Thrombosis Research 159 (2017) 33–38

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

In vitro exploration of latent prothrombin mutants conveying antithrombin resistance

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ARTICLE INFO

Keywords:
Prothrombin
Antithrombin
Mutant prothrombin
Antithrombin resistance
Residual clotting activity score

ABSTRACT

Introduction: Antithrombin resistance (ATR) prothrombinemia is an inherited thrombophilic disorder caused by missense mutations in prothrombin gene (*F2*) at Arg596 of the sodium-binding region. Previously, prothrombin mutants Yukuhashi (Arg596Leu), Belgrade (Arg596Gln), and Padua 2 (Arg596Trp) were reported as ATR-prothrombins possessing a risk of familial venous thrombosis. To identify additional *F2* mutations causing the ATR-phenotype, we investigated the coagulant properties of recombinant prothrombins mutated at amino acid residues within the sodium-binding region by single nucleotide substitutions (Thr540, Arg541, Glu592, and Lys599).

Materials and methods: We constructed expression vectors of prothrombin mutants, established stably transfected HEK293 cells, and isolated the recombinant prothrombin proteins. We evaluated procoagulant activity and ATR-phenotypes of those mutants in reconstituted plasma by mixing with prothrombin deficient plasma.

Results: The secreted quantity of all prothrombin mutants was the same as that of the wild-type prothrombin. Procoagulant activity of each mutant varied from 1.7% to 79.5% in a one-stage clotting assay and from 2.0% to 104.5% in a two-stage chromogenic assay. Most prothrombin mutants tested presented with a severe ATR-phenotype. To estimate the thrombosis risk of these mutations, we determined the residual clotting activity (RCA) after 30 min inactivation with antithrombin. RCA scores, normalized to the wild-type, revealed that prothrombin mutants Lys599Arg (5.35) and Glu592Gln (4.71) had high scores, which were comparable with prothrombins Yukuhashi (4.36) and Belgrade (5.19).

Conclusions: Mutation of prothrombin at the sodium-binding site caused ATR-phenotypes. Of those tested, Lys599Arg and Glu592Gln may possess a thrombosis risk as large as the known pathogenic prothrombins Yukuhashi and Belgrade.

1. Introduction

Venous thrombosis is a typical multifactorial disease in which acquired risks and inherited genetic predisposition play an important role in development [1]. Genetic studies of hereditary thrombophilia have revealed that deficiencies of natural anticoagulants, such as antithrombin (*SELPINC1*) [2], protein C (*PROC*) [3], and protein S (*PROS1*) [4], induce clinically significant but uncommon hypercoagulable conditions. Loss-of-function mutations in each of these factors are associated with an increased risk of thrombosis [5]. In contrast, gain-of-function mutations in factor V gene (*F5*) (*F5* c.1601G > A, Arg506Gln: factor V Leiden) [6] and prothrombin gene (*F2*) (*F2* c.*97G > A: prothrombin G20210A) [7] are more common genetic variants with mild to moderate risk of thrombosis [5]. However, factor V Leiden and prothrombin G20210A are found only in the Caucasian population [8,9].

In 2012, we reported a novel prothrombin missense mutation with familial thrombophilia as the first case of antithrombin resistance

Abbreviations: ATR, antithrombin resistance; *F2*, factor II gene symbol; *F5*, factor V gene symbol; TAT, thrombin-antithrombin complex; PT, prothrombin time; APTT, activated partial thromboplastin time; NCBI, the National Center for Biotechnology Information; HGVS, the Human Gene Mutation Society; RRTA, relative residual thrombin activity; RCA, calculated residual clotting activity; VTE, venous thromboembolism; SNP, single nucleotide polymorphism

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http://dx.doi.org/10.1016/j.thromres.2017.09.020
Received 8 August 2017; Received in revised form 5 September 2017; Accepted 19 September 2017
Available online 20 September 2017
0049-3848/

(ATR): prothrombin Yukuhashi (*F2* c.1787G > T, p.Arg596Leu) [10]. The proband carrying the heterozygous mutation developed venous thromboembolism (VTE) at a young age and continued warfarin treatment. However, she experienced recurrent VTE. The proband's family members carrying the same mutation on *F2* also had episodes of thrombosis. Many of the missense mutations in the prothrombin gene cause hypoprothrombinemia or dysprothrombinemia, leading to a bleeding tendency [11–14]. Mutant thrombin derived from prothrombin Yukuhashi displays a low procoagulant activity; however, it substantially impaired inactivation by antithrombin, resulting in a prolonged clotting function, causing recurrent thromboembolism.

Subsequently, *F2* c.1787G > A (p.Arg596Gln: prothrombin Belgrade) and *F2* c.1786C > T (p.Arg596Trp: prothrombin Padua 2) were reported as ATR-prothrombins [15,16]. Prothrombins Belgrade and Padua 2 are mutants with replacement of the Arg596 residue, same as prothrombin Yukuhashi. Moreover, our previous recombinant mutant study showed that Arg596Gly prothrombin also presented with an ATR-phenotype [17]. Two exosites of thrombin, the γ-loop and sodium-binding region, are critical domains used to form and stabilize the thrombin–antithrombin (TAT) complex [18]. Arg596 resides in the sodium-binding region, using its side chain, directly binds to Asn265 of antithrombin, suggesting that the disruption of the sodium-binding region causes the ATR-phenotype in prothrombin [10]. At other amino-acid residues, alanine altered mutants of Glu592 and Lys599 in the sodium-binding region of thrombin were previously studied and shown to impair the interactions with fibrinogen, protein C, thrombomodulin and antithrombin [19,20].

In this study, to further explore latent abnormal prothrombin conveying the ATR-phenotype, we investigated the procoagulant and ATR characteristics of recombinant prothrombins mutated by single base substitutions at the sodium-binding domain of thrombin. Furthermore, we estimated the thrombotic risk of each mutant prothrombin by calculating a novel index based on their procoagulant activity and resistance to antithrombin inactivation.

2. Materials and methods

2.1. Materials

Bovine factor Va and factor Xa were obtained from Haematologic Technologies (Essex Junction, VT, USA). Human fibrinogen was purchased from Wako Pure Chemical Industries (Osaka, Japan). Neoplastin plus and PTT-Reagent RD were purchased from Roche Diagnostics KK (Tokyo, Japan). Human antithrombin was generously provided by Mitsubishi Tanabe Pharma Co. (Osaka, Japan). The synthetic chromogenic substrate H-D-Phe-Pip-Arg-p-nitroanilide (S-2238) was obtained from Sekisui Medical Co. (Tokyo, Japan).

2.2. Prothrombin mutants

We constructed expression vectors of wild-type prothrombin and mutants with single nucleotide substitutions at Thr540, Arg541, Glu592, and Lys599 using Quik Change Lighting Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) or the overlap extension polymerase chain reaction method [21]. Amino acids were numbered by the Human Gene Mutation Society (HGVS) nomenclature with translation initiation methionine + 1. We transfected HEK293 cells with these mutant prothrombin expression vectors using the calcium phosphate method [22], subsequently established stable transfectants by G418 selection, and prepared the respective recombinant prothrombins as previously described [17]. Prothrombin antigen level was determined by enzyme-linked immunosorbent assay (Enzyme Research Laboratories, South Bend, IN, USA).

2.3. Antithrombin resistance detection assay

In this study, we used a mixture of bovine activated factor X (FXa), bovine activated factor V (FVa), phospholipid, and calcium chloride as a prothrombin activator. The optimum assay conditions were determined empirically with ACL TOP500 CTS (Instrumentation Laboratory). In the first step, 40 μL of prothrombin activating mixture was added to 100 μL diluted sample and incubated for 2 min at 37 °C. In the second step, 20 μL antithrombin solution was added and incubated for various time periods up to 30 min. In the final step, the residual thrombin activity was determined by measuring changes of absorbance at 405 nm (ΔmAbs/min) with a thrombin-specific chromogenic synthetic substrate (S-2238). Relative residual thrombin activity (RRTA) was calculated by the following formula.

$$\mathrm{RRTA}(\%) = \frac{(\text{inactivated thrombin activity at each time point})}{(\text{thrombin activity at 0 min})} \times 100$$

2.4. Measurement of thrombin activity

The prothrombin activity was measured by two different methods, a one-stage clotting assay and a two-stage chromogenic assay, as previously described [10,17]. To examine the coagulant functions of the recombinant prothrombins in plasma, we prepared reconstituted plasma by mixing prothrombin-deficient plasma (prothrombin activity, < 1%; Instrumentation Laboratory, Bedford, MA, USA) with the recombinant prothrombins as described previously [10]. The one-stage clotting assay was performed using a tissue extract prothrombin time reagent, Neoplastin Plus. For the chromogenic assay, the reconstituted plasma was diluted with 50 mmol/L Tris-HCl pH 7.5, and incubated with excess prothrombin activating mixture composed of FXa and FVa, phospholipid, and calcium chloride, for 2 min. After addition of S-2238, the change of absorbance at 405 nm (ΔAbs/min) was measured using TBA-180 (Toshiba Medical Systems Co, Tokyo, Japan). These results were compared with a standard curve generated from diluted normal pooled plasma samples.

2.5. In silico analysis of TAT formation

To predict the effect of each mutation on TAT formation, we performed *in silico* simulations. Wild-type- and mutant-thrombin structures were optimized from the crystal structure of the antithrombin–-thrombin–heparin ternary complex (Protein Data Bank ID: 1TB6) using Discovery studio 4.1 (Accelrys, San Diego, CA, USA). The docking pattern of optimized mutant thrombins to antithrombin were simulated using ZDOCK SERVER (http://zdock.umassmed.edu/), and the respective mutant TAT complex data obtained for the four amino-acid candidates (Thr540, Arg541, Glu592, and Lys599). Root mean square deviation (RMSD) between wild-type TAT complex and the mutant TAT complexes was calculated by Biopolymer structures RSMD using Discovery Studio software. Interaction energies of bonds between mutated thrombin and fragments of antithrombin were also calculated. Molecular structure models were generated using Discovery Studio 4.1 Visualizer (Dassault systemes, Tokyo, Japan). Protein disruption due to an amino-acid replacement was simulated using Polyphen-2 software (http://genetics.bwh.harvard.edu/pph2/).

3. Results

3.1. Preparation and validation of prothrombin mutants

To investigate the resistance of thrombin mutants to antithrombin inactivation associated with the sodium-binding region, we focused on the amino acid residues at Thr540, Arg541, Glu592, and Lys599 of prothrombin (Fig. 1). These residues compose the sodium-binding region in conjunction with Arg596. The single base substitutions of