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

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ABSTRACT

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

Introduction

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

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

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

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

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

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Materials and Methods

Materials

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

Recombinant Prothrombins

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

Kinetic Analysis of Thrombin Inactivation by AT

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

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

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

Demonstration of AT Resistance In Warfarinized Patient Plasma

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

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

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

Results

Assay Conditions In Kinetic Analysis of Thrombin Inactivation by AT

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

Analysis of AT Resistance for Recombinant Prothrombin

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