogen binding (VWF/Fg). When the parameter of *in vivo* bleeding tendency, BT/AG2, was plotted against VWF selectivity, VWF/Fg, there was a strong correlation between these parameters. We also assessed cell-based selectivity for VWF inhibition, and found that there was a strong correlation between bleeding tendency and VWF selectivity. Thus, inhibition of VWF binding by $\alpha_{IIb}\beta_3$ antagonists is responsible for their ability to prolong BT. This observation is in agreement with findings showing that $\alpha_{IIb}\beta_3$ ligands, fibrinogen, and VWF play functionally different roles in hemostasis. Under high flow conditions, VWF binding to $\alpha_{IIb}\beta_3$ is essential for the formation of the initial irreversible platelet adhesion. In contrast, fibrinogen binding to $\alpha_{IIb}\beta_3$ plays a key role in later thrombus growth and stabilization, including platelet aggregation [14,25]. As VWF binding is responsible for the initial arrest of bleeding, $\alpha_{IIb}\beta_3$ antagonists with stronger potency for VWF inhibition would be predicted to prolong BT.

One question raised by this study is why $\alpha_{IIb}\beta_3$ antagonists targeting the same receptor have different effects on VWF and fibrinogen binding. This difference could be explained by a difference in the binding sites within $\alpha_{IIb}\beta_3$ molecule. VWF contains an RGD motif as receptor recognition sequence, whereas fibrinogen binds to $\alpha_{IIb}\beta_3$ through a non-RGD motif in its γ-chain, indicating that the nature of binding is different between two ligands [26,27]. Several reports also indicate that $\alpha_{IIb}\beta_3$ contains two distinct and interacting ligand binding sites, and that small peptide ligands can binds to distinct sites and elicit different functional responses [28–30]. Therefore, the selectivity of $\alpha_{IIb}\beta_3$ antagonists for inhibition of fibrinogen vs. VWF binding probably originates from discrimination of binding sites within $\alpha_{IIb}\beta_3$. In fact, $\alpha_{IIb}\beta_3$ antagonists such as FK633 and xemilofiban, which have low VWF/Fg values, were modified from an RGD peptide structure, whereas FK419 and FR233229, which have high VWF/Fg values, more closely resemble the structure of the LDV peptide in the γ-chain of fibrinogen [31].

The prolongation of BT has been attributed to the effects on other RGD-dependent integrins, such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$ [32], or to the calcium ion dependence of their antagonism [33]. However, all antagonists examined in the present study were selective for $\alpha_{IIb}\beta_3$ among RGD-dependent integrins, but still have different effects on BT. In terms of calcium ion dependence for inhibition of platelet aggregation, we also determined the relationship between prolongation of BT and inhibition of aggregation measured by heparinized platelets. However, no correlation was observed between the inhibition of aggregation and prolongation of BT (data not shown). Thus, integrin specificity and calcium dependence do not explain the dissociation of prolongation of BT and inhibition of platelet aggregation.

Debate still continues regarding the measurement of BT in man and its predictive value [34, 35]. However, platelets play a central role in the process of a hemostatic plug formation, as BT is prolonged in patients with platelet disorder or taking antiplatelet agents [36]. Thus, to ensure whether there are any differences of hemorrhagic property between $\alpha_{IIb}\beta_3$ antagonists, further clinical evaluation and comparison of antagonists such as FK419 will elucidate its clinical relevance.

In conclusion, the effect of $\alpha_{IIb}\beta_3$ antagonists on BT could be discriminated from their inhibitory effect on platelet aggregation. The prolongation of BT for $\alpha_{IIb}\beta_3$ antagonists is dependent on the ability to inhibit VWF binding to $\alpha_{IIb}\beta_3$ Platelet cohesion mediated by the interaction of VWF with $\alpha_{IIb}\beta_3$ plays an important role in the process of a hemostatic plug formation at the site of the wound. Thus, our present study provides novel evidence showing that ligand selectivity of $\alpha_{IIb}\beta_3$ antagonists affects their hemorrhagic property, BT.

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Disclosure of conflicts of interest

The authors declare that they have no conflicts of interest.

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ORIGINAL ARTICLE

Impaired platelet function in a patient with $P2Y_{12}$ deficiency caused by a mutation in the translation initiation codon

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Summary. In this study, we have identified a patient (OSP-1) with a congenital $P2Y_{12}$ deficiency showing a mild bleeding tendency from her childhood and examined the role of $P2Y_{12}$ in platelet function. At low concentrations of agonists OSP-1 platelets showed an impaired aggregation to several kinds of stimuli, whereas at high concentrations they showed a specifically impaired platelet aggregation to adenosine diphosphate (ADP). ADP normally induced platelet shape change and failed to inhibit PGE_1-stimulated cAMP accumulation in OSP-1 platelets. Molecular genetic analysis revealed that OSP-1 was a homozygous for a mutation in the translation initiation codon (ATG to AGG) in the *P2Y*$_{12}$ gene. Heterologous cell expression of wild-type or mutant $P2Y_{12}$ confirmed that the mutation was responsible for the deficiency in $P2Y_{12}$. OSP-1 platelets showed a markedly impaired platelet spreading onto immobilized fibrinogen. Real-time observations of thrombogenesis under a high shear rate (2000 s^{-1}) revealed that thrombi over collagen were small and loosely packed and most of the aggregates were unable to resist against high shear stress in OSP-1. Our data suggest that secretion of endogenous ADP and subsequent $P2Y_{12}$-mediated signaling are critical for platelet aggregation, platelet spreading, and as a consequence, for stabilization of thrombus.

Keywords: $\alpha_{IIb}\beta_3$, initiation codon, mutation, $P2Y_{12}$ deficiency, platelets, thrombogenesis.

Introduction

Platelets play a crucial role not only in a hemostatic plug formation, but also in a pathologic thrombus formation, particularly within atherosclerotic arteries subjected to high shear stress [1,2]. As an initial step in thrombogenesis, platelets adhere to exposed subendothelial matrices such as von Willebrand factor (VWF) and collagen, then become activated and aggregate to each other. These processes are primarily mediated by platelet surface glycoproteins such as GPIb-IX-V, $\alpha_2\beta_1$, GPVI, and $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) [3,4]. In addition, several mediators such as adenosine diphosphate (ADP), thromboxane A_2, and thrombin cause further platelet activation and recruitment of circulating platelets to the injury sites through activation of $\alpha_{IIb}\beta_3$ and subsequent binding of VWF and fibrinogen.

Recent studies have demonstrated a critical role for ADP in arterial thrombogenesis [5–7]. ADP is actively secreted from platelet dense granules on platelet activation and is passively released from damaged erythrocytes and endothelial cells. Platelets possess at least two major G protein-coupled ADP receptors that are largely responsible for platelet responses to ADP: $P2Y_1$ and $P2Y_{12}$ [6]. $P2Y_1$ is the G_q-coupled receptor responsible for mediating platelet shape change and reversible platelet aggregation through intracellular calcium mobilization [8,9], whereas $P2Y_{12}$ is the G_i-coupled receptor responsible for mediating inhibition of adenylyl cyclase and sustained platelet aggregation [10–12]. $P2Y_{12}$ is the therapeutic target of efficacious antithrombotic agents, such as ticlopidine, clopidogrel, and AR-C compounds [5,6], and its congenital deficiency results in a bleeding disorder [13,14]. The analyses of patients with $P2Y_{12}$ deficiency as well as $P2Y_{12}$-null mice would provide more precise information about the role of $P2Y_{12}$ in platelet function than those using $P2Y_{12}$ inhibitors. To date, four different families with a defect in the expression or the function of $P2Y_{12}$ have been characterized [10,13–16]. In this study, we have described a patient with the congenital $P2Y_{12}$ deficiency due to a homozygous mutation in the translation initiation codon and analyzed the role of $P2Y_{12}$ in platelet aggregation, platelet spreading onto immobilized fibrinogen, and thrombogenesis on a type I collagen-coated surface under a high shear rate. Our present data have demonstrated a crucial role of $P2Y_{12}$ in various platelet functions.

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Materials and methods

Patient history

The proband (OSP-1) is a 67-year-old Japanese female with a lifelong history of easy bruising. She (OSP-1) was born from non-consanguineous parents who had no hemorrhagic diathesis. Although she showed massive bleeding during delivery of her son, she had no history of transfusions. Patient OSP-1 showed normal platelet count, normal coagulation tests (prothrombin time and activated partial thromboplastin time) and slightly elevated plasma fibrinogen (398 mg dL^{-1}). Ivy bleeding time of the patient was consistently prolonged (>15 min). Clot retraction by MacFarlane's method was normal (50%; normal values 40%–70%). Her son never suffered from a bleeding tendency. Informed consent for analyzing their platelet function and molecular genetic abnormalities was obtained from OSP-1, her husband and their son.

Preparation of platelet-rich plasma and washed platelet suspension

Platelet-rich plasma (PRP) for aggregation studies was prepared by a centrifugation of whole blood anticoagulated with citrate at 250 *g* for 10 min and then the platelet count was adjusted at 300×10^6 mL^{-1} by platelet-poor plasma. Washed platelets were prepared as previously described [17]. In brief, 6 volumes of freshly drawn venous blood from the patient, her husband, son or healthy volunteers were mixed with 1 volume of acid–citrate–dextrose (ACD; National Institutes of Health Formula A, NIH, Bethesda, MD, USA) and centrifuged at 250 *g* for 10 min to obtain PRP. After incubation with 20 ng mL^{-1} prostaglandin E1 (PGE_1; Sigma-Aldrich, St Louis, MO, USA) for 15 min, the PRP was centrifuged at 750 *g* for 10 min, washed three times with 0.05 mol L^{-1} isotonic citrate buffer containing 20 ng mL^{-1} PGE_1 and resuspended in an appropriate buffer.

Platelet aggregometry

Platelet aggregation using PRP was monitored by a model PAM-6C platelet aggregometer (Mebanix, Tokyo, Japan) at 37 C with a stirring rate of 1000 r.p.m. as previously described [18]. Protease-activated receptor 1-activating peptide (PAR1 TRAP, SFLLRNPNDKYEPF) and adenosine 3′,5′-diphosphate (A3P5P) were purchased from Sigma-Aldrich Corp. $P2Y_{12}$ antagonist, AR-C6993MX (2-propylthio-D-fluoromethylene adenosine 5-triphosphate) was a kind gift from AstraZeneca (Loughborough, UK).

Flow cytometry and measurement of intracellular cAMP

Flow cytometric analysis using various monoclonal antibodies (mAbs) specific for platelet membrane glycoproteins was performed as previously described [19].

For measuring intracellular cAMP levels, samples of 200 μL of washed platelets (60×10^6) in Walsh buffer (137 mM of NaCl, 2.7 mM of KCl, 1.0 mM of $MgCl_2$, 3.3 mM of NaH_2PO_4, 3.8 mM of HEPES, 0.1% of glucose, 0.1% of BSA, pH 7.4) were incubated with 1 μmol L^{-1} PGE_1 for 15 min, and then platelets were stimulated with ADP or epinephrine. After incubation for 15 min, total cellular cAMP levels were measured using the Biotrak cAMP enzyme immunoassay system from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Platelet adhesion assay

Adhesion study was performed as previously described [20]. In brief, non-treated polystyrene 10 cm dishes were coated with 100 μg mL^{-1} human fibrinogen in 5 mL of phosphate-buffered saline (PBS) at 4 C overnight. After washing with PBS, dishes were blocked with PBS containing 1% of bovine serum albumin (BSA) for 90 min at 37 C. Aliquots (1 mL) of washed platelets (25×10^6 mL^{-1}) were added to the fibrinogen-coated dishes and incubated at 37 C. After incubation for 40 min, adherent platelets were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100 and stained with TRITC-conjugated phalloidin. Platelet morphology and degrees of spreading were determined by fluorescence microscopy (Olympus, Tokyo, Japan).

Platelet thrombus formation under flow conditions

The real-time observation of mural thrombogenesis on a type I collagen-coated surface under a high shear rate (2000 s^{-1}) was performed as previously described [21]. In brief, type I collagen-coated glass coverslips were placed in a parallel plate flow chamber (rectangular type; flow path of 1.9-mm width, 31-mm length, and 0.1-mm height). The chamber was assembled and mounted on a microscope (BX60; Olympus, Tokyo, Japan) equipped with epifluorescent illumination (BX-FLA; Olympus) and a charge-coupled device (CCD) camera system (U-VPT-N; Olympus). Whole blood containing mepacrine-labeled platelets obtained from OSP-1 or control subjects was aspirated through the chamber by a syringe pump (Model CFV-3200, Nihon Kohden, Tokyo, Japan) at a constant flow rate of 0.285 mL min^{-1}, producing a wall shear rate of 2000 s^{-1} at 37 C.

Amplification and analysis of platelet RNA

Total cellular RNA of platelets was isolated from 20 mL of whole blood, and $P2Y_1$ or $P2Y_{12}$ mRNA was specifically amplified by reverse transcription-polymerase chain reaction (RT-PCR), as previously described [22]. The following primers were constructed based on the published sequence of $P2Y_{12}$ cDNA and used for the first round PCR for $P2Y_{12}$ cDNA: Y12F1, 5′-GGCTGCAATAACTACTACTTACTGG-3′ [sense, nucleotide(nt) −74 to −50]; Y12R4, 5′-CAGGACAGTGTAGAGCAGTGG-3′ (antisense, nt 85 to 105) [10].

Allele-specific restriction enzyme analysis (ASRA)

Genomic DNA was isolated from mononuclear cells using SepaGene kit (Sanko Junyaku Co Ltd, Tokyo, Japan). Amplification of the region around the initiation codon of the $P2Y_{12}$ gene was performed by using primers *Bsr*DI-GF, 5′-CTTTTGTTCTCTAGGTAACCAACAAGCAA-3′ (sense, the mismatched base is underlined), and Y12R4 (antisense described above) using 250 ng of DNA as a template. These primers can be found in GenBank accession no. AC024886.20 and the sense primer corresponds to 127558–127585. PCR products were then digested with restriction enzyme *BsrD*I. The resulting fragments were electrophoresed in a 6% polyacrylamide gel.

Construction of $P2Y_{12}$ expression vectors and cell transfection

The full-length cDNA of wild-type (WT) and mutant $P2Y_{12}$ was amplified by RT-PCR using primers Y12-*Hin*dIII-F, 5′-GAATTCAAGCTTCAAGAAATGCAAGCCGTCGACA-ACCTC-3′ (sense, nt −6 -21 for WT, *Eco*RI and *Hin*dIII sites introduced at the 5′ end were underlined) or Y12-*Hin*dIII-F2, 5′-GAATTCAAGCTTCAAGAAAGGCAAGCCGTCGAC-AACCTC-3′ (sense, nt −6 −21 for mutant), and Y12H-Not-R, 5′-TCTAGAGCGGCCGCTCAATGGTGATGGTGATGA-TGCATTGGAGTCTCTTCATT-3′ (antisense, nt 1012–1029, His × 6 were introduced before stop codon, *Not*I and *Xba*I sites introduced at the 5′ end were underlined). The amplified fragments were digested with *Hin*dIII and *Not*I, and the resulting 1059-bp fragments (nt −9 −1050) were extracted using QIAquick gel extraction kit (Qiagen, GmbH, Germany). These fragments were inserted into the pcDNA3 (Invitrogen, San Diego, CA, USA) digested with *Hin*dIII and *Not*I. The fragments inserted were characterized by sequence analysis to verify the absence of any other substitutions and the proper insertion of the PCR cartridge into the vector.

A total of 10 μg of WT or mutant $P2Y_{12}$ construct was transfected into human embryonic kidney 293 cells (HEK293 cells, 10^6 cells) using the calcium phosphate method as previously described [22]. Transfectants were lyzed by 1% Triton X-100 PBS containing protease inhibitors 2 days after transfection, and proteins were separated by 7.5% SDS-PAGE. After transferred onto a PVDF membrane, expressed proteins were detected by rabbit anti-His tag antibody.

Results

Platelet aggregation studies

We first examined the expression of platelet membrane glycoproteins in OSP-1 by flow cytometry. The patient's platelets (OSP-1 platelets) normally express GPIb-IX, $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), $\alpha_2\beta_1$, and CD36 (data not shown). Fig. 1 shows platelet aggregation of PRP in response to various agonists. The aggregation of OSP-1 platelets induced by 20 μM of ADP was markedly impaired with only a small and transient

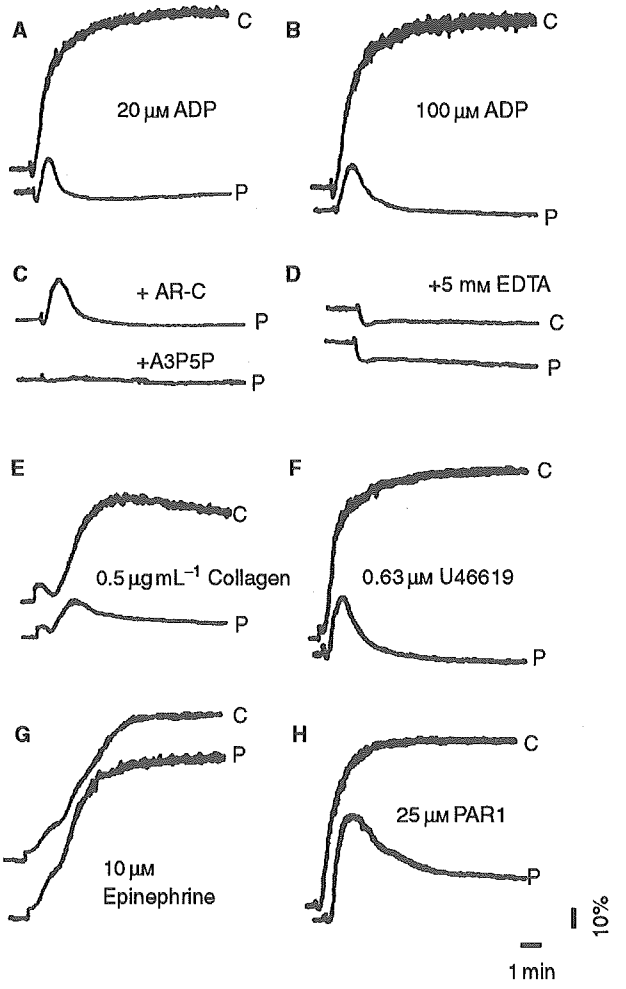

Fig. 1. Platelet aggregation induced by various agonists. Platelet aggregation was induced by various agonists in citrated PRP from patient OSP-1 (labeled P) or a control subject (labeled C). Agonists used are (A) 20 μM of ADP, (B) 100 μM of ADP, (C) 20 μM of ADP in the presence of 1 μM of AR-C69931MX (AR-C), a specific $P2Y_{12}$-antagonist, or 1 mM of A3P5P (A3P5P), a specific $P2Y_1$-antagonist, (D) 20 μM of ADP in the presence of 5 mM of EDTA, (E) 0.5 μg mL^{-1} of collagen, (F) 0.63 μM of U46619, (G) 10 μM of epinephrine, and (H) 25 μM of PAR1-TRAP.

aggregation (Fig. 1A), and the aggregation was still impaired even at 100 μM of ADP (Fig. 1B). As compared with control platelets, the aggregation of OSP-1 platelets was also impaired with a transient aggregation in response to low concentrations of collagen (0.5 μg mL^{-1}, Fig. 1E), U46619 (0.63 μM, Fig. 1F), or PAR1 TRAP (25 μM, Fig. 1H). In response to 1.3 mg mL^{-1} ristocetin (not shown) or 10 μM of epinephrine (Fig. 1G), OSP-1 platelets aggregated normally. When OSP-1 platelets were stimulated with 20 μM of ADP in the presence of 5 mM of EDTA, the light transmission decreased equivalent to control platelets suggesting that OSP-1 platelets changed shape normally (Fig. 1D). We then examined effects of ADP receptor antagonists on the aggregation of OSP-1 platelets induced by 20 μM of ADP. A total of 1 mM of A3P5P, a specific $P2Y_1$ antagonist, abolished the residual response of OSP-1 platelets to ADP, whereas 1 μM of AR-C69931MX, a specific $P2Y_{12}$

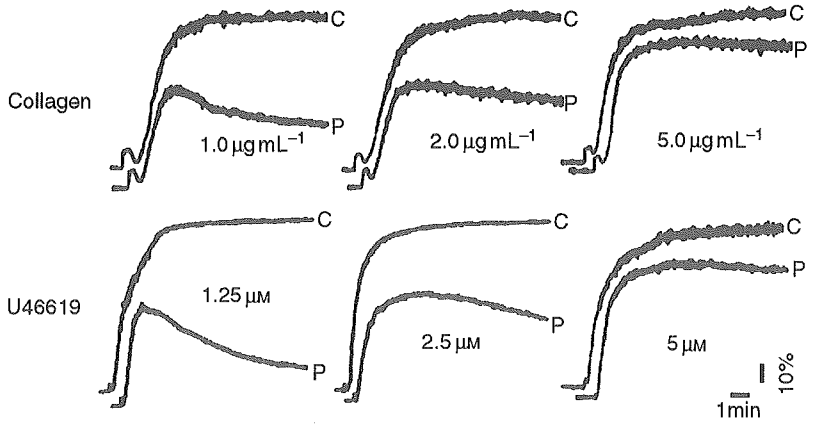

Fig. 2. Platelet aggragation induced by collagen or U46619 at various concentrations. Platelet aggregation in citrated PRP from patient OSP-1 (labeled P) or a control subject (labeled C) was induced by various concentrations of collagen or U46619. At high concentrations of collagen or U46619, OSP-1 platelets aggregate almost normally.

antagonist, did not induce an additional inhibition on the platelet aggregation (Fig. 1C). These data suggest that the impaired response of the patient's platelets may be due to an abnormality in signaling evoked by ADP and that $P2Y_{12}$-mediated signaling rather than $P2Y_1$-mediated signaling may be completely defective in patient OSP-1.

We also examined the aggregation of OSP-1 platelets induced by higher concentrations of agonists. As shown in Fig. 2, the aggregation response of OSP-1 platelets improved as the concentrations of agonists increased, and they aggregated almost normally in response to high concentrations of collagen (5 μg mL^{-1}), U46619 (5 μM), or PAR1 TRAP (100 μM) (not shown). In addition, we confirmed that 1 μM of AR-C69931MX conferred essentially the same defect on the aggregation of control platelets in response to U46619 as that of OSP-1 platelets and did not further inhibit OSP-1 platelet aggregation induced by 5 μg mL^{-1} of collagen, 5 μM of U46619, or 100 μM of PAR1 TRAP (data not shown). These data indicated that at high concentrations of agonists OSP-1 platelets showed the specifically impaired aggregation to ADP.

Effect of ADP on PGE_1-stimulated cAMP accumulation in platelets

To determine whether $P2Y_{12}$-mediated signaling is specifically impaired, we examined an inhibitory effect of ADP on 1 μM of PGE_1-stimulated cAMP accumulation in platelets from the patient, her husband, their son, and healthy unrelated controls. ADP inhibited intracellular cAMP levels in platelets from the patient's husband, son and healthy unrelated controls (not shown) by approximately 80%, whereas the inhibition was only 15% in the patient's platelets (Fig. 3). In contrast to ADP, epinephrine normally inhibited cAMP accumulation in platelets from the patient as well as her husband and son. These results strongly suggest that the defect could be due to an abnormality in G_i coupling ADP receptor, $P2Y_{12}$.

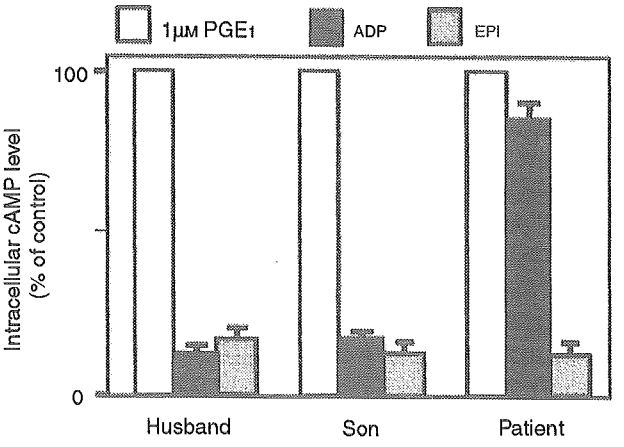

Fig. 3. E ect of ADP or epinephrine on the inhibition of PGE_1-induced cAMP accumulation in platelets. Washed platelets from patient OSP-1, husband or son were incubated with 1 μM of PGE_1 for 15 min and stimulated with 20 μM of ADP or 10 μM of epinephrine. Intracellular cAMP levels were expressed as a percent of cAMP levels in the absence of agonists. Results in OSP-1 are the mean of two experiments.

Nucleotide sequence analysis of cDNA and genomic DNA of $P2Y_{12}$

To reveal a molecular genetic defect in OSP-1, we analyzed the entire coding regions of both $P2Y_1$ and $P2Y_{12}$ cDNAs amplified from platelet mRNA by RT-PCR. A single nucleotide substitution (T → G) was identified within the translation initiation codon (ATG → AGG) in the patient's $P2Y_{12}$ cDNA (Fig. 4A). This substitution was also confirmed by reverse sequencing. No other nucleotide substitutions were detected within the coding region of either $P2Y_{12}$ or $P2Y_1$ cDNA from the patient. OSP-1 appeared homozygous for the substitution, and the substitution was not detected in 20 control subjects.

Nucleotide sequence analysis of PCR fragments from the patient's genomic DNA also suggested the homozygosity of the substitution (data not shown). To further confirm the homo-

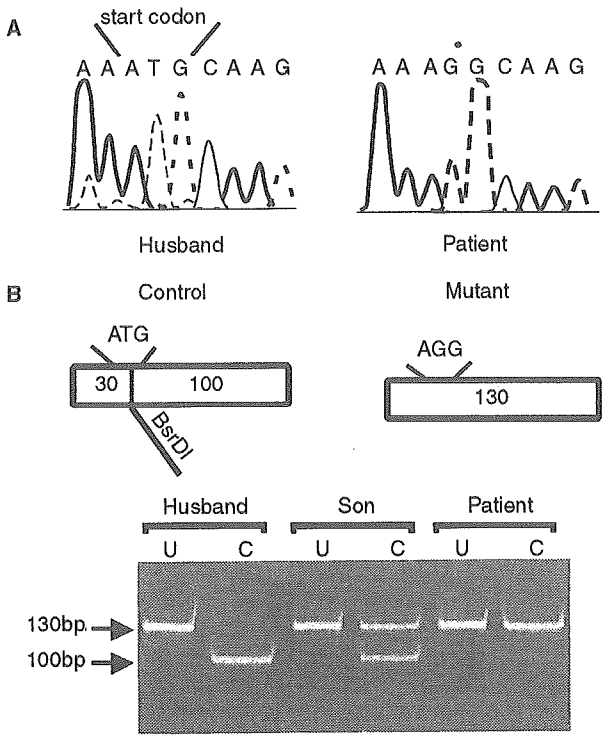

Fig. 4. Sequence analysis of $P2Y_{12}$ cDNA and restriction enzyme analysis of the $P2Y_{12}$ gene. (A) cDNA obtained by RT-PCR from platelet mRNA was analyzed by sequencing using a sense primer Y12F1. (B) PCR was performed to generate 130-bp fragments including initiation codon of $P2Y_{12}$ as described in Materials and methods. Undigested (U) or digested (C) PCR products with *Bsr*DI were analyzed on a 6% polyacrylamide gel. In patient OSP-1, the T → G mutation at position 2 abolishes a *Bsr*DI restriction site.

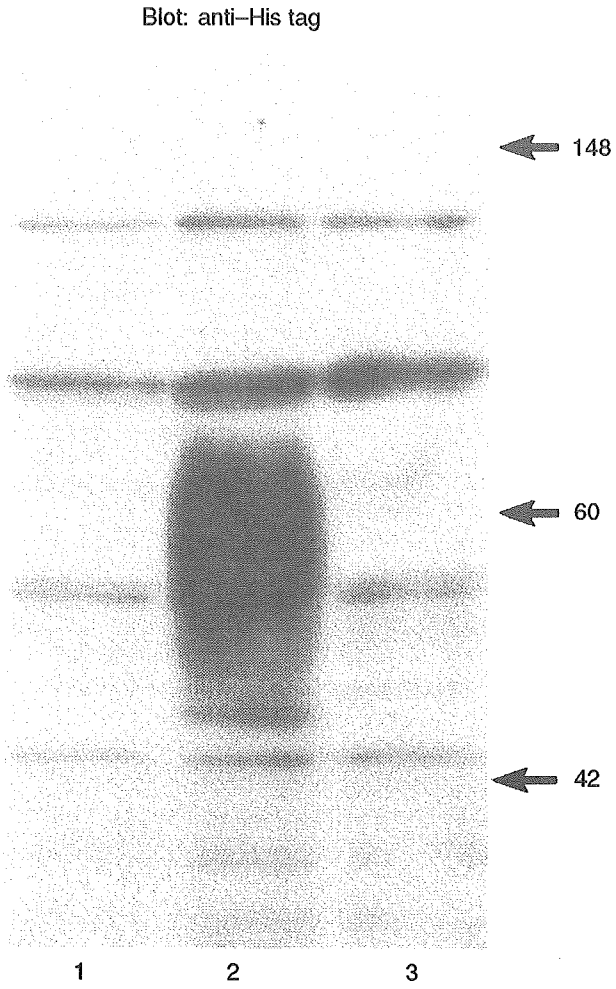

Fig. 5. Expression of $P2Y_{12}$ in HEK293 cells transfected with WT or mutant His-tag attached $P2Y_{12}$. Wild-type or mutant $P2Y_{12}$ construct was transfected into HEK293 cells using the calcium phosphate method. Transfectants were lyzed by 1% Triton X-100 PBS containing protease inhibitors 2 days after transfection. Cell lysates from mock transfectant (lane 1), cells transfected with WT $P2Y_{12}$ (lane 2) or mutant $P2Y_{12}$ (lane 3) were separated by 7.5% SDS-PAGE, and immunoblot was performed by anti-His-tag antibodies.

zygosity, allele-specific restriction enzyme analysis (ASRA) was performed. The region around the initiation codon of the $P2Y_{12}$ gene was amplified by PCR using primers *Bsr*DI-GF and Y12R4. A restriction site for *Bsr*DI would be abolished by the T → G substitution. As shown in Fig. 4B, ASRA clearly indicated that the patient and her son were homozygous and heterozygous for the substitution, respectively. These results also confirm that the substitution is inheritable.

Heterologous cell expression of WT and mutant $P2Y_{12}$

As the substitution at the translation initiation codon might induce an alternative translation starting at downstream ATGs leading to an expression of shorter form of $P2Y_{12}$, we decided to investigate effects of the substitution found in the patient on the expression of $P2Y_{12}$. Expression vectors encoding WT and mutant $P2Y_{12}$ in which His-tag was attached at the C-terminal portion of $P2Y_{12}$ were constructed as described in the Materials and methods. Wild-type or mutant $P2Y_{12}$ construct was transfected into HEK 293 cells, and then expressed proteins were analyzed 48 h after transfection in an immunoblot assay employing anti-His antibodies. As shown in Fig. 5, WT $P2Y_{12}$ protein with an apparent molecular weight of 60 KDa was expressed in 293 cells as a His-tag-positive protein. In sharp contrast, the mutant $P2Y_{12}$-expression vector failed to express any His-tag-positive protein. These results provide strong evidence that the T → G substitution at the translation initiation codon of $P2Y_{12}$ cDNA is responsible for the $P2Y_{12}$ deficiency.

Platelet spreading on immobilized fibrinogen

As it has been well documented that release of endogenous ADP is required for full platelet spreading onto immobilized fibrinogen [23], we next analyzed the patient's platelet spreading in order to evaluate the role of $P2Y_{12}$. Control platelets adhered to fibrinogen underwent morphological changes ranging from filopodia protrusion to complete spreading, and 50.5% ± 21.3% of the adherent platelets spread ($n = 3$) (Fig. 6A). In sharp contrast, the patient's platelets showed an

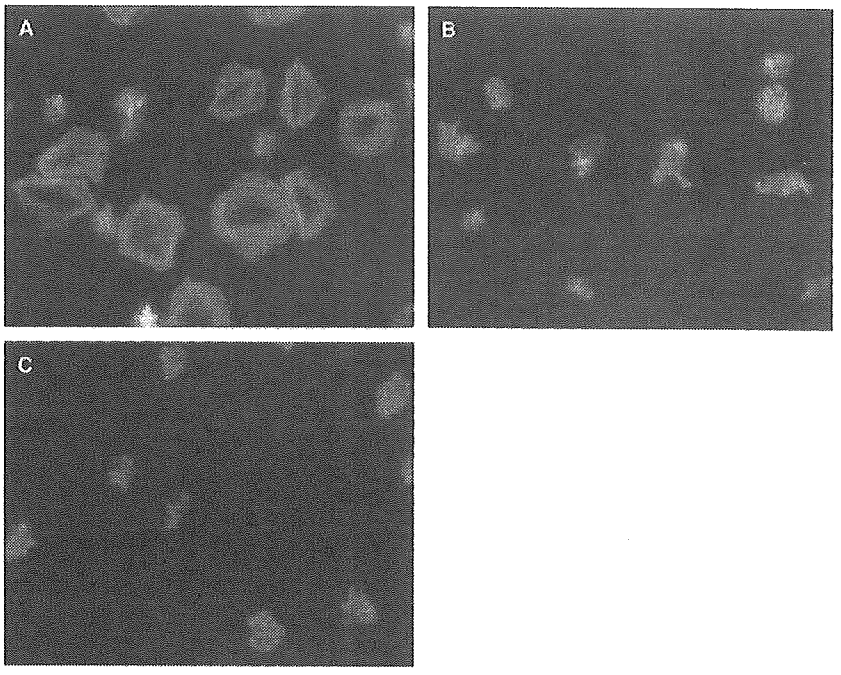

Fig. 6. Platelet spreading on immobilized fibrinogen. (A,B) Washed platelets from a control subject were applied onto fibrinogen-coated polystyrene dishes and incubated at 37 C for 40 min without any inhibitor (A) or with 1 μM of AR-C69931MX (B). (C) Washed platelets from the patient were applied onto fibrinogen-coated polystyrene dishes and incubated at 37 C for 40 min without any inhibitor. Adherent platelets were then fixed, permeabilized and stained with TRITC-conjugated phalloidin. Platelet morphology was analyzed by fluorescence microscopy.

impaired spreading and only 2.3% ± 1.4% of the adherent platelets spread ($n = 3$, $P < 0.001$, Fig. 6C). Similar results were obtained with control platelets in the presence of 1 μM of AR-C69931MX (6.2% ± 2.2%, $n = 3$, $P < 0.001$, Fig. 6B). In addition, 1 mM of A3P5P also markedly inhibited platelet spreading ($n = 3$, 10.1% ± 2.2%, $P < 0.001$, not shown). These results suggest that both $P2Y_{12}$ and $P2Y_1$ are necessary for platelet spreading.

Platelet-thrombus formation on immobilized collagen under flow conditions

To investigate the role of $P2Y_{12}$ in thrombus formation, we observed the real-time process of mural thrombogenesis on a type I collagen-coated surface under flow conditions with high shear rate (2000 s^{-1}) using the whole blood from OSP-1. Real-time observation revealed that thrombi formed on type I collagen were unstable. As platelet aggregates of the patient were loosely packed each other and unable to resist against high shear stress, most of the aggregates at the apex of the thrombi came off the thrombi constantly. On the other hand, most of thrombi formed by control platelets were densely packed with higher fluorescent intensity and were stable with constant growth during observation (Video 1 and 2).

As shown in Fig. 7A, the area covered with patient platelets after 7 min of flow was greater than that of control platelets (91.8% ± 0.3% vs. 82.2% ± 1.4%, $n = 3$, $P < 0.01$). However, thrombi formed by OSP-1 platelets were loosely packed, whereas thrombi were large and densely packed in controls. The overall fluorescent intensity of thrombi of OSP-1 platelets was lower than that of control platelets. Three-dimensional analysis revealed the striking difference in size and shape of individual thrombus formed after 10 min between the patient and control platelets (Fig. 7B). Thrombi formed by control platelets were large in size, clearly edged and surrounded by thrombus-free areas. On the other hand, individual thrombus formed by patient platelets was mostly small and appeared to be a thin layer of platelet aggregates. Thrombus height at the plateau phase was 10.2 ± 0.4 μm, which was less than half of controls (21.2 ± 0.4 μm).

Discussion

$P2Y_{12}$ coupled with $G\alpha_i$, primarily with $G\alpha_{i2}$, consists of 342 amino acid residues with seven transmembrane domains (TM), and its deficiency is responsible for congenital bleeding diathesis [10–16]. To date, five mutations responsible for a defect in the expression or the function of $P2Y_{12}$ in four different families have been demonstrated [10,15,16]. Patient ML possessed a mutation consisting of a two nucleotide deletion at amino acid 240 (near the N-terminal end of TM6), which would lead to a premature termination of $P2Y_{12}$ [10,14]. A two nucleotide deletions at amino acid 98 (next to the N-terminal end of TM3) and a single nucleotide deletion occurring just beyond TM3 were identified in other two families, both of which would lead to a premature termination of $P2Y_{12}$ [13,15]. However, in these reports expression studies had not been performed to show the direct association between these mutations and the $P2Y_{12}$ deficiency [10,15]. Patient AC, whose platelets expressed dysfunctional $P2Y_{12}$ with normal

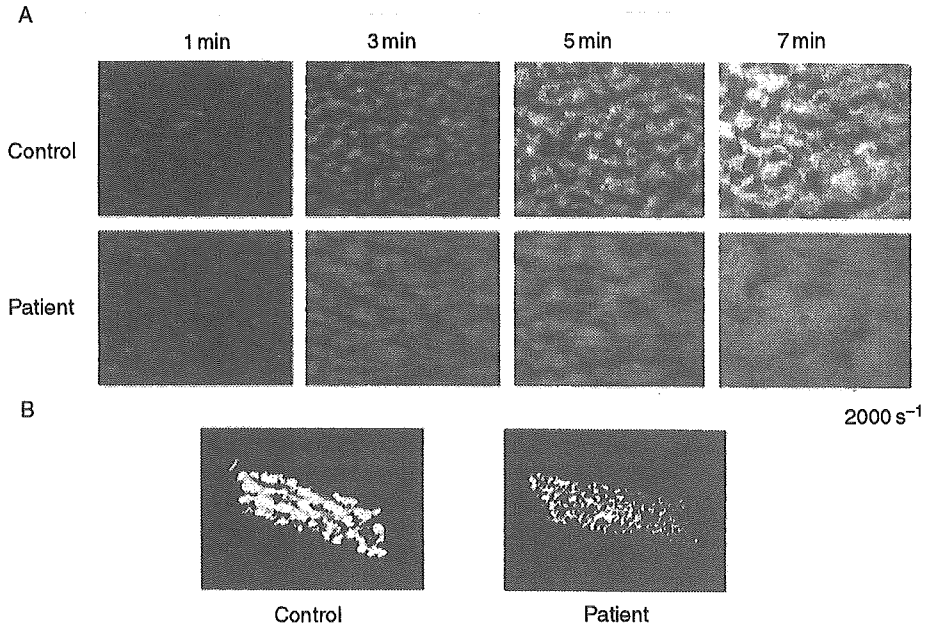

Fig. 7. Thrombus formation on immobilized collagen under flow conditions. (A) Whole blood containing mepacrine-labeled platelets obtained from the patient or control subjects was aspirated through a chamber with type I collagen-coated coverslips. Thrombi formed under a high shear rate (2000 s^{-1}) at indicated time points were observed using a microscope equipped with epifluorescent illumination and a CCD camera system. (B) Three-dimensional structure of thrombi formed after 10 min flow by platelets from the patient or a control subject was analyzed.

binding capacity of 2-methylthioadenosine 5′-diphosphate (2MeS-ADP), was compound heterozygous for Arg256 → Gln in TM6 and for Arg265 → Trp in the third extracellular loop of P2Y$_{12}$. Platelets from patient AC showed an increased platelet aggregation at high dose ADP compared with low dose ADP, suggesting the presence of residual receptor function [16]. In this study, we described a patient (OSP-1) with congenital bleeding diathesis bearing a novel homozygous mutation within the translation initiation codon (ATG → AGG) of the P2Y$_{12}$ gene. Consistent with previous studies, the aggregation of OSP-1 platelets with P2Y$_{12}$ deficiency was impaired to various agonists such as collagen, U46619, and PAR1 TRAP at low concentrations, but almost normal at high concentrations [11–14]. These findings confirmed the critical role of P2Y$_{12}$-mediated signaling evoked by endogenous ADP in platelet aggregation induced by low concentrations of agonists. In contrast to patient AC with residual P2Y$_{12}$-mediated signaling, the impaired platelet aggregation in OSP-1 in response to ADP was neither improved even at 100 μM of ADP stimulation nor reduced by adding 1 μM of AR-C69931MX, suggesting a complete loss of the P2Y$_{12}$ function. Family study confirmed that patient OSP-1 was homozygous for the mutation, and our expression study demonstrated that the mutation is responsible for the P2Y$_{12}$ deficiency.

A number of examples of mutations in the translation initiation codons have been demonstrated in various diseases [24]. Although some cases having mutations in the initiation codons did not express any related abnormal protein, Pattern *et al.* showed an abnormal Gα_s protein possibly synthesized as a result of initiation at downstream ATGs due to a mutation at an initiation codon (ATG → GTG) in patients with Albright's hereditary osteodystrophy [24,25]. In OSP-1, we detected the T → G mutation at position +2, and our expression study denied the possibility that the substitution might induce an alternative translation at downstream ATGs leading to an expression of shorter form of P2Y$_{12}$.

As to platelet spreading onto immobilized fibrinogen, OSP-1 platelets showed the impaired platelet spreading. Similarly, A3P5P inhibited the platelet spreading. It has been well documented that release of endogenous ADP is required for full platelet spreading onto immobilized fibrinogen [23], and Obergfell *et al.* [26] have demonstrated that the platelet spreading requires sequential activation of Src and Syk in proximately to $\alpha_{IIb}\beta_3$. In contrast to the ADP-induced platelet shape change shown in OSP-1 platelets in the platelet aggregometer, our data indicated that both P2Y$_{12}$ and P2Y$_1$ were necessary for the full spreading onto immobilized fibrinogen.

Employing clopidogrel or AR-C69931 MX as an inhibitor for P2Y$_{12}$, several studies examined the role of P2Y$_{12}$ in thrombogenesis under flow conditions [27–30]. However, some discrepancy between the studies was pointed out and non-specific effects of these inhibitors were not completely ruled out [28–30]. As patient OSP-1 was deficient in P2Y$_{12}$ as shown in this study, it would be informative to examine the real-time process of thrombogenesis on a type I collagen-coated surface under a high shear rate (2000 s^{-1}) employing whole blood obtained from OSP-1. Our data demonstrated that P2Y$_{12}$-deficiency led to the loosely packed thrombus and the impaired thrombus growth with enhancing adhesion to collagen, which was consistent with the study by Remijn *et al.* [30] employing patient ML's platelets. The increase in platelet adhesion to

collagen was probably due to the impaired platelet consumption by the growing thrombi [27,30]. Moreover, our real-time observation indicated that the loosely packed aggregates were unable to resist against high shear stress, and then most of the aggregates at the apex of the thrombi came off the thrombi. In contrast, Andre *et al.* [12] did not detect significant differences in *ex vivo* thrombus volume formed over human type III collagen under a shear rate of 871 s^{-1} between $P2Y_{12}^{-/-}$ and WT mice. Although Andre *et al.* used non-anticoagulated mouse blood instead of anticoagulated blood, Roald *et al.* [27] demonstrated that clopidogrel significantly reduced the thrombus volume over type III collagen employing non-anticoagulated human blood. Loosely packed platelet thrombi with swollen non-degranulated platelets were detected following clopidogrel intake, whereas densely packed thrombi with partly fused platelets were detected before clopidogrel intake by electron microscopy [27]. Thus, it is likely that differences between human and mouse, rather than those between non-anticoagulated and anticoagulated blood, may account for the discrepancy. Nevertheless, they showed that *ex vivo* thrombi were loosely packed and that only small and unstable thrombi were formed in $P2Y_{12}^{-/-}$ mice without reaching occlusive size in mesenteric artery injury model *in vivo* [12].

Our present study has demonstrated the novel mutation responsible for the $P2Y_{12}$ deficiency and suggested that secretion of endogenous ADP and subsequent $P2Y_{12}$-mediated signaling is critical for platelet aggregation, platelet spreading, and as a consequence, for stabilization of thrombus. Mild bleeding tendency observed in patient OSP-1 further emphasizes the efficacy of $P2Y_{12}$ receptor as a therapeutic target for thrombosis.

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Supplementary material

The following supplementary material is available online at http://www.blackwell-synergy.com/loi/jth:

Figure S1. Perfusion study using control platelets. A real-time movie of platelets perfused over type-I collagen shows that thrombi formed by control platelets are densely packed and stable. This 5-second movie was taken at 5-minute perfusion under a high shear rate (2000 s^{-1}).

Figure S2. Perfusion study using OSP-1 platelets. A real-time movie of platelets perfused over type-I collagen shows that thrombi formed by the patient OSP-1 platelets are loosely packed and unstable. Newly formed aggregates on the top of thrombi keep on moving toward downstream and some aggregates came off the thrombi. This 5-second movie was taken at 5-minute perfusion under a high shear rate (2000 s^{-1}).

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Measurement of ADAMTS13 activity and inhibitors

Toshiyuki Miyata, Koichi Kokame and Fumiaki Banno

Purpose of review
Acquired or congenital deficiency in the plasma von Willebrand factor-cleaving protease ADAMTS13 causes life-threatening thrombotic thrombocytopenic purpura. This condition is characterized primarily by thrombocytopenia and microangiopathic hemolytic anemia, accompanied by variable degrees of neurologic dysfunction, renal failure, and fever. Measurement of ADAMTS13 activity is important in the diagnosis of microangiopathies such as thrombotic thrombocytopenic purpura. This review introduces both established and emerging assays for ADAMTS13 activity, focusing on their impact on clinical practice.
Recent findings
Previously established assays are useful screening methods to identify suspected thrombotic thrombocytopenic purpura. Novel assays measuring ADAMTS13 activity using either recombinant peptides or synthetic substrates directly measure the activity quantitatively. These assays can also detect neutralizing autoantibodies in the plasma of patients with acquired ADAMTS13 deficiency. Although ADAMTS13 in control subjects exhibits a broad variation in activity, ranging from 30 to 200%, significant decreases in ADAMTS13 activity have been observed in several physiologic and pathologic conditions. A portion of thrombotic thrombocytopenic purpura patients, however, did not display severe ADAMTS13 deficiency, suggesting that as-yet-unidentified environmental or genetic factors may contribute to the etiology of thrombotic thrombocytopenic purpura.
Summary
New assays measuring ADAMTS13 activity will contribute significantly to the accurate diagnosis of microangiopathies, ultimately leading to improved clinical treatment of these diseases. These assays may also help to clarify the role of ADAMTS13 activity in additional thrombotic disorders, including disseminated intravascular coagulation, stroke, and myocardial infarction.

Keywords
ADAMTS13, microangiopathy, thrombotic thrombocytopenic purpura

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Abbreviations

TSP-1	thrombospondin type 1
TTP	thrombotic thrombocytopenic purpura
ULVWF	ultralarge von Willebrand factor
VWF	von Willebrand factor

1065-6251

Introduction

Thrombotic thrombocytopenic purpura (TTP) is characterized by thrombocytopenia and microangiopathic hemolytic anemia, accompanied by variable penetrance of neurologic dysfunction, renal failure, and fever [1,2,3•,4••,5••]. TTP leads to the development of systemic microvascular thrombi of platelets, largely resulting from the accumulation of ultralarge von Willebrand factor (ULVWF) multimers in plasma. The accumulation of ULVWF multimers is caused by congenital or acquired deficiency in a von Willebrand factor (VWF)-cleaving protease, ADAMTS13, a metalloprotease of the ADAMTS family (a disintegrinlike and metalloprotease with thrombospondin type I motifs) [6•–9•]. ADAMTS13 was purified and cloned by several groups in 2001 [10–15]. As the basic and clinical studies on TTP and ADAMTS13 have greatly advanced since this discovery, this review focuses on the available assays measuring ADAMTS13 activity. We also discuss the sensitivity and specificity of ADAMTS13 activity assessment in the diagnosis of TTP.

Pathogenesis of thrombotic thrombocytopenic purpura

VWF, a substrate of ADAMTS13, is a multimeric protein important for platelet adhesion to subendothelial connective tissue. In vascular endothelial cells, the primary site of VWF production, VWF dimers are initially formed through formation of a disulfide bond near the C-termini. The prosequences of the VWF dimer are cleaved, followed by formation of ULVWF multimers by generation of a disulfide bond near the N-termini. ULVWF multimers are either stored in Weibel–Palade bodies or secreted into the plasma. Under normal circumstances, ULVWF multimers are unfolded by high shear stress in the circulation, making internal sites available for proteolysis by ADAMTS13, which specifically cleaves the peptide bond between Y1605 and M1606 in the A2 domain of VWF. Cleavage depolymerizes the ULVWF complexes into smaller multimers. Functional deficiency of ADAMTS13, resulting from either genetic mutations [16•] or inhibitory autoantibody production, leads to the accumulation of ULVWF multimers in plasma. These multimers promote intravascular thrombi of platelets, resulting in platelet

consumption and hemolysis. After the formation of microvascular thrombi in the kidney or brain, patients may suffer renal failure or neurologic dysfunction [1,2,3°,4••,5••].

Recent progress of ADAMTS13

The domain structure of ADAMTS13 shares similarities with other ADAMTS proteases, but is most divergent among this family [13] that contains 19 members in humans [17°]. ADAMTS13 is synthesized predominantly in the liver [12–15], although expression has been observed in other tissues and cell types, including platelets [18°]. In sequence, the 1427 amino acid precursor contains a signal peptide, a short propeptide, a metalloprotease domain, a disintegrinlike domain, a thrombospondin type 1 (TSP-1) repeat, a Cys-rich domain, a spacer domain, seven additional TSP-1 repeats, and two CUB domains [12–15]. CUB domains are named for complement components C1r/C1s, urinary epidermal growth factor, and bone morphogenic protein-1, each of which has one or more CUB domains [2]. Although propeptides are generally necessary to preserve the latency of metalloproteases, the ADAMTS13 propeptide is not required for enzyme latency [19]. The metalloprotease domain is necessary, but not sufficient, for VWF cleavage, indicating the requirement for other domains in VWF cleavage [20,21]. A single nucleotide polymorphism within the Cys-rich domain, encoding a P475S mutation, is found in the Japanese population at an allelic frequency of approximately 5% [22]. This mutant exhibits low, but significant, VWF-cleaving activity, suggesting a role for the Cys-rich domain in proteolysis. Although this polymorphism is also found in the Chinese population at a lower frequency, it has not been identified in the white population [16°,23,24]. The spacer domain is indispensable for VWF cleaving activity. Truncation mutants lacking the spacer domain lose activity in both static and flow conditions [20,21,25°]. Inhibitory autoantibodies against ADAMTS13 in patients with acquired TTP are directed against epitopes in the Cys-rich and spacer domains [20,26••,27]. In addition, a truncated ADAMTS13 resulting from insertion of the retroviruslike element has been identified in a number of mouse strains [28••]. This truncated form, lacking the C-terminal two TSP-1 motifs and two CUB domains, exhibits the same activity levels as the full-length form. Although the exact cleavage sites have not been determined, both thrombin and plasmin can cleave ADAMTS13, resulting in the loss of activity [29••].

Extensive study has elucidated the mechanisms underlying the interaction of ADAMTS13 with VWF. VWF has three A-type domains; ADAMTS13 cleaves the central A2 domain. Homology modeling estimates that a scissile peptide bond exists inside the A2 domain [30]. The VWF A1 and A3 domains are binding sites for platelet GPIbα and collagen respectively. Although the A1 domain inhibits cleavage of the A2 domain by ADAMTS13, this inhibition is reversed on interaction of the A1 domain with platelet GPIbα or heparin [31••]. A single nucleotide polymorphism in the VWF A2 domain encoding a Y1584C mutation is found in the population of the United Kingdom with an allelic frequency of about 0.5%. This mutant VWF protein demonstrated an increased susceptibility for ADAMTS13 proteolysis [32,33].

Assays of ADAMTS13 activity

Five methods are widely used to assay ADAMTS13 activity [4••,34°]: multimer analysis by SDS-agarose gel electrophoresis [35], analysis of degraded VWF by SDS–polyacrylamide gel electrophoresis [36], two-site immunoradiometric analysis [37], residual collagen binding analysis [38], and residual ristocetin cofactor activity analysis [39].

Pioneering work using a recombinant A1-A2-A3 VWF fragment expressed in *Drosophila* cells has recently been described for measuring ADAMTS13 activity [40]. This assay requires denatured cleavage conditions and uses Western blotting to detect the products.

Investigators demonstrated that ULVWF multimers newly released from endothelial cells are anchored to the surfaces of endothelial cells in a P-selectin-dependent manner [41°] and form long stringlike structures to which fluorescence-labeled platelets adhere [42]. Based on these finding, the cleavage of endothelial-derived ULVWF multimers under flow was used to examine ADAMTS13 activity [43°]. After the cleavage of these strings on endothelial cells under flow by ADAMTS13, the numbers of ULVWF strings were quantified as a measure of enzyme activity.

Evaluation of these methods

A multicenter comparison has been organized to evaluate these assays [44]. ADAMTS13 activity was determined in 30 plasma samples, with activities ranging from less than 3 to more than 100%, from patients with congenital or acquired TTP and other unrelated conditions by four different analyses: the multimer analysis by SDS–agarose gel electrophoresis, the two-site immunoradiometric analysis, the residual collagen binding analysis, and the residual ristocetin cofactor activity analysis. All assays identified plasma samples with severe ADAMTS13 deficiencies (<5%) and those with strong inhibitory activity, with some exceptions of results observed using the collagen binding assay. Plasma samples with normal to moderately reduced activity exhibited less concordant results.

An additional international collaborative study evaluated these four methods, as well as two additional methods [45°]: a quantitative immunoblotting assay using a recombinant VWF A1-A2-A3 fragment [40] and a cleavage assay using endothelial cell-derived ULVWF stringlike structures with attached platelets under flow [43°]. In this study, measurement of six plasma samples with 0%, 10%, 20%,

40%, 80%, and 100% of ADAMTS13 activity indicated that the most consistent and reliable assays were the multimer analysis by SDS–agarose gel electrophoresis, the residual collagen binding analysis, and the residual ristocetin cofactor activity analysis.

New assays using recombinant or synthetic substrates

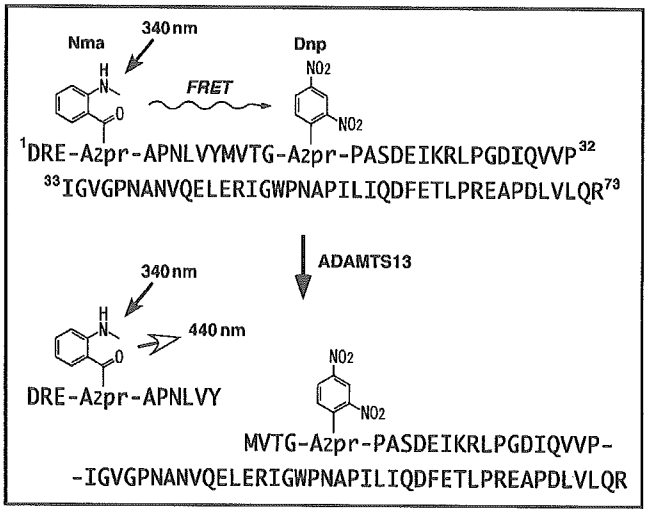
The assays described here use multimeric forms of VWF as substrates that require denaturing conditions, such as urea, for cleavage. Most of these assays measure the residual activity of VWF multimers, likely resulting in a reduced sensitivity for the quantification of ADAMTS13 activity. Therefore, more accurate and simpler methods are desired. Recently, novel ADAMTS13 assays have been developed using both recombinant and synthetic substrates. These high-throughput methods are rapid and do not require denaturing conditions or protease-free purified VWF substrates.

An assay using the minimal VWF region required to serve as a specific substrate has been attempted [46•]. The substrate peptide, designated GST-VWF73-H, consists of a 73-amino acid sequence spanning residues D1596 to R1668 of the VWF A2 domain, designated VWF73. This sequence is flanked by an N-terminal glutathione S-transferase and six C-terminal His sequences. This assay does not require a denaturing condition for cleavage, but necessitates Western blotting for detection.

To avoid the use of Western blotting, a fluorogenic peptide (FRETS-VWF73) was synthesized [47••]. FRETS-VWF73 contains the core VWF73 peptide, in which the Q1599 residue at the P7 position of the scissile bond was converted to a 2,3-diaminopropionic residue (A2pr) modified with a 2-(*N*-methyl amino)benzoyl group (Nma). The N1610 residue at the P5′ position was converted to A2pr modified with a 2,4-dinitrophenyl group (Dnp; Fig. 1). When the Nma group is exited at 340 nm, fluorescence resonance energy is transferred to the neighboring quencher, Dnp. After cleavage of the bond between Y1605 and M1606 by ADAMTS13, the quenching of fluorescence resulting from the energy transfer does not occur, allowing the emission of fluorescence at 440 nm by Nma. Thus, incubation of FRETS-VWF73 with plasma samples containing ADAMTS13 resulted in a quantitative increase in fluorescence. Neither thrombin nor plasmin increased fluorescence of FRETS-VWF73. The analysis can be completed within 1 hour using a 96-well format and commercial plate readers. FRETS-VWF73 is now commercially available from the Peptide Institute, Inc. (Minoh, Japan).

A novel enzyme immunoassay-based method using recombinant GST-VWF73-H has also been described [48•]. In this assay, GST-VWF73-H was captured onto anti-GST precoated microtiter plates and, after incubation with plasma

Figure 1. Structure of the synthetic fluorogenic ADAMTS13 substrate FRETS-VWF73



FRET, fluorescence resonance energy transfer; Nma, 2-(*N*-methyl amino) benzoyl group; Dnp, 2,4-dinitrophenyl group; A2pr, 2,3-diaminopropionic residue.

samples, the remaining amount of intact GST-VWF73-H is detected with an anti-His epitope antibody.

The recombinant A2 domain, flanked with six N-terminal His residues and a C-terminal Tag-100 sequence, was also used for an ADAMTS13 activity assay [49,50•]. The A2 peptide, captured on a Ni^{2+}-coated microtiter plate, was incubated with plasma samples. After washing, the amount of A2 peptide remaining in the well was detected with a monoclonal antibody against the Tag-100 sequence.

Detection of autoantibodies

All the assay methods described here are useful to detect ADAMTS13 autoantibodies in plasma of patients with acquired TTP. An inhibitor assay is generally carried out using mixtures of heat-inactivated plasma from patients and normal plasma at 1:1 dilution or several dilutions. The amount of activity inhibited by 1 mL of the patient's plasma was then estimated; thus, 1 U of inhibitor neutralizes the activity in 1 mL of normal human plasma [51]. Alternatively, the protease activity in the mixture was expressed as a percentage of that in control plasma. For the detection of autoantibodies against ADAMTS13, an ELISA kit is now commercially available from Technoclone GmbH (Vienna, Austria).

Precaution for measurement of ADAMTS13 activity in plasma

To measure ADAMTS13 activity, blood is normally collected into tubes containing sodium citrate as an anticoagulant. As ADAMTS13 is a metalloprotease, inclusion of EDTA completely inhibits enzyme activity. A high