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CASE REPORT

A complex genomic abnormality found in a patient with antithrombin deficiency and autoimmune disease-like symptoms

Io Kato · Yuki Takagi · Yumi Ando · Yuki Nakamura · Moe Murata · Akira Takagi · Takashi Murate · Tadashi Matsushita · Tadaaki Nakashima · Tetsuhito Kojima

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Abstract Hereditary antithrombin (AT) deficiency is an autosomal dominant thrombophilic disorder caused by *SERPINC1* abnormality. In the present study, we analyzed *SERPINC1* in a Japanese patient with AT deficiency and autoimmune disease-like symptoms. Direct sequencing and multiplex ligation-dependent probe amplification revealed that the patient was hemizygous for the entire *SERPINC1* deletion. Single nucleotide polymorphism genotyping, gene dose measurement, and long-range polymerase chain reaction (PCR) followed by mapping PCR and direct sequencing of the long-range PCR products revealed that the patient had an approximately 111-kb gene deletion from exon 2 of *ZBTB37* to intron 5 of *RC3H1,* including the entire *SERPINC1* in chromosome 1. We also found a 7-bp insertion of an unknown origin in the breakpoint, which may be a combination of three parts with a few base-pair microhomologies, resulting from a replication-based process known as 'fork stalling and template switching'. Because *RC3H1,* which encodes the protein roquin is involved in the repression of self-immune responses, the autoimmune disease-like symptoms of the patient may have resulted from this gene defect. In conclusion, we identified an entire *SERPINC1* deletion together with a large deletion of *RC3H1* in an AT-deficient patient with autoimmune disease-like symptoms.

Keywords Genome rearrangement · Antithrombin deficiency · *SEPINC1* · *RC3H1*

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I. Kato · Y. Takagi · Y. Ando · Y. Nakamura · M. Murata · A. Takagi · T. Murate · T. Kojima (✉)
Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, 1-1-20 Daiko-Minami, Higashi-ku, Nagoya 461-8673, Japan
e-mail: kojima@met.nagoya-u.ac.jp

T. Matsushita
Department of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan

T. Nakashima
Department of Cardiology, Tokuyama Central Hospital, Shunan, Japan

Introduction

Antithrombin (AT), a member of the serine protease inhibitor superfamily, functions as a major physiological anticoagulant molecule [1, 2]. AT forms a complex with serine protease type coagulation factors such as thrombin and factor Xa and, inactivates them. When heparin binds to AT, AT undergoes conformational changes and accelerates the anticoagulant efficacy by more than a 1000-fold.

Congenital AT deficiency caused by abnormality in the AT gene (*SERPINC1*) is inherited as an autosomal dominant disorder associated with predisposition to recurrent venous thromboembolism. In 1965, Egeberg described the first case of inherited AT deficiency, the incidence of which is estimated to be 1 in 500 to 1 in 5000 with no racial or sexual differences worldwide [3, 4]. It has been reported that homozygous AT-null mice result in embryonic lethality [5] and assumed that complete AT deficiency in humans could be fatal. Indeed, causative mutations in inherited AT deficiencies have usually been determined to be heterozygous [6]. Till date, extensive gene analyses in patients with AT deficiency have revealed many distinct *SERPINC1* defects such as missense, nonsense, deletion and insertion mutations. It is rare to find a large deletion

(>20 bp) in *SERPINC1*, and very few cases have been analyzed with regard to the deletion region in detail [7–10]. One of the reasons is that a large heterozygous gene deletion on an autosomal chromosome may result in normal findings by polymerase chain reaction (PCR)-mediated direct sequencing analysis.

Here, we report a large deletion of more than 100 kb in chromosome 1 involving *SERPINC1* in a Japanese patient who suffered from autoimmune disease-like symptoms associated with venous thromboembolism and was diagnosed with AT deficiency.

Materials and methods

Patient and DNA sample

The patient was a Japanese female who had a first episode of deep vein thrombosis (DVT) with pulmonary embolism (PE) at the age of 41 years. Since then, she had been treated with warfarin in another hospital. One year later, she was admitted to Yamaguchi University Hospital to be surveyed for autoimmune disease because she had autoimmune-like symptoms such as joint pain and mild fever with a positive rheumatoid arthritis (RA) test. However, she was not diagnosed with a definitive autoimmune disease. Two years later, she was readmitted to Yamaguchi University Hospital for further examination because of recurrent DVT despite warfarin treatment. Finally, she was diagnosed with AT deficiency and treated with plasma-derived AT concentrate. She was also referred to Nagoya University for examination of DNA abnormalities. She had no family history of thrombosis at that time. The study was approved by the ethics committee of each university. A genomic DNA sample was isolated from peripheral blood leukocytes of the patient after a written informed consent was obtained.

Direct sequencing and multiplex ligation-dependent probe amplification (MLPA) analysis of *SERPINC1*

All exons and intron–exon junctions were amplified by PCR and sequenced as described previously [11]. MLPA analysis of *SERPINC1* was performed using the SALSA MLPA P227 SerpinC1 kit (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions to search for intragenic deletions or duplications [12].

Identification of deletion region and breakpoint

To assess the extent of the deletion, PCR-mediated single nucleotide polymorphism (SNP) genotyping, real-time PCR, and long-range PCR were performed with primer pairs designed on the basis of the NCBI Reference Sequence (NT_004487.19) containing *SERPINC1* at chromosome 1 (Supplemental Table 1). PCRs for SNP genotyping were performed under the same conditions as *SERPINC1* sequencing, except for the annealing temperature of 55 to 68 °C. In a range covering 21–27.7 Mb of NT_004487.19, SNP genotyping and real-time PCR were performed to assess the ploidy in a certain part of the genome.

Real-time PCR was performed using SYBR Premix Ex Taq and the Thermal Cycler Dice Real Time System *II* (Takara Bio Inc., Otsu, Japan). Relative values of the interested gene dosages were calculated using the delta–delta C_t method, in which the C_t was defined by each second derivative maximum (SDM) point of the amplification curves. All relative gene dosages were revised using that of *F11* exon 15 as a reference.

Long-range PCR was performed with KOD FX Neo DNA polymerase (Toyobo Co. Ltd.) in a touch-down PCR, which involved 25 cycles of a temperature profile similar to that of *SERPINC1* sequencing, with the exception that the annealing temperature was 74 °C in the first cycle and decreased by 2 °C every 5 cycles to reach 68 °C and that the extension time was 10 min. Nested PCR following long-range PCR was performed for 25 cycles with KOD FX Neo DNA polymerase. The products were analyzed by mapping PCR and by digested patterns with some restriction enzymes, such as *Sma*I, *Eco*RI, *Sca*I, and *Sac*I (New England Biolab Japan).

Results

PCR-mediated sequencing revealed no causative mutation in *SERPINC1* in the patient (data not shown). However, MLPA analysis for *SERPINC1* revealed that the relative gene dosage values in all exons were ~50 % of normal values, suggesting that the patient had a complete *SERPINC1* deletion (Fig. 1).

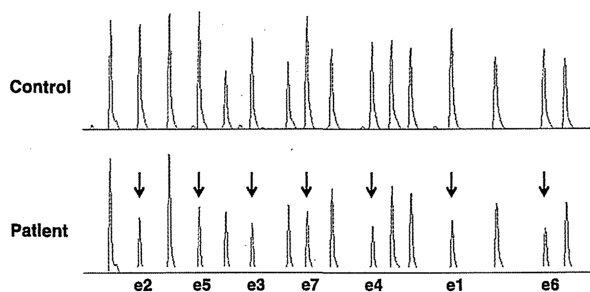

Fig. 1 Multiplex ligation-dependent probe amplification (MLPA) analysis for *SERPINC1*. MLPA analysis revealed that the relative gene dosage values in all *SERPINC1* exons of the patient were ~50 % of normal values, suggesting a complete *SERPINC1* deletion

Table 1 Locations and results of SNP genotyping and real-time PCR

Gene	Reference SNP, real-time PCR	Genotype of patient	Location (bp) (NT_004487.19)	Distance from *SERPINC1* (kb)
F5	rs6022	T/G	21,018,468	−4,357
F5	rs6029	G/A	21,018,615	−4,356
FASLG	rs10458360	G/C	24,122,617	−1,252
SLC9A11	rs7516544	G/A	24,980,073	−395
SLC9A11	rs12565753	G/A	24,980,247	−395
KLHL20	rs2273366	A/G	25,213,584	−161
DARS2	rs2295366	T/G	25,314,334	−61
DARS2	Real-time PCR (A1)	2 n	25,315,440	−60
5′-breakpoint			25,326,758	−48
ZBTB37	rs1322774	C	25,327,882	−47
ZBTB37	rs9286895	A	25,331,109	−44
ZBTB37	Real-time PCR (A2)	1 n	25,344,011	−31
SERPINC1	Real-time PCR	1 n	25,367,296	−8
SERPINC1 c.1			25,375,039	0
RC3H1	rs9425780	G	25,391,942	17
RC3H1	rs12566651	A	25,393,879	19
RC3H1	Real-time PCR (A3)	1 n	25,396,451	21
RC3H1	rs12066153	T	25,426,057	51
3′-breakpoint			25,437,835	63
RC3H1	rs1884994 (A4)	T/C	25,438,924	64
RC3H1	rs6686083	T/A	25,441,181	66
RABGAP1L	rs727279	G/A	25,953,806	579
CACYBP	rs1046439	T/C	26,457,913	1,083
RFWD2	rs10913112	G/A	27,402,470	2,027
RFWD2	rs670143	C/T	27,665,137	2,290

A1, A2, A3, and A4 are PCRs shown in Fig. 2A

We evaluated whether certain parts of the genome were lost on 1 allele by SNP genotyping or real-time PCR in 21–27.7 Mb of NT_004487.19. Locations and results of SNP genotyping and real-time PCR are shown in Tables 1, 2. In Fig. 2A, *DARS2* (A1) was diploid and *ZBTB37* (A2) was monoploid, suggesting that the breakpoint might locate between them on the centromere side. On the telomere side, the breakpoint may be located on *RC3H1* (A3–A4), next to *SERPINC1*.

We then performed nested PCR for long-range amplification over the deleted portion (Fig. 2B). We obtained an approximately 13-kb PCR product from the patient's genome; however, we did not obtain any product from the normal control (B-1). The 13-kb amplicons were purified and used as templates for mapping PCRs targeting parts of uncertain deletion regions (B-2). On the centromere side of the 13-kb mutant PCR product, mapping PCR at B1 revealed a positive signal; however, a positive signal was not revealed at B2 located on *ZBTB37* intron 3. On the telomere side, mapping PCR at A4 located on intron 4 of *RC3H1* revealed a positive signal; however, a positive signal was not revealed at B3 on intron 5.

We performed another PCR targeted from F3 to R3 (115 kb in size from the normal control), and obtained an aberrant 4-kb product from the patient; however, no product was obtained from the normal control (Fig. 2C). To assess an unknown region of the deletion, PCR products from the patient were digested with several restriction enzymes that recognize a single site in the PCR product from the normal control (C-1). The PCR products digested with either *Sma*I or *Eco*RI changed to the expected sizes, indicating that these positions were not deleted. On the other hand, those digested with either *Sca*I or *Sac*I did not change, thereby indicating that these positions were deleted (C-2).

Finally, we performed gene walking analysis for amplicons from mutant allele and found a breakpoint at both sides of the deletion (Fig. 3). The mutant allele of the patient lost an 111-kb region from *ZBTB37* exon 2 to *RC3H1* intron 5, and had a small inserted sequence of 7 bp inside the breakpoint. We searched for the origin of the 7-bp insertion on the GenBank database and found that the same sequence existed in more than 900 positions on chromosome 1. However, the same alignment as a 15-bp

Table 2 Location and name of primers in Fig. 2

Gene	Name of primer (set)	Location (bp)[a] (NT_004487.19)	Distance from *SERPINC1* (kb)	cf.	Short PCR result
DARS2 int 16	F1	25,314,224	−60.8	Fig. 2B	
DARS2 ex 18	F2	25,315,428	−59.6	Fig. 2B	
DARS2 ex 18	A1	25,315,440	−59.6	Fig. 2A	
GAS5 int 5	F3	25,323,895	−51.1	Fig. 2C	
GAS5 int 1	B1	25,324,990	−50.0	Fig. 2B	+
ZBTB37 int 1	seq	25,326,325	−48.7	Fig. 2C	
ZBTB37 ex 4	B2	25,327,667	−47.4	Fig. 2B	−
ZBTB37 ex 6	A2	25,344,011	−31.0	Fig. 2A	
SERPINC1 c.1		25,375,039	0.0		
RC3H1 ex 19	A3	25,396,451	21.4	Fig. 2A	
RC3H1 int 5	B3	25,437,502	62.5	Fig. 2B	−
RC3H1 int 4	A4	25,438,717	63.7	Fig. 2A, B	+
RC3H1 int 4	R3	25,439,134	64.1	Fig. 2C	
RC3H1 int 4	R2	25,439,413	64.4	Fig. 2B	
RC3H1 int 4	R1	25,439,479	64.4	Fig. 2B	

A1–A4 and B1–B3 are PCR primer sets

[a] Position of the centromere side

sequence, corresponding to the breakpoint and including a 7-bp insertion, was only found 1.2-kb downstream of the breakpoint on the centromere side in the reverse direction.

Discussion

Here, we reported a case with a large deletion of more than 100 kb on chromosome 1 involving the entire *SERPINC1* and most part of *RC3H1* in a Japanese patient with AT deficiency who also suffered from autoimmune disease-like symptoms. The breakpoint of the deletion would be caused by a complicated rearrangement with a 7-bp insertion, which could be explained by the Fork Stalling and Template Switching (FoSTeS) model [13, 14]. Lee et al. [15] proposed a model based on a DNA replication stage to explain the microhomology of the junctions, the putative mechanism of which is the switching of a nascent strand during DNA replication. According to this model, during DNA replication, the replication fork stalls at one position, following which the nascent lagging strand disengages from the original template, transfers, and anneals to another replication fork in physical proximity. It then "primes" and restarts the DNA synthesis. These steps could occur multiple times in series; therefore, the eventual replicated DNA sequence results in the complicated alignment. The generated alignments are characterized by the sequences at the junction juxtaposed to some sequences derived from different origins with microhomologies. These features could account for the complicated gene rearrangement that was simply and successfully determined in this case.

The observed gene rearrangement also resulted in losses of the entire coding sequence of *ZBTB37* and two-thirds sequence of *RC3H1*. *ZBTB37* encodes a protein known as "Zinc finger and BTB domain-containing protein 37", but its function remains unknown. Meanwhile, *RC3H1* encodes a protein termed "RING finger and CCCH-type zinc finger domain-containing protein 1 (Roquin-1)". Roquin-1 is an intracellular protein that is highly conserved across its full length from mammals to invertebrates, and it limits inducible T cell co-stimulator (ICOS) expression by promoting the degradation of ICOS mRNA.

Vinuesa et al. [16] identified that a methionine residue at position 199 is substituted by arginine (M199R) in Roquin-1 of mice, termed "the *sanroque* strain". In *sanroque*-female mice, homozygous M199R mutation of Roquin-1 increased ICOS expression on T cells, causing the accumulation of lymphocytes typically associated with lupus-like autoimmune symptoms [17]. On the other hand, a recent study reported that tissue-specific knockout of Roquin-1 in the hematopoietic system did not cause autoimmunity but caused defined changes in immune homeostasis, dominated by the expansion of eosinophilic granulocytes, macrophages, and CD8 effector-like T cells [18]. Leppek et al. [19] demonstrated that Roquin-1 recognizes the constitutive decay elements (CDEs) folded into an RNA stem–loop motif and that Roquin-1 proteins promote mRNA degradation. Through genome-wide investigation, it was revealed that Roquin-1 targets several immunity- and inflammation-related mRNAs. These data suggest that Roquin-1 plays an important role in the immune system.

Meanwhile, no human *RC3H1* mutation has been reported till date, and the influence of *RC3H1* mutation in humans is not clear. We do not have any evidence to explain autoimmune disease-like symptoms of this patient

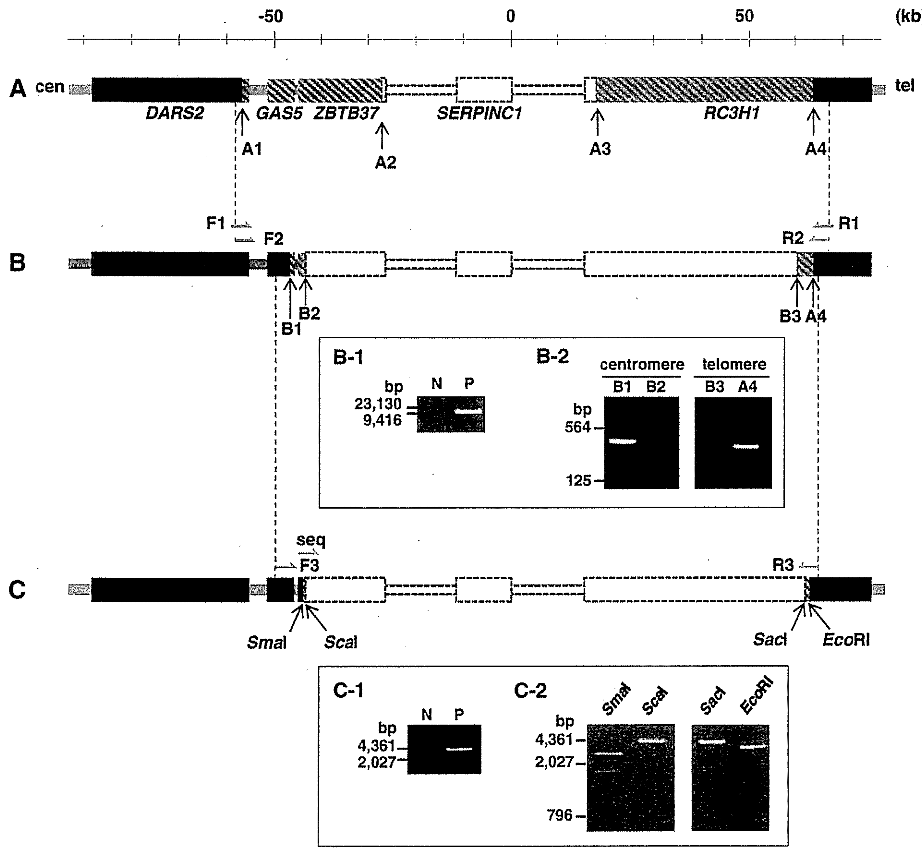

Fig. 2 Strategy to assess deletion region. Strategy schemes to assess the extent of deletion region are shown. *Closed bars* indicate no deletion regions, whereas *dashed white bars* indicate obvious 1-allele deleted regions. *Striped bars* indicate uncertain regions. *Up-pointing arrows* indicate tested regions of deletion assessment. *Horizontal arrows* (F1-3, R1-3) indicate primer sites for respective polymerase chain reactions (PCRs) and sequencing (cf. Table 2). **A** Summarized results of single nucleotide polymorphism (SNP) genotyping and real-time PCR. **B** Ranges of nested long-range PCR from *DARS2* to *RC3H1* (F1–R1 and F2–R2). Inserted B-1 and B-2 are results of nested long-range PCR (N, normal; P, patient) and mapping PCR (B1, B2, B3, and A4) for nested PCR products, respectively. **C** Another long-range PCR targeting *GAS5* to *RC3H1* (F3–R3). Inserted C-1 and C-2 are results of second long-range PCR (N, normal; P, patient) and digestion patterns by restriction enzymes, respectively

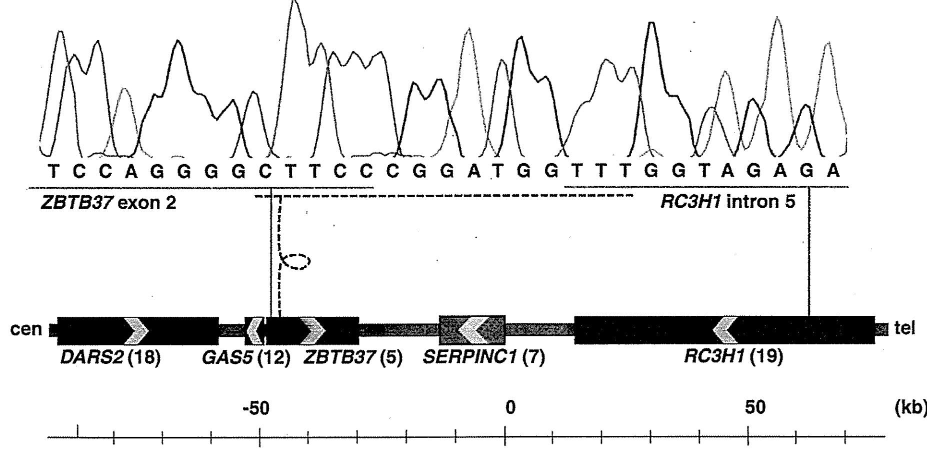

Fig. 3 DNA sequence of breakpoint junction. The sequence was connected *ZBTB37* exon 2 to *RC3H1* intron 5 with an insert of 7 bp. The *bottom column* indicates a schema of genome around *SERPINC1*. The numbers in *parentheses* indicate total exon numbers of each gene

with AT deficiency; however, we identified a large deletion including the entire *SERPINC1* together with most part of *RC3H1*. These data suggest that the *RC3H1* defect may have some effect on the immune responses of the patient.

In summary, we identified a complex genome rearrangement on chromosome 1 involving deletion of the entire *SERPINC1* and most part of *RC3H1*, which may be associated with the autoimmune disease-like symptoms together with AT deficiency in this patient.

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Conflict of interest The authors declare that they have no conflict of interest.

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VI. 特　論

新規血栓性素因アンチトロンビン抵抗性の発見と今後の展望

高木夕希　小嶋哲人

Discovery and prospects of a novel thrombophilia: antithrombin resistance

Yuki Takagi, Tetsuhito Kojima
Department of Pathophysiological Laboratory Sciences,
Nagoya University Graduate School of Medicine

Abstract

Pathogenesis of venous thromboembolism(VTE) known to be complex and multifactorial process involves the interaction of acquired factors and genetic predisposing conditions. Deficiency of natural anticoagulant factors such as antithrombin(AT), protein C and protein S increases the risk of a VTE. Recently, we have reported novel mechanism of hereditary thrombosis in a Japanese family, in which AT resistance was associated with a missense mutation(p. Arg596Leu) in the prothrombin gene named prothrombin Yukuhashi. The mutant thrombin showed a low clotting activity, but a severely impaired inactivation by AT, resulting in a susceptibility to thrombosis. We have developed a new laboratory test to evaluate AT resistance in plasma. Prothrombin mutation causing AT resistance has found in Caucasian, not only in Japanese.

Key words: VTE, DVT, PE, AT resistance, thrombophilia

はじめに

静脈血栓塞栓症(venous thromboembolism: VTE)は，遺伝的リスクと環境的リスクが重なることで発症する多因子疾患である．静脈血栓症は，欧米人に多くみられ，日本人には少ないとされてきたが，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少なくないことが明らかになってきた．静脈血栓症を招く原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．遺伝的リスクとしては，生理的血液凝固阻止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)欠損症/異常症が広く知られている．

本稿では，新たな先天性血栓性素因として報告されたアンチトロンビン抵抗性とそのスクリーニング検査法について概説する．

1．先天性血栓性素因

遺伝的リスクである先天性血栓性素因をもつ患者は，50歳以下の比較的若年で深部静脈血栓症(deep venous thrombosis: DVT)や肺塞栓症(pulmonary embolism: PE)などを発症し，繰り返すことも多い．血栓発生部位に関しても腸

名古屋大学大学院医学系研究科 医療技術学専攻 病態解析学講座

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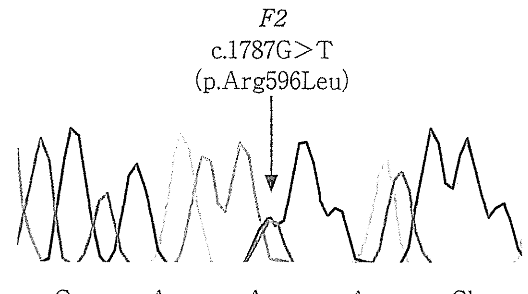

Cys Asp Arg Asp Gly
野生型 TGT GAC CGG GAT GGG
変異型 TGT GAC CTG GAT GGG
Leu

図1 AT抵抗性を呈するプロトロンビン遺伝子変異

発端者のプロトロンビン遺伝子(*F2*)において一塩基置換が同定された．アンチトロンビンとの結合部に位置するアルギニンがロイシンに置換するミスセンス変異(c.1787G>A, p.Arg596Leu：Prothrombin Yukuhashi).
(文献[1]より改変)

間膜静脈や上矢状静脈などの非定型部位での発症が多いことも特徴である．また，家族歴がみられることが多く，その原因として先に挙げた生理的血液凝固阻止因子の遺伝子異常が同定されている．しかし，いまだ原因不明な遺伝性血栓症も数多く，発症要因が不明な血栓症は特発性血栓症として難病疾患の一つに指定されている．こうしたなか，著者らのグループは長らく原因不明であった静脈血栓症家系において血栓症発症原因となる遺伝子変異を凝固因子であるプロトロンビン遺伝子に同定し，新たな血栓性素因として報告した[1,2].

2. アンチトロンビン抵抗性(antithrombin resistance：ATR)

1）プロトロンビン遺伝子変異

発端者は日本人女性であり，11歳のときにDVTを発症した．女性の家系では3世代にわたって8人の静脈血栓症患者がおり，うち3人は既に亡くなっていた．本家系においては，若年性の静脈血栓症がみられ，特に代を経るにつれてその発症率が高くなる傾向があった．以上から遺伝性血栓症が強く疑われたため，2001年当時，本家系での既知の先天性血栓性素因について検査がされたが，すべてが否定された[3]．こうしたなか，2009年ISTH Boston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プロトロンビン遺伝子異常の存在が報告された[4]．それを受け発端者のプロトロンビン遺伝子を解析したところ，プロトロンビンの活性体トロンビンに対する生理的凝固阻止因子ATとの結合部に位置する596番アルギニンがロイシンに置換するミスセンス変異(c.1787G>A, p.Arg596Leu)が，ヘテロ接合体で同定された(**図1**)．また，このミスセンス変異は本家系内の他の血栓症患者でも検出されたことから，遺伝性血栓症の原因であることが強く疑われた.

2）血栓症発症機序

2009年ISTH Bostonではプロトロンビン遺伝子異常の存在が報告されたが血栓症に至る機序は解析されておらず，著者らのグループは日本人患者での血栓症発症機序について詳細に解析した.

異常プロトロンビンで変異がみられたアルギニン(Arg596)はトロンビンへの活性化後に，AT分子のアスパラギン(Asn265)との結合に重要な水素結合を形成していることから[5]，Arg596Leu置換はATによるトロンビン不活化不全を起こす可能性が示唆された．しかし，血栓症患者は治療のためにワルファリンを服用しており患者血漿検体でのプロトロンビン機能解析は困難であったため，遺伝子工学技法を用いてリコンビナント野生型/変異型プロトロンビンを作製してトロンビンへの活性化動態，活性化後の不活化動態を比較検討した.

a. 変異型プロトロンビンの活性化動態

プロトロンビン欠乏血漿に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，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，トロンビンに特異的な発色性合成基質S-2238に対する活性を反映する合成基質法の3種を用いて野生型/変異型プロトロンビンの活性化動態を測定した．その結果，野生型疑似血漿はいずれの測定でも正常血漿と同様な活性を示したが，変

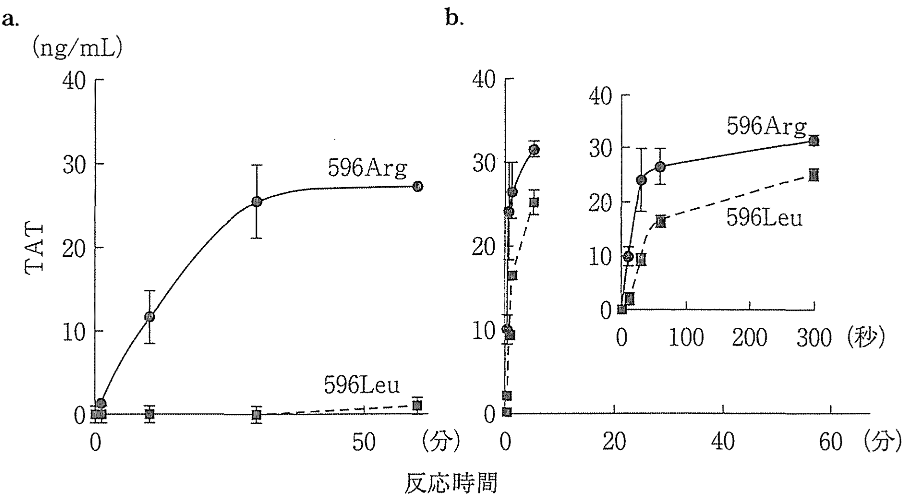

図2　変異型トロンビンのTAT複合体形成能

リコンビナントプロトロンビン由来トロンビンとATとの結合能(TAT複合体形成能)をELISA法にて測定した結果，(a)ヘパリン非存在下で，変異型(596Leu)は野生型(596Arg)に対してTAT複合体形成がほとんどみられず，(b)ヘパリン存在下でも低値を示した.

(文献[1]より改変)

異型では3つのすべての測定法で野生型を下回り，凝固一段法で最も低く(野生型の15％)，ついで凝固二段法(同32％)，合成基質法(同66％)の順で活性が大きくなった．これらの結果より，変異型プロトロンビンはトロンビンへの転換が遅延し，フィブリノゲンを基質とした凝固活性も低下することが示唆された．また，S-2238はフィブリノゲンと比較して分子量が小さいために合成基質法では変異型トロンビンの活性があまり低下しないと推察された.

一方，ウシ由来のFXa・FVaとリン脂質，カルシウムイオンからなるプロトロンビナーゼを用い，プロトロンビンからトロンビンへの活性化の様子を経時的にウェスタンブロット解析したところ，野生型と変異型でほとんど差がみられなかった．ウェスタンブロット解析で，トロンビンへの転換に要した最短時間は20秒と長く数秒単位の差を反映する凝固法による検出限界には及ばなかったことが，先述した凝固一段法，二段法での比較と一見矛盾するようにみえる要因と考えられた.

b. 変異型トロンビンの不活化動態

プロトロンビンを十分に活性化した後に生理的阻止因子ATと反応させ，TAT複合体形成能(トロンビンとATとの結合能)を比較したところ，ヘパリン非存在下で，野生型では経時的にTAT複合体の増加がみられたが，変異型では形成時間30分まで検出感度未満であり，60分後にわずかに検出されただけであった(**図2-a**).ヘパリン存在下では変異型でも野生型に似た経時的な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た(**図2-b**)．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.

更に，プロトロンビン欠乏血漿にリコンビナント変異型プロトロンビンを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，不活化の著しい遅延がみられ，結果的に測定時間内での総トロンビン活性量(活性値の持続時間の積分値)が著しく増大していた(**図3**).すなわち，患者血漿中の変異型プロトロンビンは，凝固活性は低いものの，いったん活性化されるとATによる不活化をほとんど受けず(AT

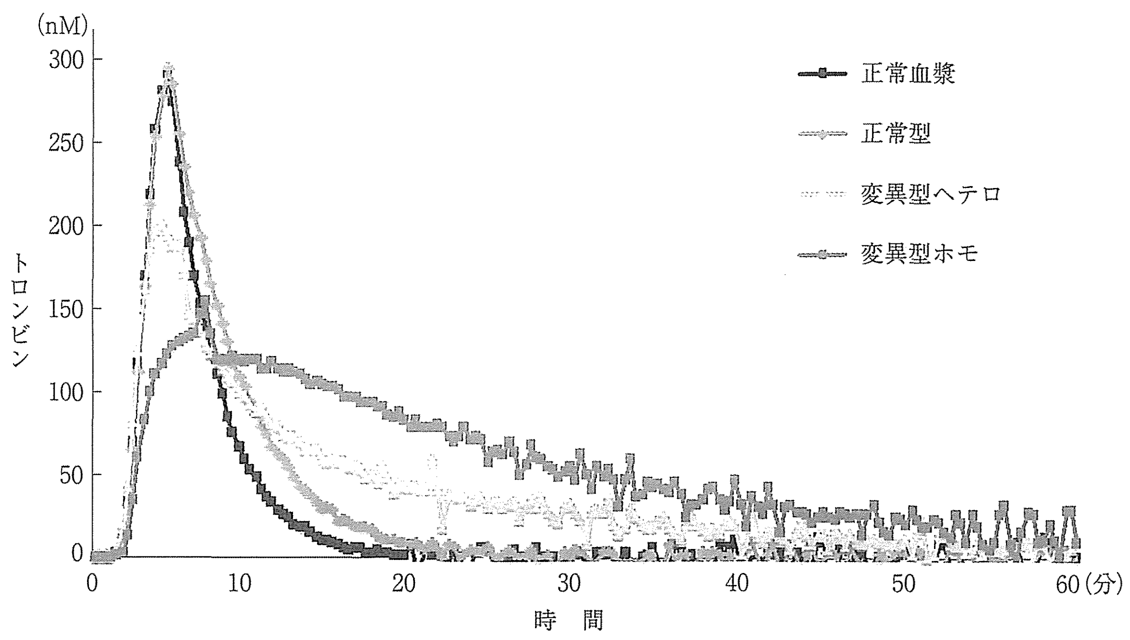

図3 プロトロンビン異常疑似血漿でのトロンビン生成試験(TGA)

プロトロンビン欠乏血漿に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ものの不活化が著しく遅く，結果として総トロンビン活性量(活性値と持続時間の積分値)の著しい増大を認めた.
(文献[1]より改変)

抵抗性)，凝固活性を保ち続けることが示唆され，これが遺伝性血栓症の原因であると判明した.

3) スクリーニング検査法の開発

著者らは，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観察することでAT抵抗性を検出する臨床検査法を考案し，報告した[6]. この臨床検査法はプロトロンビン活性化相，トロンビン不活化相，残存トロンビン活性測定相からなる. 本検査法において，正常検体では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，トロンビン活性が約10％にまで阻害されるのに対し，変異型トロンビンでは30分後に90％以上残存していた(**図4-a**). ヘパリン存在下でも正常検体は30秒程度で10％以下まで阻害されるのに対し，変異型は30秒後に約50％のトロンビン活性が残存していることから，AT抵抗性を判別できる. また，臨床検体解析を想定し，ワルファリンが本検査法に及ぼす影響を評価した結果，考案した検査法はワルファリン服用中の静脈血栓症患者の検体でもAT抵抗性が検出可能であった(**図4-b**). 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としてのAT抵抗性の関与の実態が明らかとなることが期待される.

4) AT抵抗性 報告例

現在，日本国内においては上述家系以外の報告はないが，2013年に遺伝性血栓症をもつセルビア人2家系でAT抵抗性を示すプロトロンビン遺伝子変異(c.1787G>A, p.Arg596Gln)が報告された[7]. また，この変異は同年にインド人患者で同定され報告されたものと同じ変異と思われる[8]. これらの報告から，この新規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新たな病態解明につながることが予想される.

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阻止因子の遺伝子異常が同定されているが，いまだ原因不明な家族性血栓症も数多い. 著者らは，通常多くの報告では出血傾向を示す

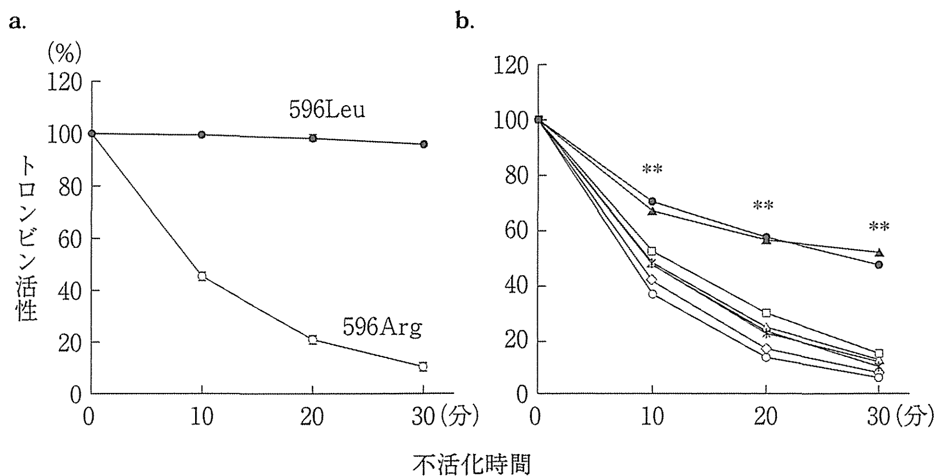

図4　スクリーニング検査法でのAT抵抗性評価

a. ヘパリン非存在下で，野生型(596Arg)は血中濃度5倍量ATとの混和30分でトロンビン活性が10％程度にまで阻害されるのに対して，変異型(596Leu)は90％以上残存がみられた.

b. ヘパリン非存在下で，プロトロンビンに異常のないワルファリン服用患者血漿(□，△，◇，米)は健常人血漿(○)と同様の不活化を示し，プロトロンビンYukuhashi患者のワルファリン服用時の血漿(●，▲)ではATの阻害を受けにくい結果を示した(** $p<0.001$).

(文献[6]より改変)

凝固因子・プロトロンビンの遺伝子変異が，正反対の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AT抵抗性を世界で初めて報告した．この血栓性素因の発見は，日本人のみでなく世界中での原因不明な家族性血栓症の発症要因の究明につながることも期待され，今後，AT抵抗性病態について更なる研究成果の蓄積が望まれる.

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ヘパリン類似物質

名古屋大学大学院医学系研究科 病態解析学講座　小嶋哲人（こじまてつひと）

point

- ヘパリン類似物質（ヘパリノイド）とは，ヘパリンと類似した糖鎖構造をもつが全く異なる分子のヘパラン硫酸のことである．
- ヘパリン類似物質（ヘパラン硫酸）を主成分とする抗凝固薬・ダナパロイドナトリウム（オルガラン®）が，日本ではDICを適応症に使用されている．
- ダナパロイドの抗Ⅹa/抗トロンビン活性比は，未分画ヘパリンや低分子量ヘパリンに比べ大きく，出血性副作用の少ないことが期待されている．
- ダナパロイドは，日本では適応症となっていないが，Ⅱ型HITでの抗凝固薬として8th ACCPガイドラインではGrade 1Bと推奨されている．
- やはり日本では適応症となっていないが，欧米ではHIT合併妊娠時での血栓症に対してもダナパロイドは有効な薬剤として評価されている．

Q　ヘパリンとは，どう違うのですか？

A　ヘパリン類似物質（ヘパリノイド）とは，ヘパラン硫酸（heparan sulfate：HS）のことで，ヘパリンと同様に，種々の分子との相互作用を介して，細胞接着，細胞増殖，血液凝固などに関わる生物学的多機能分子です[1]．ヘパラン硫酸は，ヘパリンと類似した糖鎖構造をもっていますが，ヘパリンとは生合成されるコア蛋白が異なる，全くの別分子です．ヘパラン硫酸は，ヘパリンと同様にウロン酸（グルクロン酸/イズロン酸）とグルコサミンとの2糖体の繰り返し構造を基本骨格にもち，種々の程度にO-硫酸化，グルコサミンのN-硫酸化，あるいはアセチル化を受けますが（**図1**）[2]，ヘパリンとは異なり，2糖体の繰り返し構造のうち，ウロン酸としてグルクロン酸を多く含み，O-硫酸化やグルコサミンのN-硫酸化頻度が低い特徴があります．

しかし，ヘパラン硫酸にもイズロン酸や硫酸基のクラスター（高硫酸化領域）がみられ，これらの部位はヘパリンに酷似した構造をもっています．このようなヘパラン硫酸の高硫酸化領域には，アンチトロンビン

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D-Glucuronic acid (GlcA) D-Glucosamine (GlcNH$_2$)
ヘパラン硫酸

L-Iduronic acid (IdoA) D-Glucosamine (GlcNH$_2$)
ヘパリン

図 1　ヘパラン硫酸，ヘパリンの繰り返し 2 糖単位

ウロン酸（D-GlcA または L-IdoA）とアミノ糖（D-GlcNH$_2$）からなる．
R と R′ の部分は，硫酸化されうる部位で，R は H または SO_3^-，R′ は H，$COCH_3$ または SO_3^-．
ヘパリン中にも GlcA がわずかに存在し，逆に，ヘパラン硫酸中にも IdoA が存在する．
ヘパリンの硫酸化はヘパラン硫酸に比べ高頻度．

（文献 2 より引用）

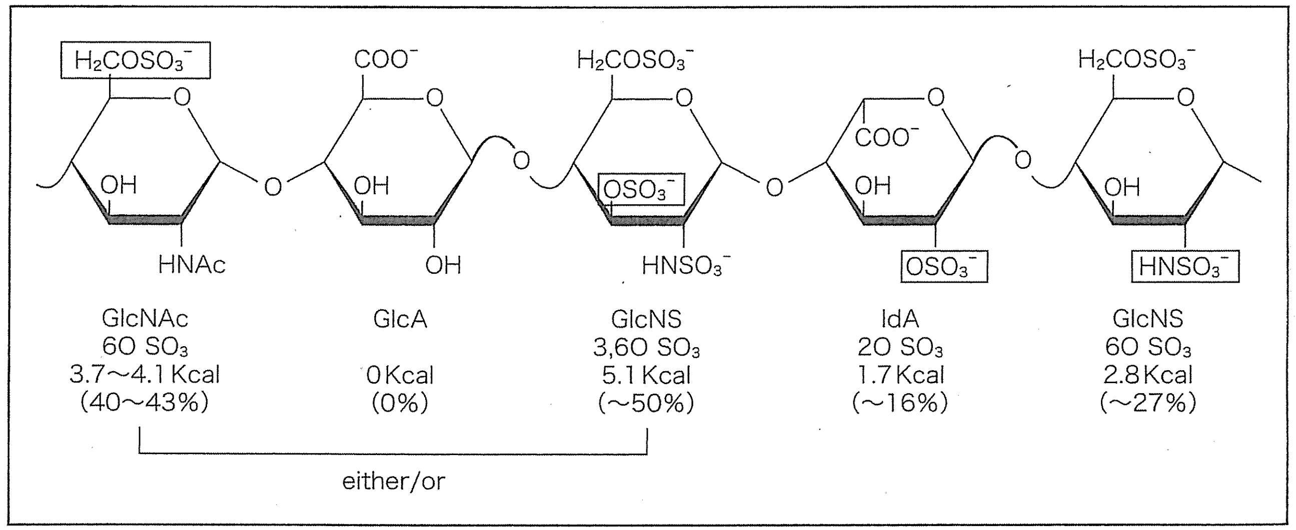

図 2　ヘパリンのアンチトロンビン結合ドメイン構造

各残基の相対的結合寄与度は，それぞれアンチトロンビンとの結合力で示した．
□で囲った O-硫酸基はアンチトロンビン結合に特に重要とされる．

（文献 3 を参照して作成）

(antithrombin：AT）と結合して AT の抗凝固活性を増強する，ヘパリンに特異な 5 糖（pentasaccharide）構造（**図 2**）[3] を含む，AT 結合ドメインを形成しています．

また，ヘパリンは通常血液中には存在しませんが，ヘパラン硫酸は生体内の血管内皮細胞でヘパラン硫酸プロテオグリカンとして産生され，血管内皮上での血液凝固反応制御，すなわち血液流動性維持に働いていると考えられています．

日本では，このヘパラン硫酸を主成分とするヘパリノイド製剤・ダナパロイドナトリウム（オルガラン注®：ヘパラン硫酸 84%，デルマタン硫酸 12%，コンドロイチン硫酸 4%）が〔disseminated intravascular coagu-

lation (DIC):播種性血管内凝固症候群〕の治療に用いられています.
一方,欧米ではこのダナパロイドが出血性副作用の少ない抗血栓症治療薬として「深部静脈血栓症の予防」あるいは「ヘパリン起因性血小板減少症 (heparin induced thrombocytopenia:HIT) での血栓症の予防・治療」に用いられています.

Q 抗凝固作用のヘパリンとの違いは何ですか?

A ヘパラン硫酸(ヘパリノイド/ヘパリン類似物質)は,前に述べたように,アンチトロンビンを介して抗凝固活性を示す点ではヘパリンと同じですが,ヘパリンに比べて抗トロンビン活性に必要な高度硫酸化領域が少ないため,抗Xa/抗トロンビン活性比が大きいことが知られています.

ダナパロイドの抗Xa活性と抗トロンビン活性の比は22/1以上で,未分画ヘパリンの1/1,低分子量ヘパリンの2~4/1に比べ格段に高く,より選択的にXa活性を抑制することが認められています(表1)[4].したがって,理論的には,ダナパロイドでは未分画ヘパリンや低分子量ヘパリンより出血の副作用が少なくなることが期待できます.実際にラットの出血モデルにおいて,同程度の出血症状を示す投与量の抗Xa活性値を比較すると,ダナパロイドは未分画ヘパリンの5~10倍ほど高く,すなわち,同じ抗Xa活性用量では,ダナパロイドでの出血性副作用が格段に少ないことが確認されています.また,静注単回投与時での血中抗Xa活性半減期は約21時間と,未分画ヘパリンの0.7時間や低分子量ヘパリンの1.7時間と比較して長く,連続投与においては投与5日目でほぼ定常状態に達し,蓄積性も認められていません.

表1 ヘパリン類製剤の比較の性質

ヘパリン類	未分画ヘパリン	低分子量ヘパリン	ヘパラン硫酸
成 分	ヘパリン	ヘパリンを分解・精製	主にヘパラン硫酸
分子量	5,000~20,000	約5,000	約5,500
抗Xa/Ⅱa活性比	1	2~4	22
血中半減期	約0.5~1時間	約2時間	約20時間
血小板への影響	強い	弱い	極めて弱い
適応症	DIC 血液凝固の防止 血栓塞栓症の治療・予防 体外循環装置使用時の血液凝固予防	DIC 体外循環装置使用時の血液凝固予防	DIC
DIC治療:用法・用量	5,000~10,000単位/day	75単位/kg/day	1,250単位×2回/day

(文献4を参照して作成)

HIT 抗体との交差反応性は？

A 抗凝固薬としてヘパリンを投与したにもかかわらず，重篤な血栓症（脳梗塞，肺塞栓症，深部静脈血栓症など）を伴う血小板減少（II型 HIT）をきたすことがあります．

これはヘパリンの重篤な副作用として知られており，活性化血小板から放出される血小板第 4 因子（platelet factor 4：PF4）と，ヘパリンとの複合体を抗原とした抗体（HIT 抗体）の出現による免疫学的病態です．HIT 抗体は，さらに血小板を活性化して，血小板凝集・血小板減少を起こし，加えて血管内皮上のヘパラン硫酸と PF4 との複合体にも反応して内皮細胞を傷害（組織因子発現）し，動静脈に血栓症を生ずる重篤な病態です．

ヘパリノイド製剤であるダナパロイドは，英国，ドイツ，フランスなどの欧州ではII型 HIT や深部静脈血栓症（DVT）に対して適応が認められ，その有用性も高く評価（8th ACCP ガイドラインでは Grade 1B と推奨）[5]されています．しかし，一部に，HIT 治療に用いられたダナパロイド療法においても血小板の増加がみられず，HIT 抗体がダナパロイドと交差反応を示したとの報告もあります[6]．このため，日本においては，使用上の注意として「ヘパリン起因性血小板減少症の既往歴があり，ヘパリン抗体と本剤との交差反応性（HIT の既往歴があり，ヘパリン抗体と本剤との交差反応性）のある患者」では原則禁忌とされています．

モニタリングは？

A ヘパリン類似物質・ヘパラン硫酸を主成分とする抗凝固薬・ダナパロイドの抗Xa/抗トロンビン活性比は，22/1 とヘパリンの 1/1 に比べ極めて大きく，低分子量ヘパリンと同様に，APTT の延長もほとんどみられないことより，通常，そのモニタリング検査は行われません．しかし，時に HIT の既往のある患者などでの血小板減少の恐れや，過量投与により出血症状が現れる恐れがあるので，血小板数，凝血学的検査（APTT を含む），便中ヘモグロビンなどの定期的検査が推奨されています．

中和薬としてプロタミンは有効ですか？

A ヘパリンには中和薬としてプロタミンがあり，出血性副作用への緊急時対応策としてプロタミン静脈内投与により，ヘパリンの作用を中和することができます．プロタミンは，AT と拮抗してヘパリンと複合体を形成することで，ヘパリンの抗凝固作用（AT コファクター活性）を中和します．ダナパロイドもヘパリンと同じく，AT コファクター活性による抗凝固作用を示します．しかしプロタミンによる中和効果はヘパリンに比べて弱く，プロタミン 100 mg 投与によるダナパロイド常用量の抗

Xa活性は約17%，抗トロンビン活性は約60%阻害されたとのデータがありますが，通常量のプロタミンでは十分には中和できません．

Q 妊娠時の使用は？

A ダナパロイドの妊娠時における使用は，日本においてはまだ適応となっていません．しかし，ヨーロッパでは数多くの妊娠時使用経験が報告されており，オランダのグループから，HITやヘパリンが無効な血栓症や流産経験83症例・91妊娠での，ダナパロイドの使用経験が報告されています[7)]．

この報告によると，生存出産成功率は90.4%と高率で，副作用も許容範囲であり，HITや低分子量ヘパリンでも無効な妊娠症例での代替抗凝固薬に，ダナパロイドは有効で安全であると報告されています．この中でダナパロイドは抗Xa活性として，1,000〜7,500U/dayを皮下注，もしくは静脈内投与され，母体の血漿中抗Xa活性は0.1〜1.2U/mL，母乳にも0〜0.07U/mLと検出されましたが，胎児・臍帯血には全く検出されていません．これは，動物実験のデータとも一致するもので，ダナパロイドの胎盤通過性は，非常に少ないものと考えられます．こうしたデータから，日本では使用上の注意として「授乳中の婦人への投与は，避けることが望ましいが，やむを得ず投与する場合には，授乳を避けさせること」と記載されています．

日本においても，臨床試験が行われたうえで，抗リン脂質抗体症候群（antiphospholipid syndrome：APS）患者における習慣性流産やヘパリンが使用できない妊娠時血栓症に対しても，ダナパロイドが適応となることが望まれています．

Q 腎機能障害時の使用は？

A ダナパロイドは，重篤な腎障害のある患者では慎重投与が必要で，血清クレアチニン値が2mg/dL以上の場合には，投与量を減らすか投与間隔を延ばす，あるいは投与の中止を考慮することが推奨され，透析患者では原則禁忌となっています．

しかし，欧州などで，ダナパロイドがヘパリンの代わりに，HIT患者の週2〜3回の透析に使われ，長期の投与が安全に実施できることが多くの文献で示されており，透析患者への投与は48時間の投与間隔をあければ可能であると思われます[8)]．

また，ダナパロイドの血中濃度が上昇し，APTTが50秒以上に延長するときは，出血リスクが増大するので，ダナパロイドによるAPTTの延長が疑われる場合には，投与を中止する必要があります．なお，ダナパロイドの血中濃度モニターについては，血中抗Xa活性を測定する必要があり

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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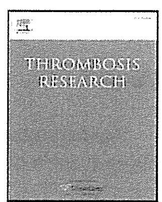

Regular Article

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

Yuki Takagi, Io Kato, Yumi Ando, Yuki Nakamura, Moe Murata, Akira Takagi, Takashi Murate, Tetsuhito Kojima *

Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, 1-1-20, Daiko-Minami, Higashi-ku, Nagoya 461-8673, Japan

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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.

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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.

* Corresponding author. Tel./fax: +81 52 719 3153.
E-mail address: kojima@met.nagoya-u.ac.jp (T. Kojima).

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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).

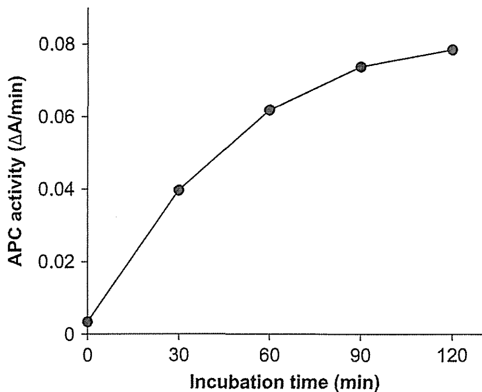

Fig. 1. Time-course of protein C (PC) activation by thrombin in the presence of thrombomodulin (TM). After wild-type prothrombin was sufficiently activated to thrombin using *Oxyuranus scutellatus* (Ox) venom, human PC was added and incubated for 0, 30, 60, 90, and 120 min in the presence of recombinant 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.

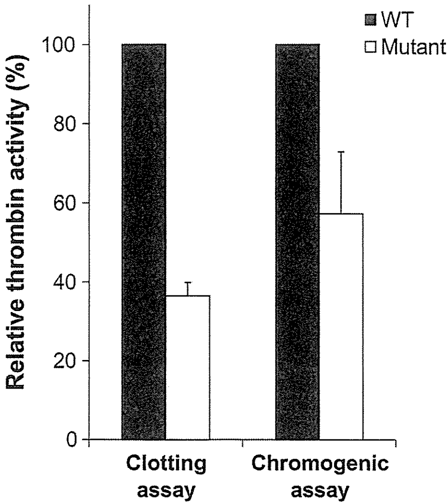

Fig. 2. Relative thrombin activity determined by 2 methods. After recombinant wild-type and mutant prothrombins were sufficiently activated to thrombins using Ox venom, thrombin activities were measured using S-2238 or human fibrinogen without thrombin inhibitor. Experiments were performed in triplicate,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

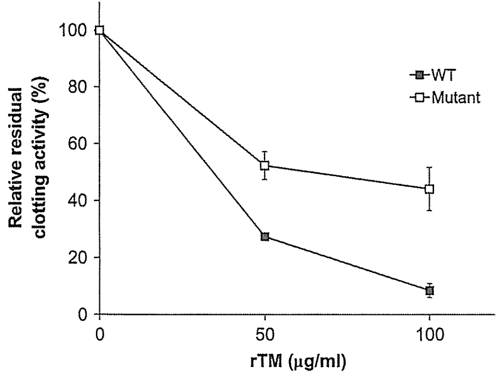

Fig. 3. Effects of rTM on fibrinogen-clotting activity of recombinant thrombins. Wild-type and mutant thrombins were incubated with rTM (0, 50, and 100 μg/mL) for a minute, and relative residual fibrinogen-clotting activities were measured. Experiments were performed in triplicate, and data were presented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

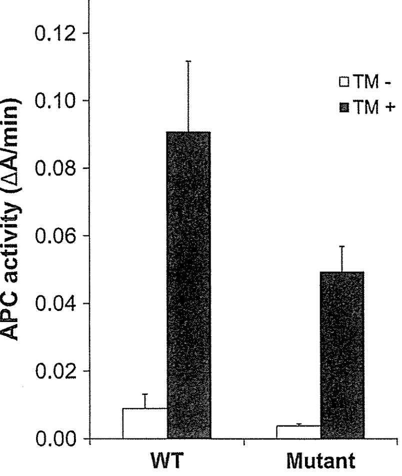

Fig. 4. APC generation assay. Recombinant prothrombins were sufficiently activated to thrombins using Ox venom, human PC was then added, and the combination was incubated for 60 min in the absence or presence of rTM. After the residual thrombin activity was blocked by Pefabloc-TH, APC activities were 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.