MATERIALS AND METHODS

Platelets are highly activated in patients of chronic thromboembolic pulmonary hypertension (CTEPH)

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Study Population
The study enrolled the patients without pulmonary hypertension (non-PH) (n=15, hypertension n=7, hyperlipidemia n=5, hypertrophic cardiomyopathies n=2 and paroxysmal atrial fibrillation n=1), the patients with pulmonary artery hypertension (PAH) (n=19, idiopathic pulmonary hypertensions n=10, collagen diseases with pulmonary hypertension n=8, congenital heart disease with pulmonary hypertension n=1) and the patients with chronic thromboembolic pulmonary hypertension (CTEPH) (n=25). All patients underwent cardiac catheterization. The diagnosis of PAH and CTEPH was made according to the ESC/ERS Guideline. Among those patients, platelets of 12 non-PH, 14 PAH and 17 CTEPH patients were stimulated with ex vivo 0.05U/ml thrombin.

Preparation of the washed platelets
At the beginning of the right cardiac catheterization, the blood samples (4 ml) were collected in a tube containing a final concentration of 0.313% sodium citrate. Platelet rich plasma (PRP) was prepared by centrifugation of blood at 200 G at 25°C for 10 min. Then, the washed platelets were prepared by gel filtration column chromatography, as described with slight modification. Briefly, 1 ml PRP was separated by 6 ml Sepharose 2B with Hepes-Tyrode Buffer without Ca²⁺ (10 mM Hepes, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂, 11.9 mM NaHCO₃, 5 mM glucose) with 0.3% bovine serum albumin. Washed platelets were obtained in 2 ml in the elution volume 2-4 ml as shown in Fig. 1A, within 1 hour after blood collection.

The flow cytometric analysis of the platelets
Washed platelets (1.25 μl) were immediately incubated with the monoclonal antibodies against PAC-1 FITC, CD62P-PE (Beckton-Dickinson) and CD61-PerCP (Beckton-Dickinson) in a tube for 20 min at 25°C in the dark. Mouse IgG1-PE control antibody (Beckton-Dickinson) and RGDS peptides (Sigma) were used to evaluate for non-specific binding. After the incubation, the samples were fixed by 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). 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 were calculated as % of total platelet numbers (n=5,000).

We also performed the flow cytometric analysis to examine the effect of weak stimulation with thrombin on platelets activation. Washed platelets of 10 non-PH, 14 PAH and 17 CTEPH patients were stimulated by 0.05 U/ml thrombin (Sigma) and 5 mM CaCl$_2$ at 25°C for 20 min and the flow cytometric analysis was performed. Washed platelets of non-PH (n=5), PAH (n=4), CTEPH patients (n=6) were also stimulated by 0.1 U/ml thrombin, 0.5 μM PAR1 agonist (SFLLRN) (Sigma) or 5 μM PAR4 agonist (AYPGFK) (Sigma) at 25°C for 20 min and the flow cytometric analysis was performed.

Preparation of the recombinant proteins for the pull down assay

The glutathione-S transferase (GST) fusion proteins for the pull down assay to evaluate GTP-bound GTPases were produced and purified as described previously$^3$ using the following plasmids. PGEX2T Rap1 binding domain of Ral GDS (amino acid residues of 801-883) for Rap1 that was cloned from cDNA of the brain of human, PGEX2T Ral binding domain of Sec5 for RalA,$^4$ PGEX2T Ras binding domain of Raf for Ras,$^5$ PGEX2T N-terminal fragment of human Daam1 for RhoA,$^6$ and PGEX2T p21 binding domain of PAK1 PBD for Rac1.$^6$

All of the purified recombinant proteins were extensively dialyzed against Buffer A (50 mM Hepes/KOH, pH 7.2, 78 mM KCl, 4 mM MgCl$_2$, 0.2 mM CaCl$_2$, 2 mM EGTA, 1 mM dithiothreitol) and stored at -80°C until use. Protein concentrations were determined by the intensities of the bands on Coomassie Brilliant Blue-stained SDS-polyacrylamide gels using bovine serum albumin as a standard.

The pull down assay for the washed platelets

Washed platelets were incubated with 0.5% Triton X-100 at 4°C for 5 min. After centrifugation of the samples at 300,000 G at 4°C for 5 min, the supernatants were obtained as the platelet lysates. Then, the platelet lysates were incubated with glutathione-Sepharose beads coated with 200 μg of each GST fusion protein at 4°C for 30 min. After washing the beads by Buffer A three times, beads-associated RalA, RhoA, Rap1, Rac1 and Ras proteins were analyzed by immunoblotting with anti-RalA (BD transduction laboratory), anti-Rap1 (BD transduction laboratory), anti-Rac1 (BD transduction laboratory) and anti-Ras (Millipore) monoclonal antibodies and anti-RhoA polyclonal antibodies (Santa Cruz Biotechnology), respectively, and were visualized with chemiluminescence.
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Densitometric analysis was performed on the blots using Image J 1.38x software (National Institutes of Health). The data were expressed as the percentage of GTP-bound GTPases of total amount.

Endothelial cell culture and stimulation with conditioned media of thrombin-activated platelets

Human lung microvascular endothelial cells (HMVECs) were purchased from Lonza and cultured with EGM™-2MV SingleQuots™ Kit (EGM-2) (Lonza). EGM-2 was exchanged to EBM™-2MV Basal Medium (EBM-2) (Lonza) 10 hours before stimulation. Washed platelets were stimulated by 0.05 U/ml thrombin (Sigma) and 5 mM CaCl₂ at 25°C for 20 min and were centrifuged at 20,400 G at 4°C for 10 min and the supernatant was collected as conditioned media. The control conditioned media was prepared in the same way with thrombin in the absence of platelets. Subconfluent HMVECs were washed with PBS and added with 1.5 ml EBM-2 and 0.5 ml either conditioned medium. The HMVECs were cultured at 37°C for 12 hours. The cells were solubilized with 1% Triton-X after washing once with PBS. Then, the supernatant was collected after centrifugation at 20,400 G for 1 min.

The expression of VCAM-1 or β-actin in endothelial cells was analyzed by immunoblotting with anti-VCAM-1 antibody (Cell Signaling) and anti-β-actin antibody (Sigma) and was visualized with chemiluminescence (ECL Prime, GE Healthcare). Densitometric analysis was performed on the blots using Image J 1.38x software (National Institutes of Health).

Western blotting for RalGAPβ

The RalGAPβ protein or β-actin in the lysate of washed platelets was analyzed by immunoblotting with anti-RalGAPβ antibody and anti-β-actin antibody (Sigma) and was visualized with chemiluminescence (ECL Prime, GE Healthcare). Densitometric analysis was performed on the blots using Image J 1.38x software (National Institutes of Health).

Statistical analysis.

The ratios of P-selectin or PAC-1 positive platelets and GTP-bound GTPases are expressed as median (interquartile ranges [IQR]) because of these were not normal distribution. All other continuous variables were expressed as the mean ±
SD. Multiple group comparison of continuous variables was analyzed by ANOVA followed by Tukey’s HSD (honest significant difference) test or Kruskal-Wallis test followed by Steel-Dwass test, as appropriate. Categorical variables were presented as frequency counts and percentages, and intergroup comparisons of categorical variables were analyzed by Fisher’s exact test.

To determine an optimal set of independent factors for the activation of RhoA and RalA, the ratios of P-selectin positive platelets or PAC-1 positive platelets, multiple regression analysis with backward elimination stepwise variable selection was utilized, which involves starting with all PH drugs, epoprostenol, oral prostaglandin I$_2$ analogue, endothelin receptor antagonist, phosphodiesterase V inhibitor.

All statistical analyses were performed using JMP Pro (SAS Institute Inc., Cary, NC) and R version 3.1.1, and P values <0.05 were considered to be statistically significant.

References