ますが，国内で実施できる状況になっていません．

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Regular Article

Antithrombin-resistant prothrombin Yukuhashi mutation also causes thrombomodulin resistance in fibrinogen clotting but not in protein C activation

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ABSTRACT

Introduction: Prothrombin Yukuhashi (p.Arg596Leu) mutation can result in thrombophilia associated with antithrombin (AT) resistance. Mutant thrombin, an active form of prothrombin Yukuhashi, demonstrated moderately lower clotting activity than the wild-type but substantially impaired the formation of the complex with AT. However, the effects of the mutation on the thrombomodulin (TM)–protein C (PC) anticoagulant system have not been previously elucidated.

Materials and Methods: We prepared recombinant wild-type and mutant prothrombins, converted to thrombins using *Oxyuranus scutellatus* venom, and performed fibrinogen-clotting assays with or without recombinant soluble TM (rTM). We also evaluated activated PC (APC) generation activity of recombinant thrombins by measuring APC activity after incubation with human PC in the presence or absence of rTM.

Result and Conclusions: rTM treatment reduced the relative fibrinogen-clotting activity of the wild-type down to 8.4% in a concentration-dependent manner, whereas the activity of the mutant was only decreased to 44%. In the absence of rTM, APC generation activity (ΔA/min at 405 nm) was fairly low (0.0089 for the wild-type and 0.0039 for the mutant). In the presence of rTM, however, APC generation activity was enhanced to 0.0907 (10.2-fold) for the wild-type and to 0.0492 (12.6-fold) for the mutant, and the relative activity of the mutant with rTM was 54% of that of the wild-type. These data suggested that the prothrombin Yukuhashi mutation may cause TM resistance in terms of inhibition of fibrinogen clotting; this may contribute to susceptibility to thrombosis, although the enhancing effect of APC generation can be maintained.

Introduction

Hemostatic disequilibrium is a pivotal mechanism in all types of thromboses. Deficiency of natural anticoagulants such as antithrombin (AT), protein C (PC), and protein S (PS) increases the risk of venous thromboembolism. Factor V Leiden and prothrombin G20210A mutations are widely known as the most frequent causes of inherited thrombophilia in Caucasians but not in Asians [1,2].

Recently, AT resistance was reported to be associated with mutant thrombin from prothrombin Yukuhashi (c.1787G > T, p.R596L) that was found in a Japanese family with inherited thrombophilia [3,4]. Mutant thrombin derived from prothrombin Yukuhashi showed moderately lower procoagulant function than the wild-type and substantially impaired inhibition by AT. The other prothrombin mutation that confers AT resistance, prothrombin Belgrade, has also been reported in 2 Serbian families with thrombophilia and is a different mutation at the same position (c.1787G > A, p.R596Q) [5]. AT-resistant thrombin may have prolonged procoagulant activity *in vivo*, resulting in predisposed thrombosis; however, the effects of the prothrombin Yukuhashi mutation on the thrombomodulin (TM)–PC anticoagulant system have not yet been analyzed.

TM, an endothelial cell receptor of thrombin, converts thrombin from a procoagulant enzyme to an anticoagulant. Thrombin bound to TM promotes rapid conversion of PC zymogen to activated PC (APC) that binds to PS and inactivates factors Va and VIIIa; it also inhibits the conversion of fibrinogen to fibrin as well as the activation of platelets [6,7]. Thus, the TM–PC system contributes to natural anticoagulant mechanisms, inhibiting fibrin clot formation and preventing excess generation of thrombin. In mice, it has been reported that the loss of TM function in endothelial cells causes spontaneous and fatal thrombosis in arterial and venous circulations, which results from unfettered activation of the coagulation system [8].

In this study, we evaluated the effects of the prothrombin Yukuhashi mutation on the TM–PC anticoagulation system.

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Materials and Methods

Materials

Purified human prothrombin, PC, and fibrinogen from fresh frozen plasma were obtained from Haematologic Technologies Inc. (Essex Junction, VT, USA) and from Wako (Osaka, Japan). PTT-Reagent RD was purchased from Roche Diagnostics KK (Tokyo, Japan). *Oxyuranus scutellatus* (Ox) venom, also known as Taipan venom, a high-molecular-weight (approximately 250 kDa) prothrombin activator, was obtained from Sigma-Aldrich (St. Louis, MO, USA). Recomodulin (ART-123), a recombinant soluble TM (rTM), was generously gifted by Asahi Kasei Pharma Co. (Tokyo, Japan). Pefabloc-TH (NAPAP), a selective inhibitor of thrombin, was purchased from Pentapharm Ltd. (Basel, Switzerland). Synthetic chromogenic substrates H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238) and Glu-Pro-Arg-*p*-nitroanilide (S-2366) were obtained from Sekisui Medical Co. (Tokyo, Japan).

Recombinant Prothrombins

We prepared recombinant prothrombins, because the proband's plasma would not be suitable for evaluation on account of warfarin treatment. We established stable transformants of the HEK293 cells that expressed wild-type and mutant recombinant prothrombins, as described previously [3]. Stable transformants were cultured for 24 h in serum-free medium including 5 μg/mL of vitamin K1 (Isei, Yamagata, Japan). The medium was collected and concentrated using Vivaspin Turbo 15 (Sartorius Stedim Biotech GmbH, Goettingen, Germany) that contained a polyethersulfone membrane with a molecular weight cut-off of 30 kDa. The concentrated medium was stored at −80 °C until use. We determined the antigen levels of prothrombin in the conditioned medium using enzyme-linked immunosorbent assay (ELISA; Enzyme Research Laboratories, South Bend, IN, USA).

Procoagulant Functional Assays for Recombinant Prothrombins

To test the procoagulant functions of recombinant prothrombins, we performed chromogenic and fibrinogen-clotting assays.

In the chromogenic assay, recombinant prothrombins were diluted to 1% of the plasma prothrombin concentration in the dilution buffer [50 mmol/L Tris–HCl (pH 8.1) with 300 mmol/L NaCl], and 500-μL aliquots of the dilutions were incubated with 100 μL of the prothrombin activator (150 μg/mL Ox venom in saline) and 100 μL of the Ca-phospholipid mixture [15 mmol/L $CaCl_2$ and 50% phospholipid (PTT-Reagent RD)] at 37 °C for 2 min to allow sufficient conversion to thrombin. We used PTT-Reagent RD dissolved in 2 mL of distilled water for the 100% phospholipid solution. Thrombin activity was measured as changes in absorbance/min (ΔA/min) at 405 nm with the spectrophotometer TBA-180 (Toshiba Medical Systems Co, Tokyo, Japan) using 200 μL of the chromogenic substrate S-2238 (0.5 mmol/L in distilled water).

In the fibrinogen-clotting assay, recombinant prothrombins were diluted to 10% of the plasma prothrombin concentration in the dilution buffer [50 mmol/L Tris–HCl (pH 7.4) without NaCl], and 10-μL aliquots of the dilutions were incubated with 10 μL of the prothrombin activator mix (50 μg/mL Ox venom in saline) and 10 μL of the Ca-phospholipid mixture [30 mmol/L $CaCl_2$ and 25% phospholipid (PTT-Reagent RD)] at 37 °C for 2 min to allow sufficient conversion to thrombin. We measured the clotting time by adding 30 μL of fibrinogen (420 mg/dL in saline). The relative residual thrombin activity was determined on the basis of the standard curve of thrombin derived from purified human prothrombin.

Inhibition of Fibrinogen-clotting Activity by TM

We performed the fibrinogen-clotting assay as described above with or without rTM. First, recombinant prothrombins were converted to thrombins using Ox venom with phospholipid and $CaCl_2$, as described above. Second, we added 10 μL of rTM solution at 3 different final concentrations (0, 50, and 100 μg/mL), and incubated each for 1 min to inhibit thrombin activity. Finally, the clotting time was measured after adding 40 μL of fibrinogen (420 mg/dL in saline). The relative residual thrombin activity was determined on the basis of the standard curve of thrombin derived from purified human prothrombin.

APC Generation Assay

To evaluate APC generation activity of mutant thrombin, we measured APC activity after incubation with human PC in the presence or absence of rTM. We performed a chromogenic assay using S-2366 specific for APC. In this assay, recombinant prothrombins were diluted to 1% of the plasma prothrombin concentration in the dilution buffer [50 mmol/L Tris–HCl (pH 8.1) and 300 mmol/L NaCl], and 500-μL aliquots of the dilutions were incubated with 100 μL of the prothrombin activator (150 μg/mL Ox venom in saline) and 100 μL of the Ca-phospholipid mixture [15 mmol/L $CaCl_2$ and 50% phospholipid (PTT-Reagent RD)] at 37 °C for 2 min to allow sufficient conversion to thrombin. Then, 100 μL of rTM (200 μg/mL in saline) and 10 μL of purified human PC (100 μg/mL in distilled water) were added and incubation was continued for 60 min at 37 °C to generate APC. We added 100 μL of Pefabloc-TH (5 μmol/L in distilled water) to the reaction solution 30 s before measuring APC activity in order to prevent nonspecific cleavage of the S-2366 chromogenic substrate by thrombin. APC activity was measured at 405 nm with TBA-180 by adding 200 μL of S-2366 (0.5 mmol/L in distilled water). We expressed APC generation activity as changes in absorbance/min (ΔA/min). Based on the data of a time-course experiment, the incubation time required for APC generation was selected as 60 min (Fig. 1).

Results

Procoagulant Functional Assays of Recombinant Mutant Prothrombins

We measured procoagulant activities of thrombins derived from recombinant wild-type and mutant prothrombins. Mutant thrombin showed relatively lower activities both in the clotting assay using fibrinogen (37% ± 3.3% of the wild-type) and in the chromogenic assay using S-2238 (57% ± 16% of the wild-type) (n = 3, mean ± SE) (Fig. 2).

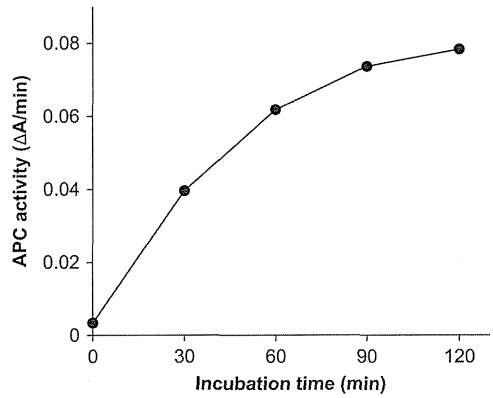

Fig. 1. Time-course of protein C (PC) activation by thrombin in the presence of thrombomodulin (TM). After wild-type prothrombin was sufficiently activated to thrombin using *Oxyuranus scutellatus* (Ox) venom, human PC was added and incubated for 0, 30, 60, 90, and 120 min in the presence of recombinant soluble TM (rTM). After the residual thrombin activity was blocked by Pefabloc-TH, activated PC (APC) activities were measured using S-2366 and expressed as ΔA/min at 405 nm.

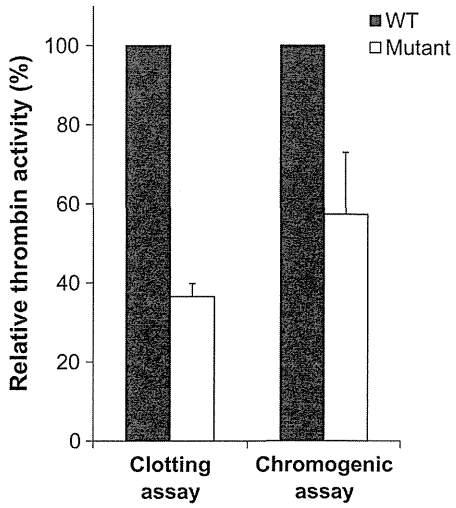

Fig. 2. Relative thrombin activity determined by 2 methods. After recombinant wild-type and mutant prothrombins were sufficiently activated to thrombins using Ox venom, thrombin activities were measured using S-2238 or human fibrinogen without thrombin inhibitor. Experiments were performed in triplicate, and data were presented as the mean ± SE.

Inhibition of Fibrinogen-clotting Activity by TM

To assess procoagulant activities of thrombins derived from recombinant prothrombins after the addition of rTM, we compared fibrinogen-clotting activities of wild-type and mutant thrombins in the absence or presence of rTM. In the presence of 50 μg/mL of rTM, fibrinogen-clotting activity of wild-type thrombin decreased by 27% ± 1.1% of the activity in the absence of rTM, and that in the presence of 100 μg/mL of rTM decreased by 8.4% ± 2.5% (n = 3, mean ± SE) (Fig. 3). On the other hand, fibrinogen-clotting activity of the mutant in the presence of 50 μg/mL of rTM decreased by 52% ± 4.9% of the activity in the absence of rTM, and that in the presence of 100 μg/mL of rTM decreased by 44% ± 7.6% (n = 3, mean ± SE) (Fig. 3). Thus, rTM treatment reduced the relative fibrinogen-clotting activity of wild-type thrombin to 8.4% in a concentration-dependent manner, whereas this treatment decreased the activity of the mutant only to 44%.

APC Generation Assay

In the absence of rTM, APC generation activities (ΔA/min at 405 nm) were 0.0089 ± 0.0024 for the wild-type and 0.0039 ± 0.0003 for the mutant (Fig. 4). However, in the presence of rTM, APC generation activities were enhanced to 0.0907 ± 0.0210 (10.2-fold) for the wild-type and 0.0492 ± 0.0076 (12.6-fold) for the mutant (n = 3, mean ± SE). We confirmed a linear relationship between APC activity and 0–0.2 ΔA/min at 405 nm in the assay using human APC donated from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) (data not shown). The relative APC generation activity of the mutant with rTM was 54% of that of the wild-type.

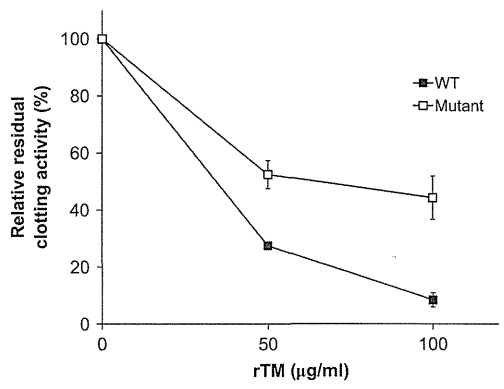

Fig. 3. Effects of rTM on fibrinogen-clotting activity of recombinant thrombins. Wild-type and mutant thrombins were incubated with rTM (0, 50, and 100 μg/mL) for a minute, and relative residual fibrinogen-clotting activities were measured. Experiments were performed in triplicate, and data were presented as the mean ± SE.

Discussion

Thrombin plays a critical role not only in blood coagulation but also in anticoagulation, because TM, an endothelial cell receptor of thrombin, converts thrombin from a procoagulant enzyme to an anticoagulant. Thrombin bound to TM promotes rapid conversion of PC to APC that cleaves and inactivates factors Va and VIIIa together with PS [6,7]. It has been reported that the prothrombin Yukuhashi mutation, which involves substitution of arginine for leucine at position 596 (p.Arg596Leu), can result in thrombophilia associated with AT resistance [3]; however, the effects of this mutation on the TM–PC anticoagulation system were not previously analyzed [4]. Therefore, we evaluated influences of the prothrombin Yukuhashi mutation on the TM–PC system in this study.

We demonstrated that rTM treatment reduced the relative fibrinogen-clotting activity more effectively in wild-type thrombin than in mutant thrombin. rTM (ART-123: recombinant human soluble TM) is composed of the active extracellular domain of TM. Similar to membrane-bound TM, ART-123 binds to thrombin and this complex converts PC into the natural anticoagulant APC [9]. Assuming that there are 100,000 copies of TM per endothelial cell, a reasonable estimate of the TM concentration in the capillaries is in the range of 100–500 nmol/L [6], which corresponds to the range of 6.4–32 μg/mL of rTM (MW: 64,000). Therefore, the rTM concentration of 50 μg/mL in this assay was slightly higher than the human TM concentration in the capillaries. Higher concentrations of rTM are needed to prolong plasma clotting time, but rTM (ART-123) is highly effective to inhibit thrombin generation at a lower dosage [10]. However, it has been suggested that at high concentrations of rTM, the prothrombin Yukuhashi mutation may cause TM resistance in terms of inhibition of fibrinogen-clotting activity of thrombin.

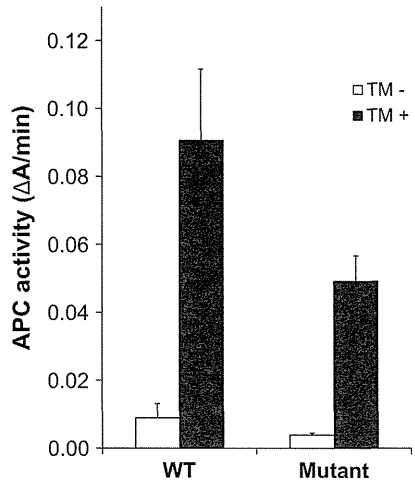

Fig. 4. APC generation assay. Recombinant prothrombins were sufficiently activated to thrombins using Ox venom, human PC was then added, and the combination was incubated for 60 min in the absence or presence of rTM. After the residual thrombin activity was blocked by Pefabloc-TH, APC activities were measured using S-2366 and expressed as ΔA/min at 405 nm. Experiments were performed in triplicate, and data were presented as the mean ± SE.

Inhibition of fibrinogen-clotting activity of thrombin by TM is more rapid than inhibition by AT. In a previous study, excess AT without heparin gradually reduced fibrinogen-clotting activity of thrombin after >30 min [11], whereas excess rTM fully diminished it within a minute in this study (data not shown). The interaction of thrombin with TM is an enzyme–cofactor interaction that should be rapid and reversible so that the cofactor can convert many allosteric enzyme molecules simultaneously [12]. Most of the thrombin formed, at least in microcirculation, is believed to be bound to TM, representing the major anticoagulant mechanism at the intact endothelial surface; AT may irreversibly inhibit thrombin while bound to TM. Thus, it is assumed that TM plays a key role in the regulation of coagulation in damaged blood vessels, although AT is a pivotal molecule that can inhibit excessive clotting over the long term.

Thrombin is an allosteric enzyme controlled by sodium binding [13, 14]. Sodium-bound thrombin (known as the fast form) is optimized for procoagulant function because of increasing substrate specificity for fibrinogen, whereas sodium-free thrombin (known as the slow form) is an anticoagulant because of increasing specificity for cleaving PC [15]. The mutation in prothrombin Yukuhashi occurred at residue Arg596 (Arg221a in the chymotrypsinogen numbering system [16]) within the sodium-binding region of thrombin and can be expected to have an influence on the binding of sodium, resulting in a change in its protease activity and specificity. In this study, we demonstrated that rTM substantially enhanced APC generation activity of mutant thrombin, although the activity was still approximately half of that of the wild-type. In chromogenic assays, procoagulant activity of mutant thrombin was 57% of that of the wild-type and APC generation activity of the mutant in the presence of rTM was 54% of that of the wild-type. This suggests that APC generation activity can be associated with the protease activity of thrombin. It is appears that rTM can form a complex with both wild-type and mutant thrombins and enhance PC activation in equal proportion, suggesting that the prothrombin Yukuhashi mutation may not be TM-resistant in terms of APC generation enhancement.

Fukudome et al. demonstrated the endothelial cell–specific expression of endothelial cell protein C–APC receptor (EPCR) *in vivo*, particularly in the aorta and large arteries but not in the capillaries [17]. EPCR was found to greatly accelerate PC activation mediated by the thrombin–TM complex. Although the high-affinity binding of PC to EPCR on the endothelial cells was a critical step for activation, TM was an essential component for activation even in the presence of EPCR. In this study, we evaluated the effect of the prothrombin Yukuhashi mutation on the activation of PC in the presence of soluble rTM, but not in the presence of both TM and EPCR. Therefore, further experiments may be needed to evaluate the mutant effect in the presence of both TM and EPCR on the endothelial cell surface taking into account *in vivo*-situation.

In conclusion, we evaluated the effects of the prothrombin Yukuhashi (Arg596Leu) mutation on the TM–PC system and demonstrated that the mutation may attenuate immediate anticoagulant activity of TM. This possibly contributes to the susceptibility to thrombosis, although its enhancing effect on APC generation can be maintained.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authorship

Y.T. performed the experiments, analyzed the data, and drafted the manuscript. I.K., Y.A., Y.N., M.M., A.T., and T.M. interpreted the data and contributed to analytical methodology. T.K. designed the project, analyzed the data, and wrote the manuscript. All authors were involved in the critical review of the manuscript prior to submission.

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トピックス臨床シリーズ

新たな血栓性素因：アンチトロンビンレジスタンス

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要　旨

静脈血栓塞栓症（VTE）は様々な先天的/後天的リスクにより発症する多因性疾患である．VTEは従来から欧米人に比べ日本人には少ないとされてきたが，診断技術の向上や食生活の欧米化などにより日本人にも決して少なくないことが判明してきている．日本人での血栓症リスクとなる先天性血栓性素因には，欧米人と同じくアンチトロンビン（AT），プロテインC（PC），プロテインS（PS）など生理的凝固抑制因子の遺伝子異常による欠乏症/異常症があり，特にPS Tokushima変異（p.K196E）は日本人特有の血栓性素因として知られている．一方，いまだに原因不明な遺伝性血栓症もあり，我々は長らく原因が不明であった静脈血栓症が多発する家系において，通常は出血症状が問題となるプロトロンビン遺伝子異常で逆に遺伝性血栓症の原因となる変異を発見し，新たな血栓性素因・アンチトロンビンレジスタンス（ATR）として報告した．本稿では，この血栓性素因・ATRについて最近の知見も踏まえて概説する．

はじめに

静脈血栓塞栓症（venous thromboembolism：VTE）は，深部静脈血栓症（Deep vein thrombosis：DVT）と肺血栓塞栓症（Pulmonary embolism：PE）の2つの概念を合わせた疾患群で，その発症要因には遺伝的リスクと環境的リスクが知られており，これらが複数重なることで発症する多因性疾患である[1]．VTEは，欧米人に多く見られ日本人には少ない疾患とされてきたが，食生活の欧米化や診断技術の向上により日本人における患者数も決して少なくはないことが明らかになってきた．VTE発症の誘因となる環境的リスクとしては，加齢，妊娠，長期臥床，ロングフライト（エコノミークラス症候群）などが挙げられる．また，その遺伝的リスクとして，生理的血液凝固抑制因子であるアンチトロンビン（antithrombin：AT），プロテインC（protein C：PC），プロテインS（protein S：PS）の欠乏症/異常症が広く知られており，日本人においてもそれぞれ数多くの遺伝子異常が報告されている．特に，PS Tokushima変異（p.K196E）は日本人特有の血栓性素因として知られ，55人に1人の頻度でヘテロが存在している[2]．一方，いまだに原因が不明な遺伝性血栓症もあり，我々は長らく原因が不明であった静脈血栓症が多発する家系において，通常は出血傾向となるプロトロンビン遺伝子異常で，逆に遺伝性血栓症の原因となることを発見し，新しい血栓性素因・アン

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チトロンビンレジスタンス（AT resistance：ATR）として報告した[3)4)]．本稿では，この新しい先天性血栓性素因・ATRとそのスクリーニング検査法の開発について，最近の知見も踏まえて概説する．

既存の先天性血栓性素因

血管損傷時には，血液が血管外に漏れ出ないよう損傷部局所ですみやかな血栓形成による止血機構が働く．逆に，健常血管内では誤って血栓が生じないように，また血管損傷部での止血血栓形成部位においては過剰に血液凝固反応が進行しないように，ともに生理的凝固抑制機構が働いている．生体防御反応の一つでもあるこれらの止血血栓形成は，血管内皮，血小板，凝固線溶因子ならびにそれらの抑制因子などの巧妙な連携制御のもとに営まれており，この制御機構の異常によりそのバランスが崩れると病的な出血症状や血栓症が発生する．これらのうち病的血栓症の原因となる遺伝的リスク要因としての先天性血栓性素因には，凝固反応の抑制制御に問題が生ずる「凝固抑制因子の遺伝子異常」と過剰な凝固反応をもたらす「凝固因子の遺伝子異常」が知られている．

1．凝固抑制因子遺伝子異常による先天性血栓性素因

生理的な凝固抑制因子であるAT，PC，PSの遺伝子異常には，それぞれの欠乏症/異常症に伴う遺伝性血栓性素因となる変異が報告されており[5)~7)]，日本人の静脈血栓症患者からも数多くの遺伝子異常が同定されている[8)]．特に，PS Tokushima (p.K196E) 変異は日本人特有の先天性血栓性素因として知られ，日本人の55人に1人がヘテロ接合体としてp.K196E変異を保有するとされる[2)]．

2．凝固因子の遺伝子異常による先天性血栓性素因

欧米人に多い遺伝的リスクとして，活性化PCレジスタンス（APC resistance）を示すFactor V Leiden (R506Q) 変異[9)]や，プロトロンビン遺伝子の3'非翻訳領域の変異により血漿中プロトロンビン濃度が上昇するプロトロンビンG20210A変異[10)]が有名である．しかし，これらは日本人を含めてアジア人には報告がなく，欧米人に血栓症の頻度が多い要因の一つとされている．また，ごく希な報告としては，凝固第IX因子（FIX）遺伝子異常が先天性出血性素因（血友病B）ではなく，逆に活性が異常高値（700%）を示すため遺伝性血栓症の原因となるFIX Padua遺伝子変異がある[11)]．

新たな先天性血栓性素因・アンチトロンビンレジスタンス（ATR）

遺伝性血栓症の中にはいまだに原因不明なものがある．我々は長らく原因が不明で静脈血栓症が多発する家系において，通常は出血傾向となるプロトロンビンの異常で逆に静脈血栓症の原因となる遺伝子異常を発見し，新しい血栓性素因・ATRとして報告した[3)4)]．これは，FV Leidenが同じく凝固因子のミスセンス変異でありながら，凝固活性は保たれ，かつ活性化PC（APC）による不活化に抵抗性を示すため，結果として血栓傾向となる病態に似ている[9)]．

1．プロトロンビンYukuhashi変異の発見

発端者の日本人女性は11歳時に深部静脈血栓症を発症していた．この家系では3世代に渡って8名の静脈血栓塞栓症患者（3名は既に死亡）がみられ，静脈血栓症は代を経るにつれ若年性に発症する傾向が見られた．2001年に既知の先天性血栓性素因について調査がされていたが，本家系には異常は検出されなかった[12)]．こうした中，2009年にBostonで開催された国際血栓止血学会（ISTH）において，ある遺伝性血栓症家系のゲノムワイド連鎖解析から，その病態は不明なもののプロトロンビン遺伝子異常の存在が報告された[13)]．この報告を受け我々も本家系発端者の解析を行ったところ，プロトロンビン遺伝子にミスセンス変異・プロトロンビンYukuhashi変異（c.1787G>A，p.R596L）を同定した．この変異は，トロンビン（プロトロンビンの活性型）がATと複合体（TAT）形成して不活化される際でのAT分子との結合部に位置するものであった（図1）．

2．本家系での血栓症発症機序解析

2009年ISTH Bostonにて報告されたプロトロンビン遺伝子異常については変異の存在の報告のみで，血栓症発症に至る病態解析の報告は未だな

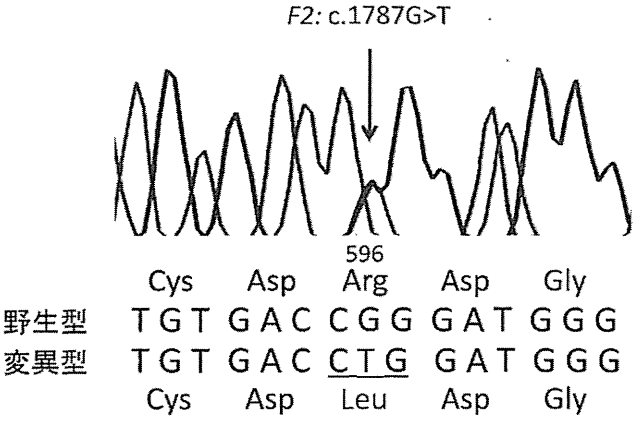


図1．アンチトロンビンレジスタンス（ATR）を示したプロトロンビン遺伝子変異
（Miyawaki, et al.：N Engl J Med, 2012から改変）
発端者のプロトロンビン遺伝子（*F2*）に，活性化後のトロンビンとATとの結合部に位置するアルギニン（596R）がロイシン（596L）に置換するミスセンス変異（c.1787G>A，p.R596L）を同定した．

されていない[13]．そこで我々は日本人家系におけるこのプロトロンビンYukuhashi変異での血栓症発症機序についての詳細な解析を行った．変異の見られたプロトロンビンのアルギニン（R596）は，活性化されたトロンビンがATと複合体（TAT）を形成して不活化される際に，ATのアスパラギン（N265）と水素結合を形成することが報告されている[14]．したがって，R596L置換はプロトロンビン活性化後の異常トロンビンの不活化不全を起こすことが予想され，また，このR596L置換変異は本家系の健常配偶者にはみられず，患者と家系内での他の血栓症患者にも検出されたことから，本家系での遺伝性血栓症の原因であることが強く疑われた．異常プロトロンビンの機能解析において，すでに血栓症治療のためにワルファリンを投与された患者血漿検体では解析困難であったため，我々は遺伝子工学的技法を用いて野生型/変異型プロトロンビンをリコンビナント蛋白としてHEK29細胞内で合成させ，その無血清培地での培養上清から濃縮し，そのトロンビンへの活性化動態，活性化後のATによる不活化動態を比較検討した．

3．トロンビンへの活性化動態

リン脂質とCaイオン存在下において，ウシ由来FXa/Fvaによるプロトロンビンからトロンビンへの転換を経時的にウェスタンブロット解析で観察したところ，変異型プロトロンビンは野生型とほぼ同様な開裂パターンを示したことから，それぞれトロンビンへの活性化はほぼ同等におこることが判明した（図2）．

次に，プロトロンビン欠乏血漿にそれぞれ野生型あるいは変異型リコンビナントプロトロンビンを添加して疑似血漿を作製し，3つの方法でプロトロンビン活性を解析した．すなわち，プロトロンビンからトロンビンへの活性化とフィブリノゲンを基質とする凝固活性を反映する凝固一段法，十分に活性化したトロンビンのフィブリノゲンに対する凝固活性のみを反映する凝固二段法，さらに，トロンビンに特異的な発色合成基質S-2238に対する活性を反映する合成基質法の，3種類の方法を用いて野生型/変異型プロトロンビンの活性を測定した（表1）．その結果，野生型疑似血漿はいずれの測定法でも正常血漿と同当な数値を示したが，変異型疑似血漿では3つ全ての方法で野生型を下回り，凝固一段法で最も低く（15%），次いで凝固二段法（32%），蛍光基質法（66%）の順で数値が大きくなった．すなわち，変異型プロトロンビンはトロンビンへの変換がやや遅延し，フィブリノゲンを基質とした活性も低下すること，フィブリノゲンと比較して分子量が小さい発色合成基質S-2238を用いて測定した活性はあまり低下しないことが観察された．ウェスタンブロット解析結果がほぼ同等の開裂パターンを示した結果と凝固一段法，二段法での明らかな差異とは一見矛盾しているようにみえるが，ウェスタンブロット法でのトロンビンへの開裂に要した最小時間は20秒と長く，数秒単位の差を反映する凝固法による際の検出限界には及ばなかったものと思われた．

4．変異トロンビンの不活化動態

変異型プロトロンビンをトロンビンに活性化した後，ATとの結合能（トロンビン・AT（TAT）複合体形成能）について野生型のそれと比較検討したところ，ヘパリン非存在下で変異型トロンビンはATにほとんど結合しないことが判明し，ほとんどATにより不活化されないことが予想さ

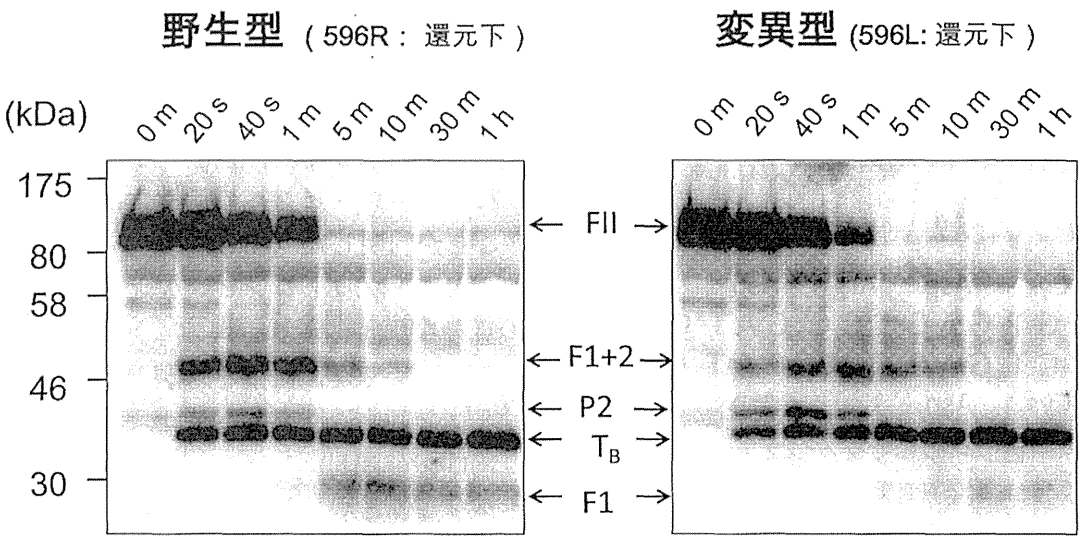


図2. リコンビナントプロトロンビンのFXa/FVaによる活性時開裂パターン
（Miyawaki, et al. : N Engl J Med, 2012から改変）
野生型（596R：左），変異型（596L：右）リコンビナントプロトロンビンのウシ由来FXa/FVaによるトロンビンへの活性化開列パターンをウェスタンブロッティング解析した．
FII：prothrombin, F1, 2：fragment 1+2, P2：prethrombin-2, T_B：B chain of thrombin, F1：fragment-1

表1. プロトロンビン活性測定

	抗原量	活性値		
		凝固一段法	凝固二段法	合成基質法
正常型（596R）	112%	91%	109%	88%
変異型（596L）	118%	15%	32%	66%

プロトロンビン欠乏血漿にそれぞれ野生型／変異型リコンビナントプロトロンビンを添加して疑似血漿とし，凝固一段法，凝固二段法，合成基質法の3種の方法を用いて野生型（596R）／変異型（596L）プロトロンビンの活性を測定した．
（Miyawaki, et al. : N Engl J Med, 2012から改変）

れた（図3）．ヘパリン存在下では変異型でも野生型に似た継時的なTAT複合体上昇を示したが，1分以内に形成されたTAT複合体は野生型の約半分程度にとどまっていた．これらの結果から，変異型トロンビンではATによるトロンビン不活化反応が強く障害されていることが予想された．

一方，プロトロンビン欠乏血漿に変異型/野生型プロトロンビンを50%ずつ添加した疑似患者血漿におけるトロンビン生成試験（Thrombin generation assay：TGA）では，野生型プロトロンビンを加えた疑似正常血漿や正常プール血漿と比較して，最高トロンビン活性がやや低いものの不活化が著しく遅延しており，結果として総トロンビン活性量に相当するETP（Endogenous thrombin potential：活性値と持続時間の積分値）の著しい増大を認めた（図4）．すなわち，患者血漿中の異常プロトロンビンは，血液凝固活性は低いものの一旦活性化されるとATRを示して凝固活性（フィブリン生成能）を保ち続けることが予想され，これが本家系の遺伝性血栓症の原因になるこ

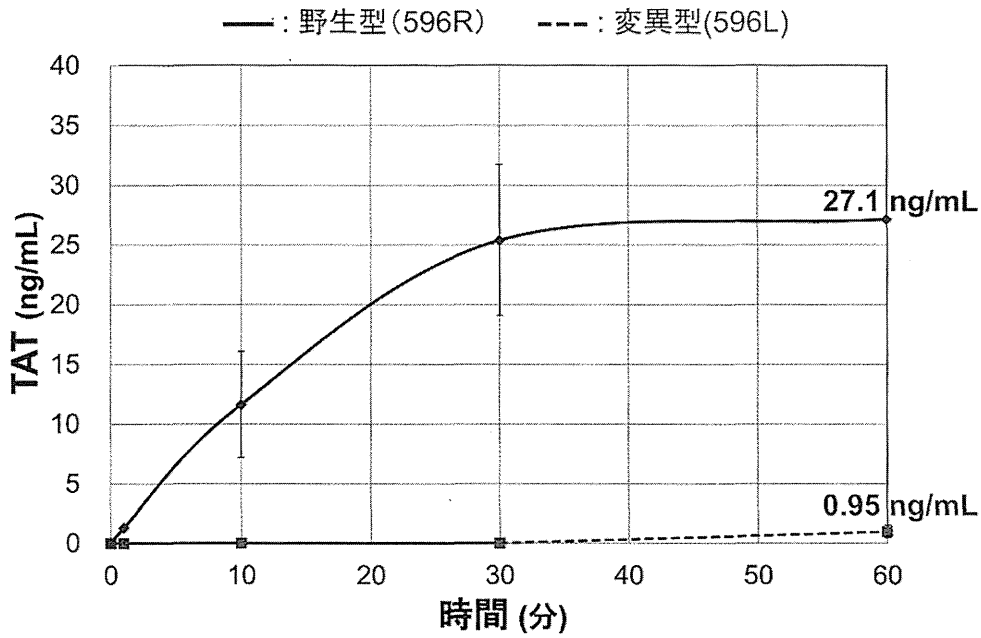
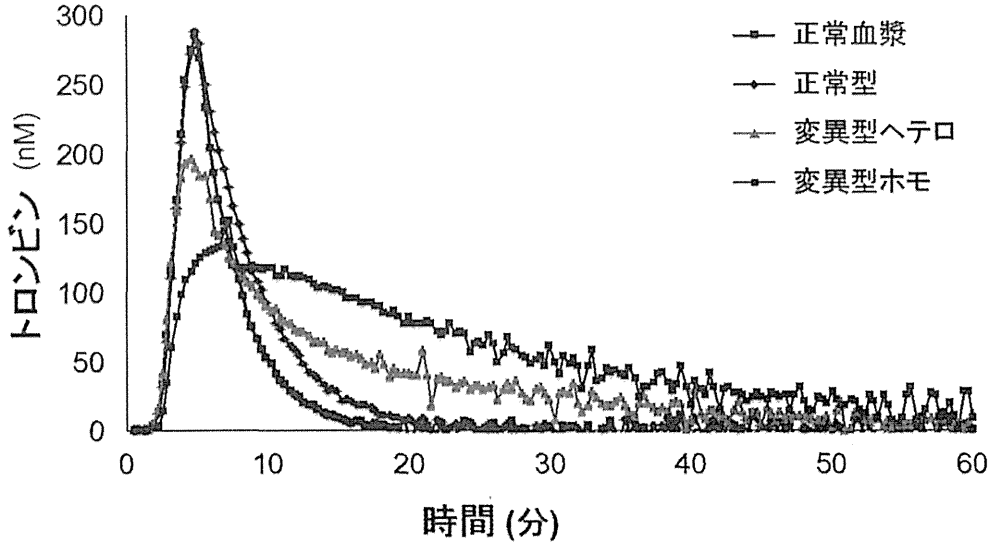


図3.　トロンビン・アンチトロンビン（TAT）複合体形成
（Miyawaki, et al.：N Engl J Med, 2012から改変）
リコンビナントプロトロンビン（正常型/596Rおよび変異型/596L）をトロンビンに変換し，それぞれアンチトロンビンとの複合体（TAT）をELISA法にて測定．正常型でのTAT形成は経時的増加が見られたが，変異型では30分まで検出限界未満，60分後でもごくわずかにしか見られなかった．

とが示唆された．

5. ATRスクリーニング臨床検査法の開発

我々は，ヘパリン存在下，非存在下でのATによるトロンビンの不活化動態を合成基質法で解析観察することでATRを検出する臨床検査法を開発し，報告した[15]．これは，血漿検体を用いてプロトロンビナーゼ様活性をもつTypan蛇毒（*Oxyuranus scutellatus* (Ox)）によるプロトロンビンを十分に活性化し，ATによるトロンビン不活化動態を各反応時間での残存トロンビン活性を測定して観察するものである．本法の開発にあたっては，反応各相での試薬，反応溶液組成，反応時間等の至適条件を検討し，ATRを検出する臨床検査法を考案した．本検査法において，プロトロンビン欠乏血漿に野生型ATを添加し作製した野生型再構成血漿検体で，ヘパリン非存在下で血中濃度5倍量のAT添加から30分で約10%にまでトロンビン活性が阻害されるのに対し，同様に作製した変異型ホモ再構成血漿検体では30分後でも90%以上残存していた（図4A）．また，静脈血栓症患者検体を想定し，ワルファリン服用検体を用いてワルファリンが本測定法に及ぼす影響についても検討した結果，考案した検査法は相対的なATによるトロンビン活性の不活化動態を観察することでワルファリン服用時にも解析可能であった．すなわち，ヘパリン非存在下でR596L変異非保有者検体は30分後にはいずれも20%程度までトロンビン活性が阻害されるのに対し，2名のR596L変異保有患者血漿ではともに30分後に約50%のトロンビン活性の残存が観察されたことから，ATR検体であると判定できる（図4B）．本検査法を用いて，原因不明であった静脈血栓塞栓症症例を解析することにより，静脈血栓塞栓症における新規血栓性素因・ATRの関与の実態が明らかとなることが期待される．ごく最近，我々はN Engl J Med誌に報告した家系とは異なる日本人静脈血栓症家系において，上述の血漿検体スクリーニング法での解析により新たなATR日本人症例を同定している[16]．



	正常血漿	正常型	変異型ヘテロ	変異型ホモ
総トロンビン活性量 (nM.min)	1276	1658	2374	3620
最高トロンビン活性 (nM)	284	283	194	144
トロンビン活性消失時間 (min)	23.5	26.5	78.0	105.0

図 4. トロンビン生成試験 (TGA)
(Miyawaki, et al. : N Engl J Med, 2012 から改変)
プロトロンビン欠乏血漿に各リコンビナントプロトロンビンを加えた疑似患者血漿 (変異型ヘテロ) では, 疑似正常血漿 (正常型) に比べ最高トロンビン活性がやや低いが消失時間が著しく長く, 結果として総トロンビン活性量 (ETP : Endogenous thrombin poteitial) の著しい増大を認めた.

6. ATR を示す新たな変異症例

2013 年 Djordjevic らは, 遺伝性血栓症をもつセルビア人 2 家系でのプロトロンビン遺伝子変異 (c.1787G>A, p.Arg596Gln) が報告したが, 我々が開発した検出法によりいずれも ATR を示すことが明らかとなった[17]. また, 同年にインド人血栓症患者においても同じ変異が同定され報告されている[18]. ごく最近, 上述のごとく我々は日本人 2 家系目の ATR 家系を血漿検体スクリーニング法により同定したが, 本家系の遺伝子解析の結果セルビア人変異と同じ c.1787G>A 変異を検出し[16], さらに別家系の日本人血栓症症例でも ATR を検出し, やはり c.1787G>A 変異を同定している (日本人 3 家系目). したがって, 血栓性素因・ATR は日本人だけでなく欧米人をはじめ他の人種にも遺伝性血栓症の原因として存在していることが明らかとなった. 血漿検体スクリーニング法の普及は, 厳密な倫理的配慮が必要な遺伝子解析に比べて利便性が高く, さらなる ATR 症例の検出にきわめて有用であると考えられる.

おわりに

遺伝性血栓症の原因として, 現在までに様々な凝固関連因子の遺伝子異常が同定されているが, いまだに原因不明な遺伝性血栓症も多くある. 我々は, 通常では出血傾向が予想される凝固因子・プロトロンビンの遺伝子変異が, 逆に静脈血栓症の原因となる詳細な分子病態を解明し, 新規血栓性素因・ATR を世界で初めて報告した. この新しい血栓性素因の発見は, 日本人だけでなく欧米人をはじめ他の人種での遺伝性血栓症においてもその病態解明による血栓症発症予防につながる

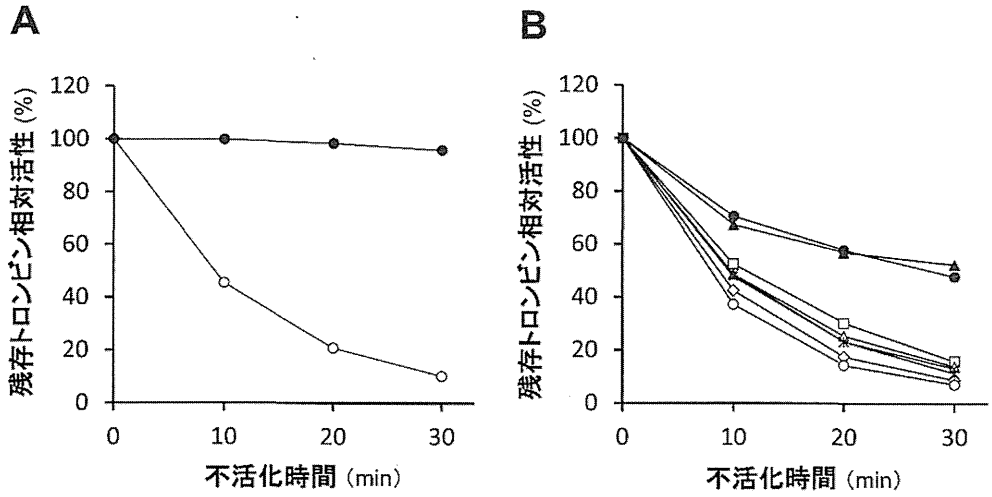


図5.　ATRスクリーニング検査法による血漿検体評価
（Murata, et al.：Thromb Res, 2014から改変）
A：ヘパリン非存在下において血中濃度5倍量ATの混和後30分でのトロンビン活性は，野生型（596R）再構成血漿では10%程度にまで阻害されるのに対して，変異型（596L）ホモ再構成血漿は90%以上の残存がみられた．
B：ヘパリン非存在下でのトロンビン活性は，プロトロンビン遺伝子に異常のないワルファリン服用患者血漿（□，△，◇，＊）では健常人血漿（○）と同様の不活化動態を示し，プロトロンビンR596L変異をヘテロにもつ2名の患者のワルファリン服用時血漿（●，▲）では明らかにATによる阻害を受けにくい結果を示した．

ことが期待でき，今後更なるATR病態についての研究成果の蓄積が望まれる．

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Abstract

A new thrombophilia: Antithrombin resistance

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Venous thromboembolism (VTE) is a multifactorial disease that develops due to a variety of congenital and/or acquired factors. Previously, it was thought that the incidence of VTE is lower in Japanese than in Caucasians, but it has now been recognized that it occurs quite frequently in Japanese based on improved diagnosis technology and the westernization of eating habits. Congenital thrombophilia due to a genetic deficiency of natural anticoagulant factors such as antithrombin (AT), protein C (PC) and protein S (PS), is known to exist in Japanese, as in Caucasians, and many of those gene defects have been identified. In particular, PS Tokushima mutation (p.K196E) is known as a thrombophilia peculiar to Japanese. On the other hand, there is still idiopathic hereditary thrombosis. In a Japanese family in whom venous thrombosis occurred frequently and the cause was unknown for a long time, we found that hereditary thrombosis was caused by a mutation in the prothrombin gene that is usually reversely involved in bleeding symptoms. We thus reported a case of antithrombin resistance (ATR) as a new thrombophilia. In this review, I will discuss an outline of this thrombophilia, ATR, based on recent findings.

Key words: Thrombophilia, Antithrombin resistance, Prothrombin, Gene mutation

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ORIGINAL ARTICLE

Candidate gene analysis using genomic quantitative PCR: identification of *ADAMTS13* large deletions in two patients with Upshaw-Schulman syndrome

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Keywords
ADAMTS13, genetic analysis, hereditary disease, mutation, quantitative PCR, thrombotic thrombocytopenic purpura, Upshaw-Schulman syndrome

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Abstract

Direct sequencing is a popular method to discover mutations in candidate genes responsible for hereditary diseases. A certain type of mutation, however, can be missed by the method. Here, we report a comprehensive genomic quantitative polymerase chain reaction (qPCR) to complement the weakness of direct sequencing. Upshaw-Schulman syndrome (USS) is a recessively inherited disease associated with severe deficiency of plasma ADAMTS13 activity. We previously analyzed *ADAMTS13* in 47 USS patients using direct sequencing, and 44 of them had either homozygous or compound heterozygous mutations. Then, we sought to reveal more extensive defects of *ADAMTS13* in the remaining three patients. We quantified copy numbers of each *ADAMTS13* exon in the patients by using genomic qPCR. Each primer pair was designed to contain at least one of the two primers used in direct sequencing, to avoid missing any exonic deletions. The qPCR demonstrated heterozygous loss of exons 7 and 8 in one patient and exon 27 in the other, and further analysis revealed c.746_987+373del1782 and c.3751_3892+587del729, respectively. Genomic qPCR provides an effective method for identifying extensive defects of the target genes.

Target exon resequencing using direct sequencing is a popular method to discover causative mutations in the candidate genes responsible for hereditary diseases. Homozygous or compound heterozygous mutations are often identified in the corresponding genes of the patients with autosomal-recessive diseases. In some cases, however, only one or no causative mutation is identified in the responsible gene: (an)other mutation(s) may be

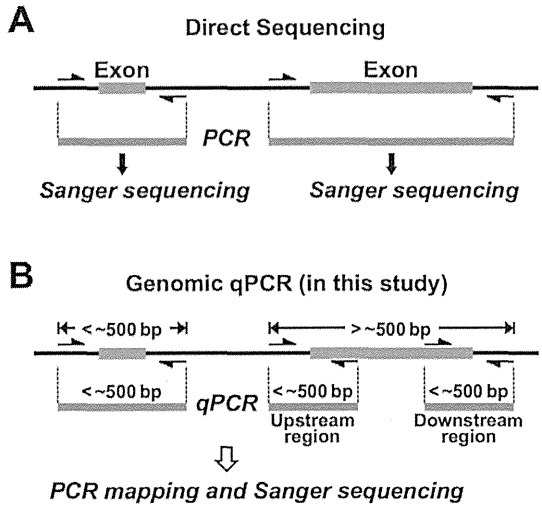


Figure 1. Principles of direct sequencing and genomic qPCR for genetic analysis. (A) In direct sequencing, target regions are amplified by PCR using primer pairs (arrows) usually designed from the intronic sequences flanking each exon, and the PCR products are directly sequenced by the Sanger method. (B) In genomic qPCR, copy numbers of target regions are quantified by real-time PCR. Each primer pair contains at least one of the two primers used in direct sequencing: common primer pairs are used for the regions smaller than ~500 bp, and, for accurate qPCR, one common and one specific primer are used for the regions larger than ~500 bp. If abnormal copy numbers are detected, PCR mapping and sequencing are performed to determine the precise sites of defects.

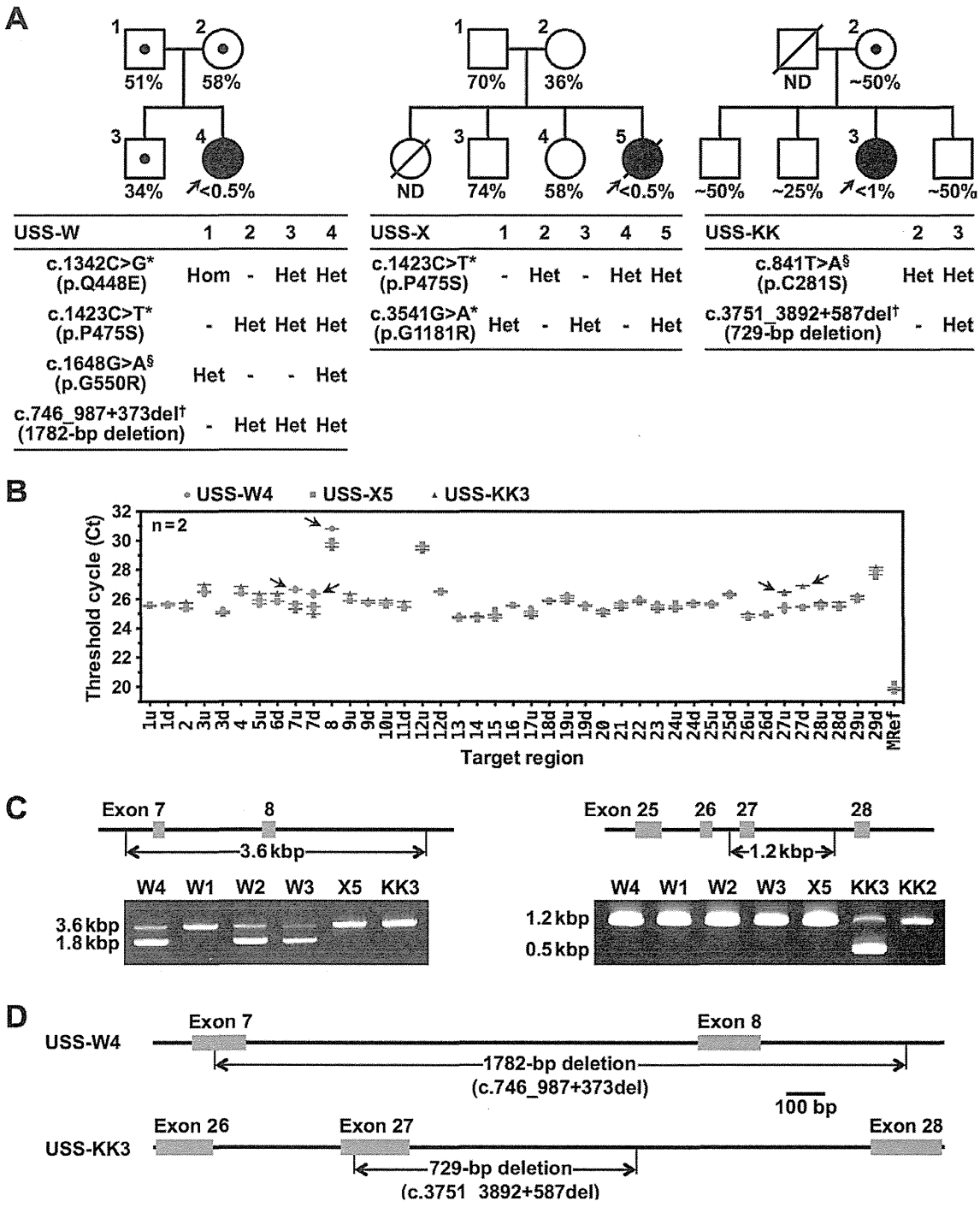
missed by the method. Although next-generation sequencing may be useful in such cases, it needs special equipments and is still expensive. In this study, we report a comprehensive genomic quantitative PCR (qPCR), which will be a powerful tool in combination with direct sequencing.

Upshaw-Schulman syndrome (USS), also called hereditary thrombotic thrombocytopenic purpura (TTP), is an autosomal-recessive trait associated with severely deficient plasma ADAMTS13 activity. Homozygous or compound heterozygous mutations in the *ADAMTS13* gene (OMIM 604134) are identified in most patients with USS (Levy et al. 2001; Kokame et al. 2002; Kokame and Miyata 2004; Matsumoto et al. 2004; Lotta et al. 2010; Fujimura et al. 2011; Hing et al. 2013). So far, more than 130 causative mutations have been identified by direct sequencing. Using that method, we previously analyzed *ADAMTS13* in 47 Japanese USS patients from 41 unrelated families (Fujimura et al. 2011). Of those, 44 patients from 38 families had either homozygous or compound heterozygous mutations in *ADAMTS13*. In the remaining three patients, however, only single missense mutations (two patients) or no mutation (one patient) was detected. In this study, we sought to reveal more extensive defects of *ADAMTS13* in these three patients by using genomic qPCR.

In general, PCR primer pairs for direct sequencing are designed to hybridize within the intronic sequences flanking each exon (Fig. 1A). Mutations such as substitutions, insertions, and deletions occurring in exons and exon–intron boundaries are identified by Sanger sequencing following genomic PCR, regardless of their heterozygosity or homozygosity (Fig. S1A). Direct sequencing, however, misses heterozygous mutations on the allele that contains no or mismatched primer target sequences: not only whole or partial deletion but also point mutations including single-nucleotide polymorphisms of primer target sequences can hamper PCR-amplification of the mutant allele, which may contain other critical mutations in the exon or exon–intron boundary (Fig. S1B). In these cases, only the target region of the other (normal) allele is PCR-amplified and sequenced, and the results are interpreted as if the regions of both alleles are normal.

Copy number analysis may overcome the limitations of direct sequencing. Multiplex ligation-dependent probe amplification (MLPA) analysis (Schouten et al. 2002) is often used for this purpose. Although MLPA is suitable for detection of genetic defects including exon deletions and duplications, it may still miss mutations that occur outside the probe target sequences. Therefore, to comple-

Figure 2. Genetic analysis of three USS families. (A) Pedigrees and genotypes of the USS patient families. Circles with arrows indicate the probands, USS-W4, -X5, and -KK3. Clinical data of the patients and the basis of diagnosis were described previously (Fujimura et al. 2011); the description of USS-KK3 being the second of three children needs to be corrected. Plasma ADAMTS13 activities were measured by us (USS-W and -X) or by Dr. Miha Furlan at University of Bern in 1999 (USS-KK), and are shown as a percentage of the normal control. ND, not determined. No subjects had ADAMTS13 inhibitors. Squares and circles with numbers indicate the subjects for genetic analysis. Each mutation was assigned a name for cDNA according to the nomenclature recommendations of the HGVS (http://www.hgvs.org/mutnomen/) based on the reference sequences AB069698.2 (cDNA) and NC_000009.11 (genomic). *·§Missense substitutions identified by direct sequencing. †Deletions identified by genomic qPCR in this study. *Pathologically unrelated missense polymorphisms. (B) Identification of exon deletions in *ADAMTS13*. Ct values of genomic qPCR are plotted by dots with lines at the mean (n = 2) for each target region. The letters u and d following the exon numbers indicate *u*pstream and *d*ownstream region of each exon, respectively. Red circles, USS-W4; green squares, USS-X5; blue triangles, USS-KK3. Arrows indicate the dots with Ct values higher than those of the other two patients. (C) *Left:* PCR-amplification of the 3.6-kbp band from the normal *ADAMTS13* allele produced a 1.8-kbp band from USS-W4, her mother (W2) and her brother (W3), but not from her father (W1). *Right:* PCR-amplification of the 1.2-kbp band from the normal *ADAMTS13* allele produced a 0.5-kbp band from USS-KK3, but not from her mother (KK2). (D) Sequencing of the 1.8- and 0.5-kbp bands in (C) indicated a 1782-bp deletion in USS-W4 and a 729-bp deletion in USS-KK3, respectively.



ment direct sequencing, we selected genomic qPCR (Aldape et al. 2002; Kuramitsu et al. 2012), using primer pairs containing at least one of the two primers used in direct sequencing (Fig. 1B). Combining direct sequencing and genomic qPCR should reveal any defects occurring within or between primer target sequences.

The study protocol was approved by the ethical committee of the National Cerebral and Cardiovascular Center; only subjects who provided written informed consent for genetic analyses were included. This study involved three USS families, USS-W, -X, and -KK (Fig. 2A). Clinical data of the patients (USS-W4, -X5, and -KK3) and the basis of diagnosis were described previously (Fujimura et al. 2011). Plasma ADAMTS13 activities for patients and family members are shown in Figure 2A. No subjects had ADAMTS13 inhibitors. The results of direct sequenc-

ing are also shown in Figure 2A. USS-W4 was a heterozygote with paternal c.1648G>A (p.G550R), USS-X5 had no causative mutations, and USS-KK3 was a heterozygote with maternal c.841T>A (p.C281S). Pathologically unrelated missense polymorphisms (p.Q448E, p.P475S, p.G1181R) (Kokame et al. 2011) were also identified in them (Fig. 2A).

Genomic DNA was prepared from blood and subjected to real-time PCR to quantify the copy numbers of each *ADAMTS13* exon. Each primer pair was designed, using Primer-BLAST (NCBI), to contain at least one of two primers used in direct sequencing (Table S1). A primer pair for the qBiomarker Multicopy Reference Copy Number Assay (MRef, Qiagen, Valencia, CA), which recognizes a stable sequence that appears >60 times throughout the human genome, was used to precisely normalize sample DNA input (~4 ng/reaction). PCR was performed using the QuantiFast SYBR Green PCR Kit (Qiagen) for all regions except exon 7 and the KOD SYBR qPCR Mix (Toyobo, Osaka, Japan) for exon 7. Dimethyl sulfoxide was added (final concentration, 5%) for amplification of exon 8. Fluorescence intensities were detected using the Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA), and each threshold cycle (Ct) value was calculated using the MxPro software (Agilent Technologies).

In genomic qPCR, the difference in Ct among subject DNAs is important information. An increase in Ct value of 1.0 indicates a heterozygous deletion of the target region, whereas a decrease of 0.58 indicates a heterozygous duplication. Ct values of the *ADAMTS13* qPCR indicated that exons 7 and 8 were heterozygously absent in USS-W4 and that exon 27 was heterozygously absent in USS-KK3 (Fig. 2B). By contrast, genomic qPCR revealed no abnormalities in USS-X5.

To confirm the deletions and narrow the deleted regions, we performed PCR using primer pairs specific to regions surrounding the deleted exons. Primers 5′-CACCTCCCCACAGACTCCTA-3′ (intron 6) and 5′-AGGCGGGCAAATCATGAGG-3′ (intron 8) amplified a 3.6-kbp band from the normal allele and a 1.8-kbp band from the mutant allele of USS-W4 (Fig. 2C, *left*). Thus, ~1.8 kbp was deleted within the region straddling exons 7 and 8 in USS-W4. The precise sites where the deletions occurred were determined by sequencing the lower PCR band, which revealed that loss of exons 7 and 8 was caused by a 1782-bp deletion ranging from the 60th nucleotide of exon 7 to the 373rd nucleotide of intron 8 (c.746_987+373del1782) (Figs. 2D, S2A). We confirmed the compound heterozygosity of p.G550R and c.746_987+373del1782 in USS-W4 by genomic PCR of the family members. The patient's mother and brother, but not father, had c.746_987+373del1782 (Fig. 2C, *left*). Direct sequencing indicated that the patient's father, but not mother and brother, had p.G550R (Fig. 2A, *left*).

On the other hand, primers 5′-AGTCACATAGCCAGCAGTGG-3′ (intron 26) and 5′-GCACTGAGCAGAGTGGTCTT-3′ (intron 27) amplified a 1.2-kbp band from the normal allele and a 0.5-kbp band from the mutant allele of USS-KK3 (Fig. 2C, *right*). Thus, ~0.7 kbp was deleted within the region straddling exon 27 in USS-KK3. Sequencing the lower band revealed that loss of exon 27 was caused by a 729-bp deletion ranging from the 36th nucleotide of exon 27 to the 587th nucleotide of intron 27 (c.3751_3892+587del729) (Figs. 2D, S2B). Although the patient's father could not be genetically analyzed, her mother had p.C281S (Fig. 2A, *right*), but not c.3751_3892+587del729 (Fig. 2C, *right*). Thus, it was likely that USS-KK3 was a compound heterozygote of p.C281S and c.3751_3892+587del729.

In conclusion, this study identified two USS patients carrying *ADAMTS13* alleles bearing exon deletions. Extensive defects of *ADAMTS13* may be more common than we expect, and genomic qPCR analysis will be effective for identifying such defects in USS patients. Of the three patients we examined, one did not exhibit abnormalities detectable by either direct sequencing or genomic qPCR. Because these combined analytical methods cannot detect large-scale events such as inversions and translocations that do not affect sequences or copy numbers of target regions, the patient may carry such a defect in *ADAMTS13*. Alternatively, plasma ADAMTS13 deficiency in the patient may be brought about by defects other than *ADAMTS13*, for example, genes involved in synthesis, folding, or secretion of ADAMTS13. Finally, we propose well-designed comprehensive genomic qPCR to complement the weakness of direct sequencing of candidate genes.

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Conflict of Interest

M. M. is a clinical advisory board for Alexion Pharmaceuticals. Y. F. is a clinical advisory board for Baxter Bioscience and for Alexion Pharmaceuticals.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer pairs for *ADAMTS13* genomic qPCR.

Figure S1. Combinatorial analysis of direct sequencing and genomic qPCR should catch any defects occurring on and between the primer target sequences. Direct sequencing detects mutations such as point mutations (including substitutions, insertions, and deletions), short insertions, and deletions in the exons and exon–intron boundaries (A), but misses mutations on the allele that contains no or mismatched primer target sequences (B). Genomic qPCR for quantifying the copy numbers of target regions complements the results of direct sequencing.

Figure S2. Deleted regions and flanking sequences of *ADAMTS13* identified in two patients with USS. The 1782- and 729-bp regions (red letters) were deleted in patients USS-W4 (A) and USS-KK3 (B), respectively. Lowercase and uppercase sequences indicate introns and exons, respectively. Underlined sequences adjacent to the breakpoints may cause microhomology-mediated end joining (MMEJ) (McVey and Lee. Trends Genet. 2008;24:529–538).

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ORIGINAL ARTICLE

Effects of factor VIII levels on the APTT and anti-Xa activity under a therapeutic dose of heparin

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Abstract In pregnant women, activated partial thromboplastin time (APTT) does not precisely reflect the anticoagulant effect of a therapeutic dose of heparin. However, the measurement of anti-Xa activity can be used to monitor the anticoagulant effect of heparin, since the plasma concentrations of coagulation factors increase in pregnant women. We evaluated the in vitro effects of increased concentrations of fibrinogen and other coagulation factors (FVII, FVIII, and FIX) on the results of assays of APTT and anti-Xa activity in plasma samples with various therapeutic concentrations of unfractionated heparin (UFH). In the presence of UFH, APTT was shortened by increased concentrations of fibrinogen, FVII, or FVIII, and this effect was much stronger when the FVIII concentration was increased. In the plasma samples containing 0.5 or 0.7 U/mL of UFH, the APTT was shortened by approximately half or one-third, respectively, when 6 U FVIII/mL was added to the sample. The anti-Xa activity was not influenced by increased concentrations of the coagulation factors. In the present study, we also evaluated the sensitivities to UHF of four APTT reagents, and found a 1.65-fold difference in the sensitivity to UFH among APTT reagents. Our results demonstrate that increased FVIII concentration shortens APTT under therapeutic doses of UFH, and that APTT thus underestimates the anticoagulant effect of UFH in pregnant women, mainly due to the increased FVIII concentration.

Keywords Anticoagulant monitoring · Anti-Xa activity · APTT · Heparin · Pregnancy

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Introduction

Heparin is widely used as an anticoagulant for the treatment and prevention of thromboembolic disease. Since the anticoagulant response to heparin varies among patients, the anticoagulant effects are routinely monitored using the activated partial thromboplastin time (APTT), and the dose is adjusted to target a therapeutic range of 1.5–2.5 times the control APTT value obtained from normal plasma [1–4].

The APTT test is a global clotting assay that primarily reflects the function of the intrinsic and common pathways of the coagulation cascade. The test is thus potentially affected by changes in the concentrations of coagulation factors including factor VIII (FVIII) and

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fibrinogen, and coagulation inhibitors such as lupus anticoagulants [5–7]. Since the APTT is shortened due to the increased concentrations of coagulation factors during pregnancy, the therapeutic range of heparin obtained in non-pregnant individuals is not applicable in pregnant women [8, 9]. Because the bleeding risk increases with the heparin dose [10], the management of heparin therapy is problematic in pregnant women. In addition, the APTT value has a high degree of variability from one reagent to another [3, 11].

Anti-Xa activity specifically determines the anticoagulant activity of heparin by measuring the ability of heparin-bound antithrombin to inhibit factor Xa [12, 13]. Therefore, increased concentrations of coagulation factors (such as FVIII and fibrinogen) during pregnancy would not interfere with the anti-Xa activity. It is recommended that the APTT goal range be based on the corresponding heparin concentration of 0.3–0.7 U/mL determined by the anti-Xa activity [4].

During pregnancy, plasma concentrations of coagulation factors are greatly changed [14, 15]. The FVIII level increases during pregnancy, beginning at 16–20 weeks, peaking at 36–40 weeks with a mean value of 212 % of the non-pregnant reference value (95 % range 79–570 %), and persisting for up to 3 days postpartum. By weeks 13–20, 50 % of pregnant women have FVIII levels above the upper non-pregnant reference value. Fibrinogen, which is approximately 250 mg/dL in normal plasma, also increases significantly during pregnancy, peaking at 36–40 weeks with the mean value of 423 mg/dL (95 % range 290–615 mg/dL). Factor VII (FVII) and factor IX (FIX), 0.5 and 5 μg/mL in normal plasma, respectively, are also increased during pregnancy, but their increments are not pronounced compared to those of FVIII and fibrinogen [15].

It is known that the anticoagulant effect of heparin, measured by determining the APTT, often decreases in the third trimester, leading to an apparent need for higher doses of heparin [16]. An infusion of cryoprecipitate including a high concentration of FVIII to rabbits led to significant shortening of the APTT [17].

The measurement of anti-Xa activity is an effective method for monitoring the heparin dose in patients with venous thromboembolic disease whose APTT remains subtherapeutic despite treatment with a high dose of heparin [8]. However, a detailed in vitro analysis of the effects of increased concentrations of coagulation factors on the APTT and the anti-Xa activity under a therapeutic dose of heparin has not yet been undertaken. In the present study, we evaluated the in vitro effects of fibrinogen, FVII, FVIII, and FIX on the assay results of APTT and the anti-Xa activity in plasma samples with various therapeutic concentrations of unfractionated heparin (UFH).

Materials and methods

Preparation of plasma samples with increased concentrations of coagulation factors

To examine the effects of fibrinogen, FVII, FVIII, and FIX on the APTT and the anti-Xa activity in the presence of heparin, we prepared plasma samples with various concentrations of fibrinogen, FVII, FVIII, and FIX, as described below. The following proteins and heparin were purchased: Human Fibrinogen Plasminogen Depleted (Enzyme Research Laboratories, South Bend, IN, USA), Coagulation Factor VII and Coagulation Factor IX (Biopur, Reinach, Switzerland), and UFH, Heparin Sodium Injection-Tanabe (Mitsubishi Tanabe Pharma, Osaka, Japan). Plasma-derived human FVIII was from the Chemo-Sero Therapeutic Research Institute, Kumamoto, Japan. This study was approved by the Ethics Review Committee of the National Cerebral and Cardiovascular Center of Japan. Written informed consent was obtained from each participant.

Blood samples were drawn from an antecubital vein of healthy volunteers (4 males, 4 females) through a needle into disposable, siliconized, evacuated plastic tubes containing 0.1 volume of 3.13 % trisodium citrate. The plasma samples were centrifuged at 3,430*g* for 10 min at room temperature and stored at −80 °C until analysis. The plasma samples contained 250 mg/dL of fibrinogen, 0.5 μg/mL of FVII, 1 U/mL of FVIII, and 5 μg/mL of FIX. The frozen plasma samples were thawed at 37 °C and mixed to make pooled plasma. We mixed nine volumes of pooled plasma and one volume of coagulation factor solution, and prepared plasma samples with 500 mg/dL of fibrinogen (corresponding to a twofold increase in plasma concentration), 1.0 μg/mL of FVII (corresponding to a twofold increase), 2, 4, and 6 U/mL of FVIII (corresponding to twofold, fourfold, and sixfold increases), or 10 μg/mL of FIX (corresponding to a twofold increase). As a control, we prepared a normal plasma sample mixed with nine volumes of pooled plasma and one volume of saline. Subsequently, 99 volumes of each plasma sample were mixed with 1 volume of UFH to give a final concentration of 0.0, 0.3, 0.5, 0.7 or 1.0 U UFH/mL.

We measured the fibrinogen concentrations of the normal plasma sample and plasma samples with the twofold increase in fibrinogen concentration and found that they were 243 and 544 mg fibrinogen/dL, respectively. We also measured the FVIII and von Willebrand factor (VWF) concentrations of the normal plasma sample and plasma samples with twofold, fourfold, and sixfold increases in FVIII concentration, and we found that they were 1.0, 2.0, 4.0, and 5.9 U FVIII/mL, and 88, 362, 1,026, and 1,786 % VWF, respectively.