Thrombin-activatable Fibrinolysis Inhibitor in Chronic Thromboembolic Pulmonary Hypertension

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Materials and Methods

The study protocol was approved by the Ethical Committees of Tohoku University and all patients provided a written informed consent (No. 2011-197).

Study Subjects

We enrolled patients who were older than 20 year-old, and performed right heart catheterization in our institute from August 2011 to June 2013. They were diagnosed as having PAH, CTEPH, or no PH. PH was defined as mean pulmonary artery pressure (mPAP) >25 mmHg at rest.\(^1,2\) The definition of PAH adds the criteria that pulmonary arterial wedge pressure (PCWP) must be \(\leq 15\) mmHg.\(^1-3\) Connective tissue disease and liver disease were diagnosed clinically and by blood tests, as defined by each criteria.\(^4,5\) CTEPH was diagnosed by ventilation-perfusion scintigraphy, computed tomography (CT), optimal coherence tomography and pulmonary angiography after the treatment with anticoagulants for 6 months.\(^2,6-9\) Pulmonary function tests, arterial blood gases, chest X-ray and CT scan were used to diagnose lung disease and hypoxia. When all the above-mentioned abnormalities were ruled out, the patient was diagnosed as having idiopathic PAH.\(^2,3,10\)

We enrolled 27 patients with CTEPH, 22 with PAH (idiopathic PAH in 14, connective tissue disease-associated PAH in 4 and congenital heart disease-associated PAH in 4) and 19 patients without PH (non-PH) as controls (connective tissue disease without PH in 9, systemic hypertension in 4, diastolic cardiomyopathy in 2, congenital heart disease without PH in 1 and others in 3) (Table). Among those patients, we enrolled 25 CTEPH, 22 PAH and 10 without
PH in the plasma clot-lysis assay.

Data Collection

Baseline demographic information (age and sex), clinical diagnosis, comorbidities (connective tissue diseases, liver diseases and congenital heart diseases), drugs (angiotensin convert enzyme inhibitor/angiotensin receptor blocker, Ca channel blocker, epoprostenol, oral prostaglandin I\textsubscript{2} analogue, endothelin receptor antagonists, phosphodiesterase (PDE) - V inhibitors and warfarin) and hemodynamic data from cardiac catheterization were recorded for each patient (Table). Hemodynamic parameters examined included PCWP, mPAP, systolic pulmonary arterial pressure (sPAP), diastolic PAP (dPAP), right atrial pressure (RAP), cardiac output (CO), cardiac index (CI), systolic blood pressure (sBP), diastolic BP (dBP), mean BP (mBP), pulmonary vascular resistance (PVR) and systemic vascular resistance (SVR) (Table).

Whole Blood Clot Lysis Assay

The whole blood clot-lysis assay was performed, using a modification of the methods by Frenkel et al.\textsuperscript{11} Briefly, during the right heart catheterization, we obtained 10 ml of whole blood without anticoagulants from pulmonary artery and divided the blood into 1 ml in each tube. Then, the tubes were incubated at room temperature for 4 hours. Our preliminary data showed that the whole blood was completely coagulated in 4 hours at room temperature. We examined the weight of whole blood clot and then added 10 and 100 ng/ml of tissue plasminogen activator (tPA, monteplase, Eisai, Tokyo, Japan) or 1.0 and 10 U/ml of urokinase (Calbiochem, LA Jolla, CA) with the clot. After the clot was incubated at 37°C for 4 hours, we finally measured the weight of undissolved clot and calculated the extent of the clot remaining after 4 hours. To
examine the effects of the inhibition of activated platelets or activated TAFI (TAFIa), 35.4 ng/ml of prostaglandin E$_1$ (PGE$_1$, Calbiochem, LA Jolla, CA) or 250 and 1250 μg/ml of a carboxypeptidase R inhibiting peptide (CPI-2KR), an inhibitor of activated TAFI composed of 9 amino acids (Cys-Lys-Pro-Ala-Lys-Asp-Ala-Arg-Cys), was added at the moment of blood withdrawal before coagulation started. Then, we performed the whole blood clot-lysis assay with 100 ng/ml of monteplase.

**Plasma Clot-Lysis Assay**

The plasma clot-lysis assay was performed in a 96-well microtiter plate, using a modification of the previous methods. During right heart catheterization, we obtained 5 ml of whole blood with 0.313 % citrate acid, which was centrifuged at 1,100 G for 10 min to obtain plasma. Human plasma (100 μl) from each patient was mixed with 60 μl assay buffer A (100 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4, 0.8 mmol/L CHAPS) at room temperature. Then, 500 ng/ml monteplase, 10 mmol/L CaCl$_2$ and 2.5 U/ml human thrombin (Sigma Aldrich, St. Louis, MO) were mixed and clot formation and lysis were monitored at 405 nm every 5 min at 37 °C, using SpectraMAX M2e microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA). The clot-lysis time was defined as the time from peak to the half of the peak and bottom. To evaluate the direct role of human TAFI to clot-lysis time, we added human TAFI (Hematologic Technologies Inc., Essex Junction, VT) at the concentration of 3.0 μg/ml or 6.0 μg/ml to 6 different plasmas of healthy volunteers.

**Laboratory Testing**

During right heart catheterization, we obtained 5 ml of whole blood with 0.313 % citrate acid,
which was centrifuged at 1,100 G for 10 min to obtain plasma. Plasma levels of plasminogen, tPA/PAI-1 complex (total PAI-1) and $\alpha_2$-antiplasmin/plasmin complex (PIC) were measured by the SRL Laboratory Co (Tokyo, Japan). Plasma levels of TAFI were measured in Clinical Pharmaceutics Educational Center, Nihon Pharmaceutical University, using an enzyme-linked immunosorbent assay (ELISA).\textsuperscript{16,17} Ninety-six-well microtiter plates were coated with 6 $\mu$g/ml of a monoclonal antibody (2A16) against TAFI (Protein Science Co. Ltd., Nagoya, Japan). After washing with PBS in 0.05% tween20, 50 $\mu$l of 1/600 diluted plasma was added and the plates were left at room temperature for 1 hour. Following an additional PBS in 0.05% tween20 washing, 50 $\mu$l of another horseradish peroxidase (HRP)-conjugated monoclonal antibody against proCPR (10G1) was added. After 1 hour at room temperature, the plates were washed and 100 $\mu$l of $o$-phenylenediamine-$H_2$O$_2$ and 100 $\mu$l of 3N H$_2$SO$_4$ were added to stop the reaction and then measured at 492 nm using 680 XR (Bio Rad, Hercules, CA). Plasma TAFI levels of patients were measured using the solution of human TAFI (Hematologic Technologies Inc., Essex Junction, VT). Plasma levels of TAFI were measured at the first catheterization and 3 months after percutaneous transluminal pulmonary angioplasty.

We calculated the intra-assay coefficient variation and inter-assay coefficient variation in ELISA of TAFI and found that the intra-assay coefficient variation was 3.0% and the inter-assay coefficient variation was 5.3%.

**Platelet Preparation and Assay of Platelet-released TAFI**

All the isolation steps were carried out at room temperature. Blood from the pulmonary artery of patients was collected with 0.313 % citrate acid. The citrate-anticoagulated blood was
centrifuged at 150 G for 10 min to prepare platelet-rich plasma (PRP). Then, 21.2 ng/ml PGE$_1$ was added to the PRP, which was centrifuged at 1,100 G for 10 min to sediment platelets. The supernatant (the plasma) was discarded and platelet pellet was gently re-suspended in 10 ml isotonic citrate (IC) buffer (50 mmol/L sodium citrate, 100 mmol/L NaCl and 138 mmol/L dextrose). After 10 min incubation, platelets were centrifuged at 1,100 G for 10 min, discarded the supernatant (the first supernatant) and re-suspended in 10 ml IC buffer, which procedure was repeated (the second supernatant). The platelets were re-suspended with HEPES-Tyrode buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 2.68 mmol/L KCl, 0.42 mmol/L NaH$_2$PO$_4$, 1.7 mmol/L MgCl$_2$, 11.9 mmol/L NaHCO$_3$, 2mmol/L CaCl$_2$ and 5 mmol/L glucose) in the concentration of 2.5×10$^9$ platelets/ml and activated by the addition of 0.5 U/ml human thrombin (Sigma Aldrich, St. Louis, MO, USA) for 20 min at 25°C with occasional mixing; this was followed by pelleting of activated platelets by centrifugation for 5 min at 3,000 G and 25°C and the supernatant was collected (the third supernatant). Hence, the TAFI content in the second supernatant and the third supernatant can be directly compared with immunoblotting using an anti-TAFI monoclonal antibody (provided by Clinical Pharmaceutics Educational Center, Nihon Pharmaceutical University).16

**Flow Cytometric Analysis of Platelets**

Whole blood without citrate acid was incubated with a monoclonal antibodies of PAC-1 FITC (Becton Dickinson and Company, Franklin Lakes, NJ), a marker of activated α2b-β3 complex,19 CD62P-PE (Becton Dickinson and Company, Franklin Lakes, NJ), a marker of P-selectin,19 and CD61-PerCP, an activation-independent platelet marker19 (Becton Dickinson and Company, Franklin Lakes, NJ) in a tube for 20 min at 25°C in the dark with tapping every 3 min. Mouse
IgG1-PE control antibody (Becton Dickinson and Company, Franklin Lakes, NJ) and RGDS peptides (Sigma Aldrich, St. Louis, MO) were used to evaluate non-specific binding. After the incubation, the samples were fixed with 500 µl of 1% paraformaldehyde in phosphate-buffered saline at 4°C. Within 24 hours, the samples were analyzed with FC-500 flow cytometer (Beckman Coulter, Fullerton, CA). The platelets were identified by size on forward and side scatter plots and the presence of the CD61 antigen. PAC-1 binding and P-selectin expression was calculated as % of total platelet numbers. Five thousand gated (platelets identified by CD61-positive staining) events were collected per tube.

**Genotyping of CPB2**

We obtained DNA from white blood cells from the SRL Laboratory CO and genotyped the single nucleotide polymorphisms (SNPs) of CPB2 (rs7999168, rs1926447 and rs1087) by the BEX Co., Ltd (Tokyo, Japan). For PCR, we used the primers, including ATTCCCATTGGTTAATGCAC and TTCTCCACATATGAGCAGAC for rs7999168, GCCACCCAATTGTGATTGCC and GCTTCACTCAACTAGTATGATGCC for rs1926447, and TTGACAGTACCTAGAGCCAC and TTTACTGATTCCAGCAAGACC for rs1087. The PCR reaction system (50 µL) contained 10 µL reaction buffer, 0.5 µL PrimeSTAR® HS DNA polymerase (Takara, Ohtsu, Japan), 0.2 mM dNTP, and 0.3 µM upstream primer and downstream primer. DNA (50 ng) was supplemented and PCR systems was employed with the reaction conditions as follows: 98 °C for 2 min; 98 °C for 10 sec, 58 °C for 10 sec, and 72 °C for 1 min with 30 repeats. We used the primers for sequence, including ACTCTGTAGCCCAAGCTG for rs7999168, GCTTCACTCAACTAGTATGATGCC for rs1926447, and TTGACAGTACCTAGAGCCAC and TTTACTGATTCCAGCAAGACC for rs1087. We used the BigDye® Terminators v1.1 Cycle Sequencing Kit (Applied
Biosystems, Foster City, CA, USA) and ABI Prism 3130xl Genetic Analyzer (Applied Biosystems) was employed.

**Statistical Analysis**

All statistical analyses were performed using JMP Pro (SAS Institute Inc., Cary, NC). The clot-lysis time by plasma clot-lysis assay are expressed as “medians (interquartile ranges)” as these values were not normally distributed. All other continuous variables are expressed as the mean ± SD. Multiple comparisons of continuous variables were analyzed by Tukey-Kramer test after one-way ANOVA. Since the clot-lysis time by plasma clot-lysis assay and the levels of total PAI-1 were not normally distributed, they were log-transformed in order to use Tukey-Kramer test. The relation between plasma levels of TAFI and the extent of clot remaining after 4 hours, clot-lysis time or the improvement of the extent of clot remaining after 4 hours was analyzed with Spearman's rank correlation coefficient. Categorical variables were presented as percentages, and intergroup comparisons of categorical variables were analyzed by Fisher's exact test. The comparisons of the extent of clot remaining after 4 hours in the absence of any drug and in the presence of TAFIa inhibitor (250 or 1250 µg/ml), PGE₁ (35.4 ng/ml) and the change in the plasma levels of TAFI after PTPA and clot-lysis time in the absence of human TAFI and in the presence of human TAFI were performed by paired t-test. Comparisons of means between 2 groups were performed by Student’s t-test. P-value less than 0.05 was considered to be statistically significant.
Supplemental References


