新潟県中越地震被災者でも7年後の検診結果においてDVT陽性者で震災後の脳梗塞，心筋梗塞が有意に多かった[9]．こうしたことから震災後のVTEは震災直後の致死的肺塞栓症の予防のためだけでなく，長期間にわたる二次的健康被害予防のためにもまさにpreventable diseaseとして予防すべきものであり，ロンドン地下鉄避難所の教訓から日本でも早急に簡易ベッドを基本とした避難所にすべきであろう．

謝辞

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Regular Article

Development of a new laboratory test to evaluate antithrombin resistance in plasma

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ABSTRACT

Introduction: We recently reported a variant prothrombin (p.Arg596Leu: prothrombin Yukuhashi) that confers antithrombin resistance to patients with hereditary thrombosis. To detect antithrombin resistance in plasma, we devised a laboratory test analyzing the kinetics of thrombin inactivation using antithrombin.
Materials and Methods: After incubation with prothrombin activator components (phospholipids, $CaCl_2$, and snake venom), samples were treated with excess antithrombin in the presence or absence of heparin for various time periods. Subsequently, H-D-Phe-Pip-Arg-p-nitoranilide was added and changes in absorbance/min (ΔA/min) were measured at 405 nm.
Results: After 1 min inactivation using antithrombin and heparin, the relative residual thrombin activity of recombinant mutant prothrombin (34.3% ± 2.2%) was higher than that of the wild-type (6.3% ± 1.2 %). After 30 min without heparin, the relative residual thrombin activity of recombinant mutant prothrombin (95.8% ± 0.4%) was higher than that of the wild-type (10.1% ± 1.7%), indicating that this assay could detect antithrombin resistance of the variant 596Leu prothrombin. Moreover, warfarinized plasmas from 2 heterozygous patients with prothrombin Yukuhashi mutation clearly showed higher values of the relative residual thrombin activity than those from 5 thrombosis patients lacking the mutation in the presence or absence of heparin.
Conclusions: We have devised a laboratory test to detect antithrombin resistance in plasma by analyzing the kinetics of thrombin inactivation using antithrombin. This assay may be useful for detecting antithrombin resistance in plasma, even in warfarinized patients.

Introduction

Venous thromboembolism (VTE) is a multifactorial disease resulting from a complex interaction between circumstantial and genetic factors. VTE risks can be identified in >80% patients; however, a common environmental or hereditary thrombophilic defect has not been found [1].

Genetic studies of hereditary thrombophilia have revealed 2 genetic defect types, namely, loss-of-function mutations in the natural anticoagulants antithrombin (AT), protein C, and protein S, and gain-of-function mutations in procoagulant factors V (factor V Leiden) and II (prothrombin G20210A: *F2* c.*97G > A) [2–4]. In the typical Caucasian population, the gain-of-function mutations in procoagulant factors are more prevalent than abnormalities in the anticoagulant proteins; however, this is not the cause in the Asian population [5].

To date, numerous genetic defects have been identified in families with hereditary thrombophilia, but several causative mutations still remain undiscovered. We recently reported a case of hereditary thrombosis induced by a novel mechanism of AT resistance, which is a gain-of-function mutation in the gene encoding the clotting factor prothrombin (prothrombin Yukuhashi) [6]. However, current conventional laboratory tests are unable to easily detect AT resistance in a patient's plasma, although the thrombin generation assay (TGA) could detect AT resistance in the reconstituted plasma with recombinant wild type and mutant prothrombins.

In this study, we have developed a relatively simple test to detect AT resistance that may be valuable in the diagnosis and detection of hereditary thrombophilia.

Abbreviations: AT, antithrombin; VTE, venous thromboembolism; Ox, *Oxyuranus scutellatus*; ELISA, enzyme-linked immunosorbent assay; APC, activated protein C; TGA, thrombin generation assay; PT-INR, prothrombin time-international normalized ratios; SD, standard deviation.

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Materials and Methods

Materials

Purified human prothrombin from fresh frozen plasma was purchased from Enzyme Research Laboratories (South Bend, IN, USA). PTT-Reagent RD was purchased from Roche Diagnostics KK (Tokyo, Japan). Prothrombin-deficient plasma (prothrombin activity <1%) was purchased from Mitsubishi Chemical Medience Co. (Tokyo, Japan). The chromogenic substrates, H-D-Phe-Pip-Arg-p-nitoranilide (S-2238) was purchased from Sekisui Medical Co. (Tokyo, Japan). *Oxyuranus scutellatus* (Ox) venom, also called as taipan venom, which is a high-molecular-weight (approximately 250 kDa) prothrombin activator, was obtained from Sigma-Aldrich (St. Louis, MO, USA). Human AT was obtained from Mitsubishi Tanabe Pharma Co. (Osaka, Japan). Heparin was obtained from Mochida Pharmaceutical Co. (Tokyo, Japan). Other reagents used were of analytical grade. We tested plasmas from 2 patients (Pat1 and Pat2) who were on warfarin with prothrombin Yukuhashi. Their prothrombin time-international normalized ratios (PT-INRs) were 2.8 and 1.2, respectively. We also tested plasma from 5 thrombosis patients (W1, W2, W3, W4, and W5) without the prothrombin Yukuhashi mutation, who were on warfarin. Their PT-INRs were 2.7, 2.2, 1.4, 2.5, and 1.9, respectively.

Recombinant Prothrombins

As described previously, wild-type and mutant (p.R596L: prothrombin Yukuhashi) prothrombin expression vectors and stable transformants were established by selection with geneticin (G418) after transfection into human embryonic kidney cells (HEK293) [6,7]. In brief, we transfected HEK293 cells with 30 μg of the recombinant prothrombin expression vectors using the calcium phosphate method. The transformed cells were selected in a culture medium containing 700 μg/mL G418 (Gibco BRL, Rockville, USA), and the stable transformants highly expressing the respective recombinant prothrombin molecules were obtained. Conditioned media containing stable transformants expressing recombinant prothrombins incubated for 24 h were collected in a serum-free medium containing 10 μg/mL of vitamin K1 (Isei, Yamagata, Japan), concentrated using Centriprep (Millipore, Billerica, USA), and stored at −80 °C until use. Antigen levels of the prothrombins were determined using an enzyme-linked immunosorbent assay (ELISA; Enzyme Research Laboratories).

Kinetic Analysis of Thrombin Inactivation by AT

The assay consisted of 3 steps, namely prothrombin activation, thrombin inactivation, and measurement of the residual thrombin activity. In the prothrombin activation step, Ox venom was used as a prothrombinase (FXa/FVa)-like prothrombin activator. First, the optimal pH of Tris–HCl buffer was determined in the prothrombin activation step as follows; 500 μL of 1:100 normal plasma dilutions in buffer (50 mmol/L of Tris-HCl, 0.3 mol/L of NaCl, pH7.2-8.9), 100 μL of a phospholipid (50% PTT-Reagent RD)/$CaCl_2$ (12.5 mmol/L) mixture, and 100 μL of Ox venom (0.2 mg/mL), were mixed and incubated for 4 min at 37 °C. The remaining assay conditions were determined sequentially (Fig. 1).

In the second step, thrombin was inactivated by adding 100 μL of AT solution in the presence or absence of heparin. To determine the assay conditions in this thrombin inactivation step, purified human prothrombin solution containing equivalent activity as normal plasma was used. Inactivation times with and without heparin were 0–5 min and 0–30 min, respectively. In the presence of heparin, 3 heparin concentrations with 30 μg/mL of AT (Fig. 2A) and 6 AT concentrations with 5 U/mL of heparin (Fig. 2B) in 100 μL of AT solution were used for various time periods up to 5 min.

In the final step, the residual thrombin activity was determined using a chromogenic synthetic substrate [200 μL of S-2238 (0.5 mmol/L)] to measure changes in absorbance/min (ΔA/min) at 405 nm with TBA-180 (Toshiba Medical Systems Co, Tokyo, Japan). Because the initial changes in absorbance were unstable (data not shown), a 5-s lag time for measurement of stable changes in absorbance was included. Relative residual thrombin activities compared with the 0-min data were calculated, to overcome differences in the original prothrombin levels in the samples.

Demonstration of AT Resistance In Warfarinized Patient Plasma

We tested the samples for AT resistance using the optimized procedures as follows:

First, 500 μL of 1:100 dilutions in buffer (50 mmol/L of Tris–HCl, 0.2 mol/L of NaCl, pH8.1), 100 μL of a phospholipid (50% PTT-Reagent RD)/$CaCl_2$ (15 mmol/L) mixture and 100 μL of Ox venom (0.1 mg/mL) were added simultaneously, and the samples were incubated for 2 min at 37 °C. Next, 100 μL of human AT (75 μg/mL) with or without heparin (5 U/mL) was added, and the samples were incubated for various time periods. To determine the residual thrombin activity, 200 μL of S-2238 (0.5 mmol/L) was added to the sample as a chromogenic thrombin substrate and changes in absorbance/min (ΔA/min) were measured at 405 nm. Relative residual thrombin activities were calculated by comparison with 0-min data. Using this optimized procedure, normal and warfarinized patient plasmas were analyzed.

We also tested warfarinized plasma from thrombosis patients with or without prothrombin Yukuhashi mutation for AT resistance using the optimized procedures in the presence or absence of heparin. We detected statistically significant differences between the data from thrombosis patients with and without the prothrombin Yukuhashi mutation using Student's t-test and one-way factorial analysis of variance and multiple comparison tests (Fisher's method).

Results

Assay Conditions In Kinetic Analysis of Thrombin Inactivation by AT

Optimal assay conditions in the prothrombin activation step, such as pH of 50 mmol/L Tris–HCl buffer; concentrations of NaCl, phospholipids, $CaCl_2$, and Ox venom; reaction time for Ox venom treatment; and dilution folds of plasma were demonstrated (Fig. 1A-G) and used in the assay. In the thrombin inactivation step with heparin, we tested 3 heparin concentrations (2.5–10 U/mL) with 30 μg/mL of AT (Fig. 2A) and 6 AT concentrations (8–75 μg/mL) with 5 U/mL of heparin (Fig. 2B) in 100 μL of AT solution. Finally, we chose the optimal concentrations of heparin (5 U/mL) and AT (75 μg/mL) in 100 μL of AT solution in the presence of heparin. Amount of AT in 100 μL of AT solution (75 μg/mL of AT) was 7.5 μg, whereas that in 500 μL diluted (1:100) plasma was 1.5 μg because the AT concentration would be 30 mg/dL in normal plasma. Thus, 5-fold excess AT was added to the diluted plasma to minimize the influence of endogenous AT differences during thrombin inactivation of this assay. We also used the same concentration of AT solution in the absence of heparin.

Analysis of AT Resistance for Recombinant Prothrombin

Recombinant prothrombin samples containing chromogenic activity equivalent to that of normal plasma were prepared, and AT resistance in the presence or absence of heparin was analyzed. We performed triplicate measurements (intra-assay) for recombinant prothrombins and obtained very good reproducibility of data, as shown in Fig. 3. After 1-min inactivation using AT with heparin, the relative residual thrombin activity of the recombinant mutant prothrombin was 34.3% ± 2.2% [n = 3, mean ± standard deviation (SD); intra-assay], whereas that of the wild-type was 6.3% ± 1.2 % (Fig. 3A). After 30-min inactivation

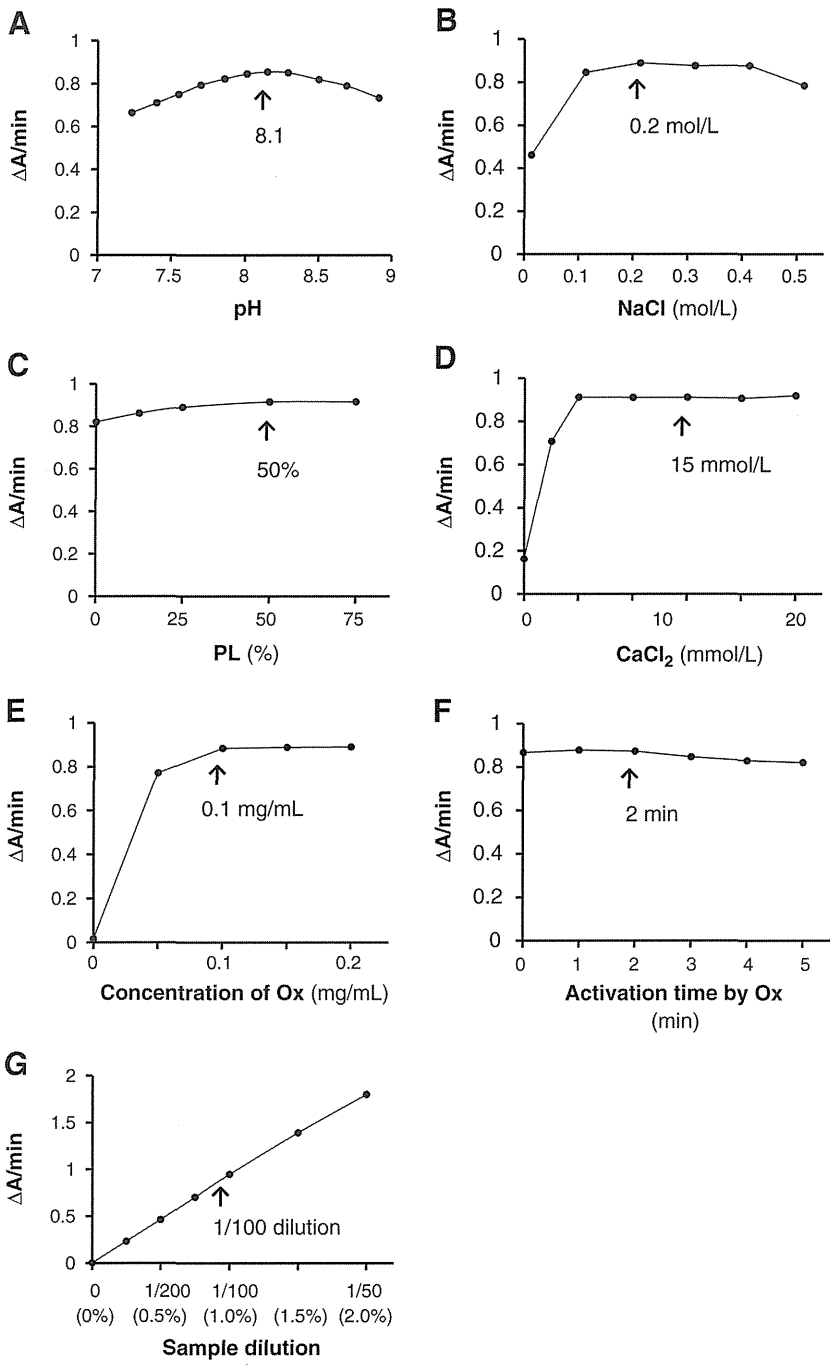


Fig. 1. Optimal assay conditions in the prothrombin activation step. Diluted plasma samples were incubated with prothrombin activator components (phospholipids, $CaCl_2$, and Ox venom) at 37 °C, S-2238 was added, and changes in absorbance/min (ΔA/min) were measured at 405 nm. Several parameters of the assay in the thrombin generation step, such as pH (A) of 50 mmol/L Tris–HCl buffer; concentrations of NaCl (B), phospholipids (C), $CaCl_2$ (D), and Ox venom (E); reaction time for Ox venom (F); and dilution folds of plasma (G) were determined. The values with arrows indicate optimal conditions.

using AT without heparin, the relative residual thrombin activity of the recombinant mutant prothrombin was 95.8% ± 0.4% (n = 3, mean ± SD; intra-assay), whereas that of the recombinant wild-type prothrombin was 10.1% ± 1.7% (Fig. 3B), indicating an obvious AT resistance of the recombinant mutant prothrombin.

Kinetic Assay Profile of Normal Plasma

We analyzed normal plasma with and without heparin repeatedly (inter-assay) (Fig. 4). The relative residual thrombin activity of normal plasma decreased rapidly in the presence of heparin by AT inactivation and slowly decreased in the absence of heparin.

Analysis of AT Resistance for Warfarinized Plasma from Thrombosis Patients with or without the Trothrombin Yukuhashi Mutation

We also analyzed warfarinized plasmas from 5 thrombosis patients without the prothrombin Yukuhashi mutation (Fig. 5). Again, relative residual thrombin activities decreased rapidly in the presence of heparin by AT inactivation and slowly in the absence of heparin, although a small variation was observed. Higher PT-INR plasma appeared to exhibit a higher relative residual thrombin activity.

Plasma samples of 2 patients with the prothrombin Yukuhashi mutation (Pat1 and Pat2; PT-INR = 2.8 and 1.2, respectively), 5 thrombosis patients without the prothrombin Yukuhashi mutation (W1-5;

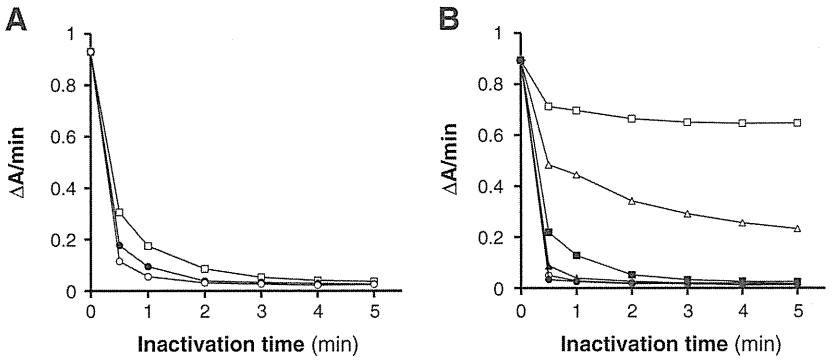


Fig. 2. Optimal assay conditions in the thrombin inactivation step. Purified human prothrombin was used to optimize heparin (**A**) and AT (**B**) concentrations in the thrombin inactivation step. A: Under optimal conditions of prothrombin activation (Fig. 1), the samples were treated with 3 heparin concentrations [2.5 U/mL (□); 5.0 U/mL (●); 10 U/mL (○)] in the presence of AT (30 μg/mL). B: Under optimal conditions of prothrombin activation (Fig. 1), the samples were treated with several AT concentrations [8 μg/mL (□), 15 μg/mL (△), 30 μg/mL (■), 45 μg/mL (▲), 60 μg/mL (○) and 75 μg/mL (●)] in the presence of heparin (5 U/mL).

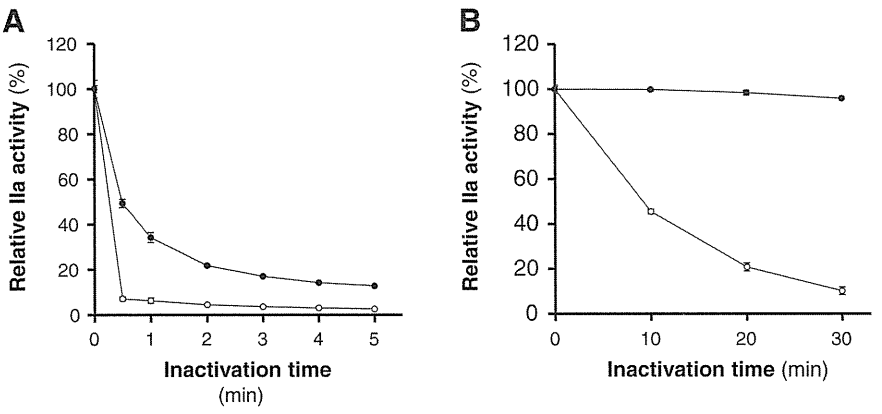
PT-INR = 2.7, 2.2, 1.4, 2.5, and 1.9, respectively), and a healthy normal subject were analyzed in the presence or absence of heparin (Fig. 5).

The initial thrombin activities of the warfarinized patient plasmas were very low, but inactivation using AT of the plasmas containing prothrombin Yukuhashi was very slow compared with that of the plasmas lacking prothrombin Yukuhashi (data not shown). Analysis of the relative residual thrombin activities after inactivation using AT revealed higher AT resistance in the plasmas of the 2 patients with prothrombin Yukuhashi than in the plasmas of the patients without prothrombin Yukuhashi, both in the presence and absence of heparin (Fig. 5). After 1-min inactivation using AT with heparin, the relative residual thrombin activities in the plasma of the 2 patients with prothrombin Yukuhashi showed significantly higher values [32% (Pat1) and 31% (Pat2)], than those in the plasma of the 5 thrombosis patients without prothrombin Yukuhashi [4%–11% (W1–5)] (Fig. 5A). There were statistically significant differences between the data of the patients with and without the prothrombin Yukuhashi mutation after 1-, 2- and 3-min inactivation, but not at 4- and 5-min inactivation.

After 30 min inactivation using AT without heparin, the relative residual thrombin activities in the plasma of patients with prothrombin Yukuhashi showed higher values [48% (Pat1) and 52% (Pat2)] than those in the plasmas of patients without prothrombin Yukuhashi [9%–16% (W1–5)] (Fig. 5B). There were also statistically significant differences between the data of the patients with and the patients without the prothrombin Yukuhashi mutation after 10-, 20- and 30-min inactivation.

Discussion

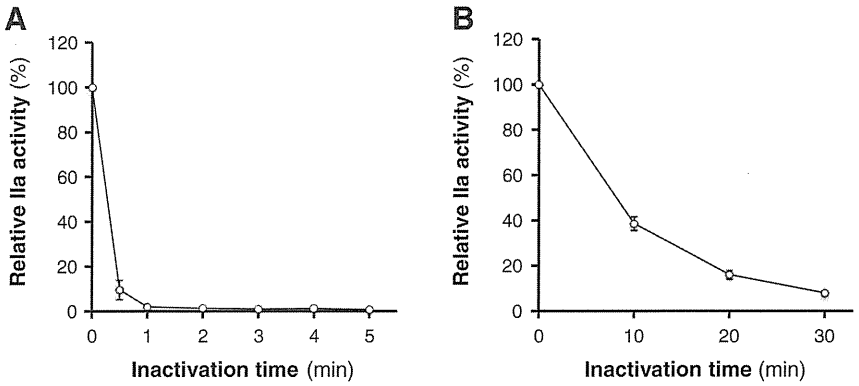
We recently reported a case of hereditary thrombosis associated with a novel mechanism of AT resistance, a gain-of-function mutation in the gene encoding the clotting factor prothrombin (prothrombin Yukuhashi) [6]. We did not initially identify any known causes of hereditary thrombophilia in this family. In 2009, at the XXII Congress of the International Society on Thrombosis and Haemostasis held in Boston, it was reported that a genome-wide linkage scan to detect genes



% \ min	0	0.5	1	2	3	4	5
rWT	100.0	7.2	6.3	4.5	3.7	3.1	2.7
SD	1.5	0.9	1.2	0.0	0.1	0.1	0.1
CV%	1.5	12.4	19.1	0.6	1.2	1.7	2.4
rMut	100.0	49.4	34.3	21.9	17.1	14.3	12.9
SD	3.9	1.9	2.2	0.6	0.2	0.3	0.2
CV%	3.9	3.8	6.4	2.6	1.3	2.0	1.4

% \ min	0	10	20	30
rWT	100.0	45.4	20.8	10.1
SD	1.2	1.0	1.8	1.7
CV%	1.2	2.3	8.6	16.9
rMut	100.0	99.8	98.4	95.8
SD	1.8	0.6	1.1	0.4
CV%	1.8	0.6	1.1	0.4

Fig. 3. AT resistance analysis for recombinant prothrombin. AT resistance for recombinant prothrombin with or without heparin was analyzed. A: Activated thrombins from recombinant prothrombins by Ox venom were inactivated using AT with heparin for 0–5 min, and relative residual thrombin activities were determined. rWT (○) and rMut (●) indicate wild type and mutant recombinant prothrombins, respectively (n = 3, mean ± SD; intra-assay). The lower table shows actual numerical data. B: Activated thrombins from recombinant prothrombins by Ox venom were inactivated using AT without heparin for 0–30 min, and relative residual thrombin activities were determined. rWT (○) and rMut (●) indicate wild type and mutant recombinant prothrombins, respectively (n = 3, mean ± SD; intra-assay). The lower table shows actual numerical data. Relative residual thrombin activities were calculated by comparison with the 0-min data. Respective values indicate the final % of the relative residual thrombin activities.



% \ min	0	0.5	1	2	3	4	5
Nor	100	9.66	1.95	1.27	1.00	1.08	0.77
SD	-	4.34	0.62	0.24	0.33	0.42	0.74
CV%	-	44.92	31.80	18.76	32.55	38.41	96.34

% \ min	0	10	20	30
rWT	100	38.72	16.06	7.99
SD	-	3.01	1.90	0.94
CV%	-	7.79	11.80	11.80

Fig. 4. Kinetic assay profile of normal plasma. Normal plasma was analyzed using the new AT resistance assay with or without of heparin. A: Activated thrombin from normal plasma prothrombin by Ox venom was inactivated using AT with heparin for 0–5 min, and the relative residual thrombin activity was determined (n = 4, mean ± SD). The lower table shows actual numerical data. B: Activated thrombin from normal plasma prothrombin by Ox venom was inactivated using AT without heparin for 0–30 min, and the relative residual thrombin activity was determined (n = 8, mean ± SD). The lower table shows actual numerical data. Relative residual thrombin activities were calculated as described in Fig. 3.

associated with a susceptibility to thrombosis, identified a prothrombin missense mutation (Arg > Trp; no description of the position); however, the detailed molecular mechanism for thrombophilia remains unknown [8]. Therefore, we analyzed the prothrombin gene as a candidate for causing thrombophilia in this family, and found the mutation [6].

Several laboratory tests are available to evaluate thrombotic diathesis, however, none of the current conventional laboratory tests can detect AT resistance in plasma easily. We developed a new laboratory test that evaluates the thrombin inactivation response in plasma to the added AT. To our knowledge, such an assay has not been previously used to analyze patients with thromboembolism, with the exception of an activated protein C (APC) resistance test measuring the anticoagulant response in plasma to the added APC [9]. The developed assays were designed to measure the residual activity of plasma-derived thrombin after the addition of excess AT and were based on the hypothesis that a poor response to AT is a predisposition to thrombosis.

The assay consists of 3 steps: prothrombin activation, thrombin inactivation using AT, and measurement of the residual thrombin activity. In the first prothrombin activation step, Ox venom, also called as Taipan venom, was used as a FXa/FVa-like prothrombin activator, which only requires Ca^{2+} and negatively charged phospholipids for maximal activity [10]. Ox venom consisting of enzymatic and non-enzymatic subunits is stable, unlike the FXa/FVa complex, which is transient. Ox venom could probably be replaced by FXa and FVa in the assay. This modification is currently being tested.

Inhibition of thrombin using AT, forming a covalent complex in a 1:1 molar ratio, is relatively slow, but is markedly enhanced in the presence of the glycosaminoglycan heparin [11]. Plasma AT plays a key role in the natural hemostatic balance to maintain blood fluidity, and patients with AT deficiency are susceptible to thromboembolic diseases, particularly deep vein thrombosis of the lower limbs and pulmonary embolism [12]. Similarly, patients with AT resistance are susceptible to thromboembolic diseases [6]. During thrombin inactivation of this assay, 5-fold excess AT was added to the diluted plasma to minimize the influence of endogenous AT differences with or without heparin. AT resistance was detected in using both methods. During the measurement of the residual thrombin activity, the assay without heparin appeared to be more effective in plasma than the assay with heparin, although a longer time was required.

Because warfarin treatment decreased the levels of vitamin K-dependent coagulation factors, including prothrombin, the initial residual thrombin activity in the warfarinized patient's plasma containing

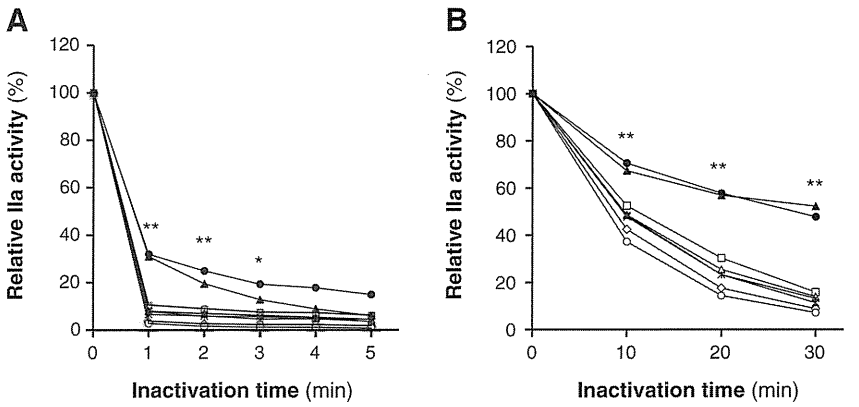


Fig. 5. Analysis of AT resistance for warfarinized plasma from thrombosis patients with or without the prothrombin Yukuhashi mutation. Warfarinized plasmas from 2 patients with the prothrombin Yukuhashi mutation (Pat1, ● and Pat2, ▲) and 5 thrombosis patients without the prothrombin Yukuhashi mutation (W1, □; W2, △; W3, ; W4, ; W5, ✳), and normal plasma (○) were analyzed. Their PT-INRs were 2.8 (Pat1), 1.2 (Pat2), 2.7 (W1), 2.2 (W2), 1.4 (W3), 2.5 (W4), and 1.9 (W5), respectively. Activated thrombin from plasma prothrombins by Ox venom was inactivated using AT with heparin for 0–5 min (A) or without heparin for 0–30 min (B) and relative residual thrombin activities were determined as described in Fig. 3. There were statistically significant differences between the data of the patients with and the patients without the prothrombin Yukuhashi mutation (*, $p < 0.01$; **, $p < 0.001$).

prothrombin Yukuhashi was lower than that in normal pooled plasma; however, a definite AT resistance was observed when converted to the relative residual thrombin activities.

We have developed a method for analyzing thrombin inactivation kinetics in plasma to detect AT resistance. Although identification of more patients with prothrombin Yukuhashi will be required to fully characterize this new assay, AT resistance was successfully detected in the warfarinized plasma obtained from the patient with prothrombin Yukuhashi. To date, plasma samples from several unrelated Japanese patients with VTE of an unknown cause have been analyzed, but none have demonstrated AT resistance. The availability of the new assay may identify more patients with AT resistance, including those with prothrombin Yukuhashi or other mutation.

In conclusion, we have devised a new laboratory test to detect AT resistance in plasma obtained from patients with prothrombin Yukuhashi. Although it is necessary to improve the efficacy and convenience of the assay, it can be used as a research tool to find AT resistance in undiagnosed patients with thromboembolism.

Authorship

M.M. and A.T. equally contributed to this work sharing first authorship, designed and performed the research, analyzed the data, and drafted the manuscript; A.S., E.O., and Y.T. performed the experiments, analyzed the data, and contributed to the analytic methodology; Y.A. I.K., and Y.N. interpret the data and contributed to the analytic methodology; T. Murate and T. Matsushita developed the project and collected the clinical samples; H.S. supervised the project and edited the manuscript; and T.K. designed the project, analyzed the data, and wrote the manuscript. All authors were involved in critical reading of the manuscript prior to submission.

Conflict of Interest Statement

The authors declare no competing financial interests.

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Paxillin is an intrinsic negative regulator of platelet activation in mice

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Abstract

Background: Paxillin is a LIM domain protein localized at integrin-mediated focal adhesions. Although paxillin is thought to modulate the functions of integrins, little is known about the contribution of paxillin to signaling pathways in platelets. Here, we studied the role of paxillin in platelet activation in vitro and in vivo.

Methods and results: We generated paxillin knockdown (Pxn-KD) platelets in mice by transplanting bone marrow cells transduced with a lentiviral vector carrying a short hairpin RNA sequence, and confirmed that paxillin expression was significantly reduced in platelets derived from the transduced cells. Pxn-KD platelets showed a slight increased in size and augmented integrin αIIbβ3 activation following stimulation of multiple receptors including glycoprotein VI and G protein-coupled receptors. Thromboxane A_2 biosynthesis and the release of α-granules and dense granules in response to agonist stimulation were also enhanced in Pxn-KD platelets. However, Pxn-KD did not increase tyrosine phosphorylation or intracellular calcium mobilization. Intravital imaging confirmed that Pxn-KD enhanced thrombus formation in vivo.

Conclusions: Our findings suggest that paxillin negatively regulates several common platelet signaling pathways, resulting in the activation of integrin αIIbβ3 and release reactions.

Keywords: Platelet, Glycoprotein, Platelet aggregation, Release reaction

Background

A breakdown of normal platelet function results in either unexpected bleeding or thrombotic events [1]. Platelets are inactive in the intact vasculature under physiological conditions. However, once the platelets encounter an injured region of the endothelium, they attach through an interaction between von Willebrand factor and the glycoprotein (GP) Ib/IX/V complex [2], and then collagen receptor GPVI triggers platelet activation. Activated platelets release several classes of agonists, including ADP and thromboxane (Tx) A_2, which promote further platelet activation [3]. These steps ultimately increase the affinity of integrin αIIbβ3 for its ligands and induce platelet aggregation [4]. The intracellular signaling that increases the affinity of integrins is known as inside-out signaling [4]. Multiple signal transduction pathways from various receptors share common inside-out signaling cascades. For example, phosphoinositol hydrolysis, which leads to calcium mobilization and protein kinase C activation [5], and Rap1b activation are well-known signaling pathways that regulate integrin-mediated platelet functions [6].

To increase the affinity of integrin αIIbβ3, inside-out signaling pathways induce a drastic conformational change of the integrin [7]. Direct interactions between cytoskeletal proteins (e.g., talin and kindlin) and cytoplasmic β integrin are essential for inducing the conformational change of integrins [7]. Indeed, the loss of talin or kindlin in platelets dramatically reduces integrin αIIbβ3-mediated platelet aggregation, despite normal expression levels of the surface receptors [8,9]. Selective blockade of talin binding by a single amino acid substitution in β3 integrin also impairs integrin αIIbβ3-dependent platelet responses [10]. Although a number of integrin-associated proteins have been reported [11], the identities of proteins and their roles in regulating integrin signaling in platelets have not been fully characterized. It is also unknown whether

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additional molecules, other than talin and kindlin, are capable of regulating integrin signaling pathways.

Paxillin is a LIM domain protein that was originally identified as a substrate for oncogene *v-src* [12]. Paxillin contains two conserved structural domains, the N-terminus and C-terminus, which consist of four LIM domains [13,14]. Two other family members have also been identified, Hic-5 and leupaxin [13,14]. Paxillin is ubiquitously expressed alongside these variants [13,14], except in human platelets that predominantly express Hic-5 [15,16]. Conversely, mouse platelets express paxillin and leupaxin in addition to Hic-5 [17]. Considering the multiple interaction motifs located within its structure, paxillin appears to serve as a signaling platform for the recruitment of numerous regulatory proteins near integrins [13,14]. Paxillin directly interacts with the cytoplasmic domain of integrin α4 and α9, but not αIIb, and these interactions controls integrin-mediated cell migration and spreading [18,19].

Integrin αIIbβ3 in platelets is suitable for studies of integrin receptors because its ligand binding and signal transduction pathways are well characterized. Elucidating the intracellular proteins involved in the activation of integrin αIIbβ3 can provide a better understanding of the functions of integrins and might result in the discovery of new antithrombotic targets [20]. We previously reported that lentiviral vector-mediated short hairpin RNA (shRNA) expression in hematopoietic stem cells greatly reduces the expression of the target protein in platelets [21]. This method enables functional analyses of target proteins that modulate platelet activation in anucleate platelets [21]. In the present study, we used this method to investigate the roles of paxillin in platelet activation, and found that paxillin negatively regulates platelet signaling pathways including the activation of integrin αIIbβ3 and release reactions.

Materials and methods

Materials

All mouse cytokines were purchased from PeproTech (London, UK). The following antibodies and agonists were obtained from the specified suppliers: PAC-1 monoclonal antibody (mAb), anti-mouse P-selectin mAb (RB40.34), anti-paxillin mAb (clone 349), and anti-Hic-5 mAb (BD Biosciences, San Jose, CA); horseradish peroxidase-conjugated anti-green fluorescent protein (GFP) polyclonal antibody (Acris Antibodies, Himmelreich, Germany); phycoerythrin (PE)-Cy7-conjugated anti-mouse IgM (eBioscience, San Diego, CA); anti-talin mAb (clone 8D4); anti-phosphotyrosine mAb (clone 4G10), and BAPTA-AM (Millipore, Billerica MA); human fibrinogen and epinephrine (Sigma-Aldrich, St. Louis, MO); anti-vinculin mAb (V284) (Chemicon, Billerica, MA); anti-mouse GPVI mAb (Six.E10), anti-mouse GPIbα mAb (Xia.G5), and anti-mouse integrin αIIbβ3 mAb (Leo.D2 and clone JON/A) (Emfret Analytics, Eibelstadt, Germany); anti-α-actin mAb (D6F6), anti-FAK polyclonal antibody, and anti-Src mAb (32G6) (Cell Signaling Technology, Danvers, MA); anti-Rap1b polyclonal antibody and anti-protein kinase Cα mAb (M4) (Upstate Cell Signaling Solutions, Lake Placid, NY); allophycocyanin (APC)-conjugated anti-rat IgG polyclonal antibody (R& D Systems, Minneapolis, MN); convulxin (ALEXIS Biochemicals, Plymouth Meeting, PA); AYPGKF (Invitrogen, Carlsbad, CA); ADP (MC medical, Tokyo, Japan); U46619 (Cayman Chemical, Ann Arbor, MI).

Lentiviral vector and virus production

A lentiviral vector plasmid for expression of shRNA sequences and GFP (LentiLox vector) was purchased from the American Type Culture Collection (Manassas, VA) [22]. To efficiently express GFP in platelets, the cytomegalovirus promoter of the LentiLox vectors was substituted with the platelet-specific GPIbα promoter (LentiLox-GPIbα) [21]. Putative shRNA sequences were designed using web-based software provided by Thermo Scientific Molecular Biology (http://www.thermoscientificbio.com/design-center/). Three shRNA sequences were synthesized for mouse paxillin and then cloned into a LentiLox vector plasmid (Additional files 1 and 2). Lentiviruses were produced as described previously [23].

Transplantation of mouse bone marrow cells

All animal procedures were approved by the Institutional Animal Care and Concern Committee of Jichi Medical University, and animal care was performed in accordance with the committee's guidelines. Mouse bone marrow cells (C57BL/6 J) were isolated and resuspended in StemPro®-34 SFM medium (Invitrogen) supplemented with 100 ng/mL each of stem cell factor, thrombopoietin, interleukin-6, and fms-like tyrosine kinase 3 ligand, and 200 ng/mL soluble interleukin-6 receptor. The lentiviral vector was added at 12–16 h after cell isolation (multiplicity of infection [MOI] = 5), and the cell culture was continued for 21–22 h. Each recipient mouse (8–12 weeks of age) was irradiated with a single lethal dose of 9.5 Gy and then intravenously injected with 2×10^6 lentivirus-transduced bone marrow cells. After transplantation, about 50% of platelets expressed GFP (Figure 1). Mice with 70% of their platelets exhibiting GFP positivity were used in experiments that could not distinguish GFP-positive platelets, *i.e.*, light transmission aggregometry, clot retraction, release concentration, calcium mobilization, and intravital microscopy.

Immunoblotting

Immunoblotting with the specific antibodies was performed as described previously [21]. To assess protein tyrosine phosphorylation, washed platelets were pretreated

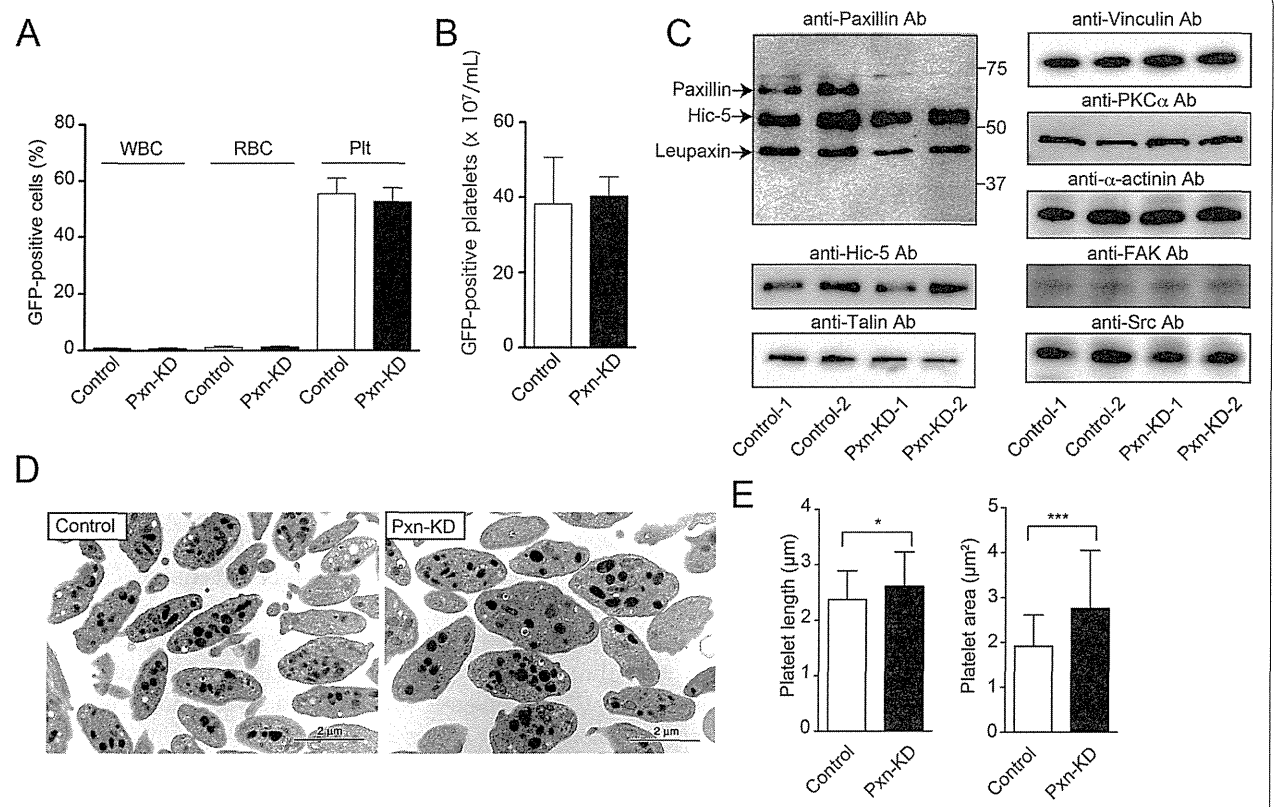

Figure 1 Characterization of Pxn-KD platelets. Bone marrow cells transduced with LentiLox-sh-control-GPIbα (Control) or LentiLox-sh-paxillin-GPIbα (Pxn-KD) at an MOI of 5 were transplanted into lethally irradiated recipient mice. **(A)** The numbers of GFP-positive cells (%) among white blood cells (WBCs), red blood cells (RBCs), and platelets (Plts) in peripheral blood at 30 days after transplantation were assessed by flow cytometry. Columns and error bars represent the mean ± s.d. ($n = 10$). **(B)** Absolute number of GFP-positive platelets in peripheral blood at 30 days after transplantation. Columns and error bars represent the mean ± s.d. ($n = 10$). **(C)** Platelet lysates were immunoblotted with the indicated antibodies. Data are for two mice with >80% of platelets expressing GFP in each experiment. **(D)** The morphology of control and Pxn-KD platelets was examined by transmission electron microscopy. Bars = 2 μm. **(E)** Platelet length (left panel) and area (right panel) were quantified by ImageJ software. Columns and error bars represent the mean ± s.d. ($n = 53–72$). Statistical significance was determined by the Student's *t*-test. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. control.

with 1 mmol/L EDTA, 5 U/mL apyrase, and 10 μmol/L SQ29548 to exclude the effects of aggregation, released ADP, and TxA_2.

Transmission electron microscopy

Mouse platelet pellets were fixed in 2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 60 min at 4°C. The samples were washed, post-fixed with 1% osmium tetroxide in 0.1 mol/L phosphate buffer for 60 min at 4°C, dehydrated with a graded ethanol series, and then embedded in Epon (TAAB Laboratories, Aldermaston, UK) as described previously [24]. Ultra-thin sections were prepared, stained with uranyl acetate and lead citrate, and then examined under a JEM1010 transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV. The length and area of platelets were quantified using ImageJ Ver. 10.2 for Macintosh (NIH, Bethesda, MD).

Preparation of washed mouse platelets and flow cytometry

A blood sample (100–400 μL) was drawn from each mouse through the right jugular vein using a 30 G syringe containing 1/10 sodium citrate, and then diluted with 3 mL Hepes/Tyrode buffer (138 mmol/L NaCl, 3.3 mmol/L NaH_2PO_4, 2.9 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mg/mL glucose, and 20 mmol/L Hepes, pH 7.4). The diluted blood was centrifuged at $120 \times g$ for 8 min, and the platelets obtained from the platelet-rich fraction were washed and resuspended in Hepes/Tyrode buffer. Just prior to centrifugation, a 15% acid-citrate-dextrose A solution and 0.1 μmol/L prostaglandin I_2 were added to inhibit platelet activation. The final platelet suspensions were adjusted to 1×10^7 platelets/mL and supplemented with 1 mmol/L $CaCl_2$. To assess the binding of JON/A, a monoclonal antibody (mAb) that recognizes activated mouse αIIbβ3 [25], to platelets, 30 μL of washed platelets was incubated with 4 μL of agonist solution, 4 μL of phycoerythrin (PE)-