Thrombin-Activatable Fibrinolysis Inhibitor in Chronic Thromboembolic Pulmonary Hypertension

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Objective—The pathogenesis of chronic thromboembolic pulmonary hypertension (CTEPH) remains to be elucidated. Thrombin-activatable fibrinolysis inhibitor (TAFI) inhibits fibrinolysis. It remains to be elucidated whether TAFI is directly involved in the pathogenesis of CTEPH. We examined potential involvement of TAFI in the pathogenesis of CTEPH in humans.

Approach and Results—We enrolled 68 consecutive patients undergoing right heart catheterization in our hospital, including those with CTEPH (n=27), those with pulmonary arterial hypertension (n=22), and controls (non–pulmonary hypertension, n=19). Whole blood clot lysis assay showed that the extent of clot remaining after 4 hours was significantly higher in CTEPH compared with pulmonary arterial hypertension or controls (41.9 versus 26.5 and 24.6%, both $P<0.01$). Moreover, plasma levels of TAFI were significantly higher in CTEPH than in pulmonary arterial hypertension or controls (19.4 ± 4.2 versus 16.1 ± 4.5 or 16.3 ± 3.3 μg/mL, both $P<0.05$), which remained unchanged even after hemodynamic improvement by percutaneous transluminal pulmonary angioplasty. Furthermore, the extent of clot remaining after 4 hours was significantly improved with CPI-2KR (an inhibitor of activated TAFI) or prostaglandin E_1 (an inhibitor of activation of platelets). Importantly, plasma levels of TAFI were significantly correlated with the extent of clot remaining after 4 hours. In addition, the extent of clot remaining after 4 hours was improved with an activated TAFI inhibitor.

Conclusions—These results indicate that plasma levels of TAFI are elevated in patients with CTEPH and are correlated with resistance to clot lysis in those patients. (*Arterioscler Thromb Vasc Biol*. 2016;36:1293-1301. DOI: 10.1161/ATVBAHA.115.306845.)

Key Words: angioplasty ■ fibrinolysis ■ hemodynamics ■ plasma ■ pulmonary hypertension

Chronic thromboembolic pulmonary hypertension (CTEPH) is one of the distinct disease entities of pulmonary hypertension (PH), characterized by obstruction of major pulmonary artery by organized thrombus and pulmonary vascular remodeling.[1–4] CTEPH leads to increased pulmonary vascular resistance, progressive PH, and right heart failure to death.[2,5]

CTEPH has been considered to occur after acute pulmonary embolism or recurrent pulmonary embolism; however, it is also known that few patients have deep vein thrombosis or episodes of acute pulmonary embolism.[6] There are few reports about the association between CTEPH and coagulation abnormality, such as antiphospholipids antibody[7] and fibrinogen mutation.[8,9] Furthermore, it has been reported that plasma levels of tissue-type plasminogen activator (tPA) and those of plasminogen activator inhibitor (PAI) were not related to the severity of CTEPH.[10] Thus, it remains to be examined whether

fibrinolysis capacity is impaired in CTEPH and if so, what molecular mechanism(s) is involved.

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma carboxypeptidase inhibitor produced by liver. TAFI is activated by thrombin, thrombin/thrombomodulin complex, and plasmin, whereas activated form of TAFI (TAFIa) removes the C-terminal lysines from fibrin and reduces the binding of tPA and plasmin to fibrin.[11] It has been demonstrated that activated platelets support the generation of thrombin,[12] which will cause activation of TAFI. TAFI has been reported to be a risk factor of recurrent deep vein thrombosis,[13] although it remains unknown whether TAFI is involved in the pathogenesis of CTEPH.

Current guidelines recommend that patients with CTEPH should be treated with life-long anticoagulation and some drugs specific for pulmonary arterial hypertension (PAH).[2] Recently, it has been reported that riociguat, a stimulator of soluble guanylate cyclase, significantly improves exercise capacity

Nonstandard Abbreviations and Acronyms	
CTEPH	chronic thromboembolic pulmonary hypertension
PAH	pulmonary arterial hypertension
PAI	plasminogen activator inhibitor
PH	pulmonary hypertension
PTPA	percutaneous transluminal pulmonary angioplasty
TAFI	thrombin-activatable fibrinolysis inhibitor
tPA	tissue-type plasminogen activator

and pulmonary vascular resistance in patients with CTEPH.[14] Although pulmonary thromboendarterectomy is an established surgical treatment for central-type CTEPH,[15] recent studies have demonstrated that percutaneous transluminal pulmonary angioplasty (PTPA) is effective for distal-type CTEPH.[16,17] However, as the precise mechanisms of CTEPH have not been fully elucidated, fundamental therapy remains to be developed.

In this study, we thus examined whether fibrinolysis capacity is impaired in CTEPH patients with a special reference to TAFI and if so, whether TAFI is directly involved in the pathogenesis of CTEPH in humans.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Clinical Characteristics of Study Subjects

Clinical characteristics of the study subjects are shown in Table. There were no significant differences in the baseline characteristics among the 3 groups except body mass index and the use of pulmonary vasodilators (epoprostenol, oral prostaglandin I$_2$ analog, endothelin receptor antagonists, and phosphodiesterase-V inhibitors) and warfarin (Table). Although body mass index was higher in patients with CTEPH than in PAH or non-PH patients, the prevalence of obesity (body mass index >25) was comparable among the groups ($P=0.25$). Endothelin receptor antagonists were used more in patients with PAH than in patients with CTEPH. In pulmonary hemodynamics, pulmonary arterial pressure and pulmonary vascular resistance were significantly higher in PAH and CTEPH than in non-PH, which were comparable between PAH and CTEPH (Table). Systolic blood pressure was significantly lower in PAH and CTEPH than in non-PH (Table).

Resistance to Fibrinolysis in Whole Blood Clot Lysis Assay

The whole blood clot lysis assay was performed using a modification of the method by Frenkel et al.[18] In this study, 8 non-CTEPH patients were treated with warfarin, and 33 non-CTEPH patients were not treated with warfarin. The extent of clot remaining after 4 hours with 100 ng/mL monteplase was comparable between the 2 groups (24.9±13.0 versus 27.8±7.4%, $P=0.31$; Figure I in the online-only Data Supplement). Thus, the results were not affected by the treatment with warfarin in this study. There was no significant difference in the extent of

Table.   Clinical Characteristics of Study Subjects

	Non-PH (n=19)	PAH (n=22)	CTEPH (n=27)
Clinical characteristics			
Age, y	59±12	48±14	57±16
Female, %	78.9	90.1	85.2
Body mass index, kg/m²	21.1±3.2	21.6±3.4	24.8±4.5**,†
Obesity (body mass index >25), %	15.8	13.6	33.3
Diabetes mellitus, %	5.2	0	11.1
History of smoking, %	21	23	30
Antiphospholipid antibody, %	…	…	3.7
Protein C or S deficiency, %	…	…	0
Red blood cell, ×10⁴/µL	422±53	421±44	412±52
Hemoglobin, g/dL	12.6±1.8	12.5±1.5	12.7±1.9
Platelet, ×10³/µL	234±71	193±64	219±77
Mean platelet volume, fl	8.4±1.0	8.7±0.9	8.7±1.0
ACE inhibitor/ARB, %	21	14	15
Calcium-channel blocker, %	21	14	15
Epoprostenol, %	0	41*	11
Oral PGI$_2$ analog, %	5	50**	44**
Endothelin receptor antagonist, %	0	77**	22*,†
PDE-V inhibitor, %	0	77**	70**
Warfarin, %	21	18	100**,†
Hemodynamic data			
mRAP, mm Hg	3.8±2.5	5.2±2.2	6.5±3.9*
Systolic PAP, mm Hg	25.1±5.2	65.7±17.2**	63.7±20.1**
Diastolic PAP, mm Hg	9.2±4.0	27.5±7.2**	22.6±7.3**
Mean PAP, mm Hg	15.5±3.4	41.4±10.8**	37.1±10.1**
PCWP, mm Hg	6.5±2.8	9.4±2.8*	9.1±3.1*
Systolic BP, mm Hg	137.3±24.5	104.5±15.4**	113.9±16.4**
Diastolic BP, mm Hg	73.8±13.8	61.4±13.8*	63.3±10.8*
Mean BP, mm Hg	98.0±15.4	76.3±14.9**	82.3±9.7**
PVR, dyn/s per cm⁵	179.6±74.2	585.6±270.0**	585.9±288.3**
SVR, dyn/s per cm⁵	1872.9±286.6	1297.5±336.3**	1571.8±443.0
CO, L/min	4.05±0.39	4.61±0.93	4.34±1.26
CI, L/min per m²	2.74±0.34	2.98±0.55	2.75±0.77

Results are expressed as mean±SD. ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; BP, blood pressure; CI, cardiac index; CO, cardiac output; CTEPH, chronic thromboembolic pulmonary hypertension; mRAP, mean right atrial pressure; PAH, pulmonary artery hypertension; PAP, pulmonary arterial pressure; PCWP, pulmonary wedge pressure; PDE, phosphodiesterase; PGI$_2$, prostaglandin I$_2$; PH, pulmonary hypertension; PVR, pulmonary vascular resistance; RAP, right arterial pressure; and SVR, systolic vascular resistance.

*$P<0.05$, **$P<0.01$ vs non-PH, and †$P<0.05$ vs PAH.

clot remaining after 4 hours in response to urokinase among the 3 groups, although both CTEPH and PAH tended to be resistant to 1.0 U/mL of urokinase compared with non-PH (Figure 1A). In contrast, importantly, in the whole blood clot lysis assay with monteplase, the extent of clot remaining after 4 hours was significantly higher in CTEPH than in other 2 groups (Figure 1B); the extent of clot remaining after 4 hours in response to 100 ng/mL of monteplase in non-PH, PAH, and CTEPH was 26.5±12.8, 24.6±11.4, and 41.9±15.2%, respectively (non-PH versus CTEPH, $P<0.01$; PAH versus CTEPH, $P<0.01$; Figure 1B). These results suggest that the clot of CTEPH is more resistant to fibrinolysis than that of non-PH or PAH.

Resistance to Fibrinolysis in Plasma Clot Lysis Assay

The plasma clot lysis assay was performed using a modification of the previous methods.[19,20] In the plasma clot lysis assay, the clot lysis time of non-PH, PAH, and CTEPH was 616 (514–714), 647 (502–844), and 788 (657–1116) seconds (median [interquartile ranges]), respectively (Figures 1C–1F), indicating that the plasma clot of CTEPH tended to be more resistant than that of non-PH or PAH. Wet weight of the whole blood clot was comparable among the 3 groups (Figure 1G).

Elevated Plasma Levels of TAFI in CTEPH

Plasma levels of total PAI-1 and plasminogen were comparable among the 3 groups (Figure 2A and 2B). In contrast, plasma levels of TAFI were significantly higher in CTEPH than in non-PH or PAH (Figure 2C; Figure II in the online-only Data Supplement). Furthermore, plasma levels of TAFI positively correlated with the extent of clot remaining after 4 hours in all patients ($P=0.02$, $R=0.30$; Figure 2D) and positively correlated with the clot lysis time ($P=0.01$, $R=0.42$) in all patients (Figure 2E). Furthermore, we analyzed the correlation between plasma levels of TAFI and clot lysis time in patients with CTEPH. Interestingly, plasma TAFI levels tended to be positively correlated with clot lysis time ($P=0.07$; Figure III in the online-only Data Supplement). Moreover, addition of human TAFI to 6 different plasmas of healthy volunteers, significantly increased the clot lysis time compared with those without human TAFI ex vivo (Figure 2F).

Elevated Platelet–Derived TAFI in CTEPH

Blood from the pulmonary artery of patients was collected and was centrifuged to obtain platelet pellet. The supernatant (the plasma) was discarded, and platelet pellet was resuspended in isotonic citrate buffer. Platelets were centrifuged,
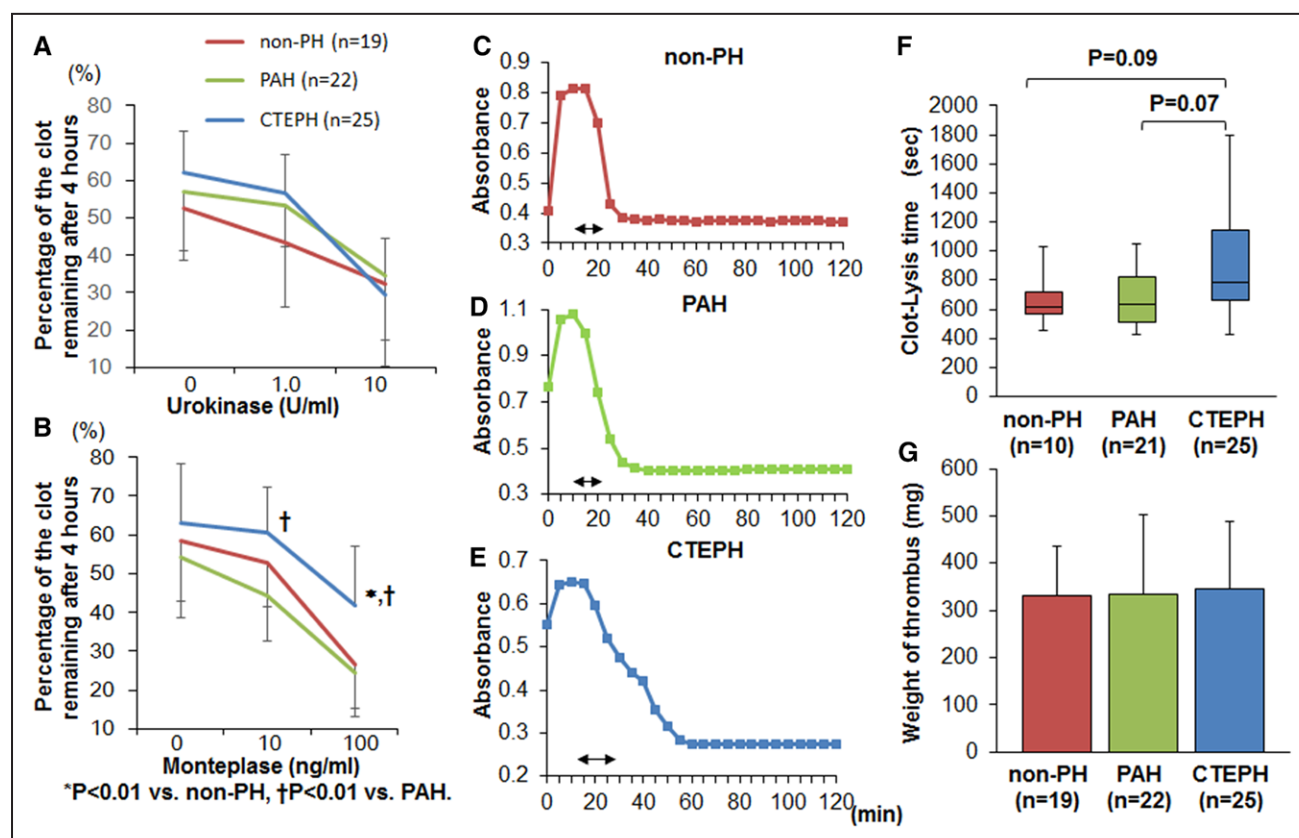

Figure 1. Clot lysis assay with blood from the pulmonary artery. **A–C**, The whole blood clot lysis assay with urokinase or monteplase in patients with non–pulmonary hypertension (PH) (n=19), patients with pulmonary arterial hypertension (PAH, n=22), and patients with chronic thromboembolic PH (CTEPH, n=25). **A**, Percentage of the clot remaining after 4 hours (mean±SD) with normal saline as a control and 1.0 and 10 U/mL urokinase. **B**, Percentage of the clot remaining after 4 hours (mean±SD) with normal saline as a control and 10 and 100 ng/mL monteplase. **C** and **D**, The clot lysis time (↔; clot lysis time) with plasma clot lysis assay in patients with non-PH (n=10), patients with PAH (n=21), and patients with CTEPH (n=25). Representative results of plasma clot lysis assay in patients with non-PH (**C**), patients with PAH (**D**), and patients with CTEPH (**E**). **F**, The results of clot lysis time in 3 groups are shown with box plot. **G**, The weight of the clots (mean±SD) was comparable among the 3 groups. Statistical significance was determined with Tukey–Kramer test. *$P<0.01$ vs non-PH, †$P<0.01$ vs PAH.

and the supernatant (the first supernatant) was discarded and resuspended in isotonic citrate buffer, which procedure was repeated (the second supernatant). TAFI was detected in the plasma and the first supernatant, but not in the second supernatant, by immunoblotting method (Figure 3A), indicating that TAFI from the plasma was completely removed by washing platelet pellet twice. After stimulation with 0.5 U/mL of thrombin at 25°C for 20 minutes, TAFI was detected in the supernatant (the third supernatant), which was considered as platelet-derived TAFI. Although TAFI was released from platelets in all the 3 groups, the TAFI levels in PAH and CTEPH were 1.4 and 2.1 folds compared with non-PH, respectively (non-PH versus CTEPH, $P<0.01$; PAH versus CTEPH, $P<0.05$), indicating that the levels of platelet-derived TAFI were significantly higher in CTEPH than in non-PH and PAH (Figure 3B and 3C).

Effects of PGE$_1$ and TAFIa Inhibitor on Resistance of Clot to Fibrinolysis in CTEPH

To confirm that the resistance to monteplase of the clot in patients with CTEPH was related to the TAFI pathway, we examined the effects of PGE$_1$ (a platelet inactivator) and CPI-2KR (TAFIa inhibitor)[21] in the whole blood clot lysis assay. First, we confirmed that PGE$_1$ was able to inhibit platelet activation (in response to tapping every 3 minutes for 20 minutes at 25°C) by the flow cytometric analysis (Figure 4A and

4B). Treatment with PGE$_1$ significantly decreased the extent of clot remaining after 4 hours when cotreated with 100 ng/mL of monteplase (29.5 ± 13.8 versus $37.8\pm13.4\%$, $P<0.01$; Figure 4C), indicating that the resistance to monteplase of the clots from patients with CTEPH is related to platelet activation. Next, we examined the effects of CPI-2KR (1.25 mg/mL) on the extent of clot remaining after 4 hours. When cotreated with monteplase (100 ng/mL), the TAFIa inhibitor dose dependently improved the extent of clot remaining after 4 hours (Figure 4D). Then, the plasma levels of TAFI positively correlated with the improvement rate of the extent of clot remaining after 4 hours ($P=0.02$, $R=0.52$) (Figure 4E). Moreover, after the treatment with CPI-2KR (1.25 mg/mL), the extent of clot remaining after 4 hours (change ratio) was significantly higher in patients with CTEPH compared with the controls (15.3 ± 11.0 versus $3.1\pm7.0\%$, $P<0.05$; Figure IV in the online-only Data Supplement). These results indicate that the resistance to monteplase of the clot from patients with CTEPH is directly related to the TAFI activation in the plasma.

No Change in Plasma Levels of TAFI After Hemodynamic Improvement by PTPA

Mean pulmonary arterial pressure was significantly reduced after PTPA (39.0 ± 7.9 mm Hg versus 24.9 ± 4.4 mm Hg, $P<0.01$; Figure 5A). In contrast, the plasma levels of TAFI did not change significantly before and after the treatment
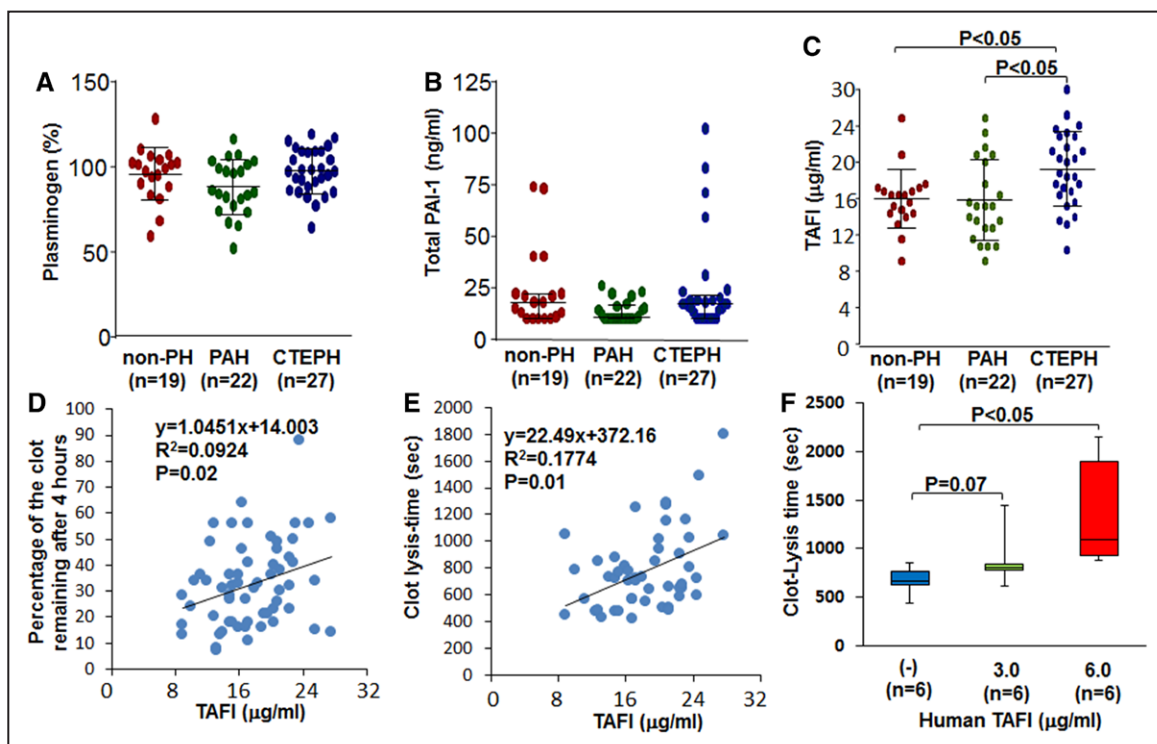

Figure 2. Plasma levels of the antigens related to fibrinolysis. The levels of the antigens related to fibrinolysis in the plasma of patients with non–pulmonary hypertension (PH), patients with pulmonary arterial hypertension (PAH), and patients with chronic thromboembolic PH (CTEPH). The levels of plasminogen (mean±SD) (**A**), plasminogen activator inhibitor (median [interquartile range], **B**), and thrombin-activatable fibrinolysis inhibitor (TAFI, mean±SD, **C**) are shown in patients with non-PH (n=19), patients with PAH (n=22), and patients with CTEPH (n=27). **D**, The correlation between the levels of TAFI and the extent of clot remaining after 4 hours of thrombus is shown. **E**, The correlation between the levels of TAFI and the clot lysis time is shown. **F**, The change of clot lysis time with human TAFI ex vivo was shown with box plot. Statistical significance was determined with Tukey–Kramer test in **A**–**C**), with Spearman rank correlation coefficient in **D** and **E** and with paired *t* test in **F**. PAI-1 indicates plasminogen activator inhibitor-1.
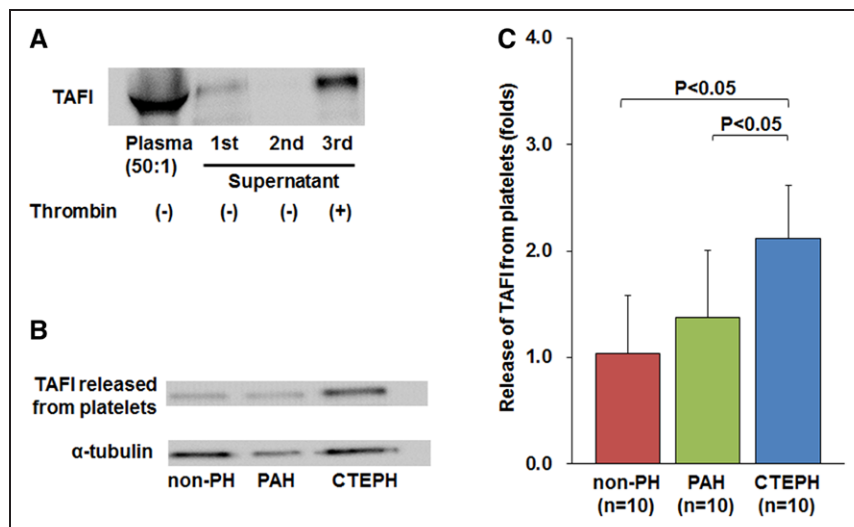
Figure 3. Thrombin-activatable fibrinolysis inhibitor (TAFI) released from platelets. **A**, TAFI was confirmed in the plasma and first supernatant, but not in the second supernatant after the stimulation with 0.5 U/mL thrombin at 20°C for 20 minutes. TAFI was reconfirmed in the third supernatant, which was defined as the levels of TAFI released from platelets. **B**, Representative results of TAFI released from non–pulmonary hypertension (PH), pulmonary arterial hypertension (PAH), and chronic thromboembolic PH (CTEPH) patients. **C**, Quantitative analysis of the levels of TAFI (mean±SD) released from platelets in non-PH, PAH, and CTEPH patients (n=10 each). Statistical significance was determined with Tukey–Kramer test.

(19.9±5.4 versus 20.0±5.1 µg/mL, P=0.92; Figure 5B). These results suggest that the increased plasma levels of TAFI in patients with CTEPH are not the results of increased pulmonary arterial pressure.

Genotype of *CPB2*

It has been reported that single-nucleotide polymorphisms of *CPB2* (eg, −1120 G/T, rs799916836 and −1583 A/T, rs108737) were related to the elevation of plasma levels of TAFI.[22–24] Indeed, it has been demonstrated that Thr325/Ile (rs192644738) is related with the active state of TAFI.[24]

We examined these single-nucleotide polymorphisms in 17 patients with CTEPH. Interestingly, we found the minor allele of rs7999168 in 4 patients with CTEPH (24%), the minor allele of rs1926447 in 4 patients (24%), and the minor allele of rs1087 in 6 patients (35%).

Discussion

The novel findings of this study were as follows: (1) the clot from patients with CTEPH was resistant to fibrinolysis with monteplase, (2) plasma levels of TAFI were elevated in patients with CTEPH and were not changed after hemodynamic improvement with PTPA, (3) the levels of TAFI released from platelets were increased in patients with CTEPH, and (4) the resistance to monteplase was improved by PGE_1 and TAFIa inhibitor. Taken together, these results indicate that TAFI is substantially involved in the resistance to thrombus fibrinolysis in patients with CTEPH. To the best of our knowledge, this is the first study demonstrating that plasma levels of TAFI is high in patients with CTEPH and are correlated with the resistance to clot lysis in CTEPH.

Baseline Characteristics of Patients With CTEPH

Endothelin receptor antagonists were used more in patients with PAH than in patients with CTEPH. There are few reports that these are effective in CTEPH.[25] Endothelin receptor antagonists improve the hemodynamics in CTEPH but do not improve the exercise capacity,[26] which was the reasons for the limited use of endothelin receptor antagonist in patients with CTEPH. Although body mass index was higher in patients with CTEPH

than in PH or non-PH patients in this study, the prevalence of obesity was comparable among the groups. Thus, we consider that the present results were not affected by the difference in the prevalence of obesity although obesity is related to the plasma levels of TAFI.[27] Furthermore, there were no patients with CTEPH who had protein C or S deficiency. Although we have no data for Factor V Leiden, there was no patients with CTEPH who had Factor V Leiden in the previous study of Japan.[28]

Effects of Warfarin on Fibrinolysis

In this study, all patients with CTEPH were treated with warfarin. Thus, we examined the effects of warfarin to fibrinolysis. We found that fibrinolysis was unaffected by the treatment with warfarin. It was previously reported that warfarin promotes fibrinolysis.[29] The control range of prothrombin time-international normalized ratio (PT-INR) is lower in Japan than in other countries. Indeed, in this study, the range of PT-INR in patients with warfarin was 1.63±0.39. This might be the potential reason why the treatment with warfarin did not affect fibrinolysis in this study.

Resistance to Fibrinolysis in Patients With CTEPH

Although a few studies examined fibrinolysis capacity in patients with CTEPH,[8,9] this study clearly demonstrates that patients with CTEPH show enhanced resistance to fibrinolysis compared with non-PH and PAH and that TAFI is directly involved in the resistance to fibrinolysis in CTEPH. Importantly, both CTEPH and PAH tended to be resistant to urokinase compared with non-PH, suggesting that the fibrin of CTEPH and PAH was resistant to plasmin, as previously reported by Miniati et al.[30] Furthermore, we demonstrated that fibrinolysis activity to urokinase and monteplase was different in CTEPH, where the clot was resistant to fibrinolysis with tPA, but not to urokinase, a consistent finding with the previous report that plasma levels of tPA antigen were elevated ≤53.0 ng/mL after 15 minutes of experimentally induced venous occlusion.[10]

Plasma clot lysis assay also showed that the clot of CTEPH was more resistant to fibrinolysis compared with those of non-PH and PAH. Maximum absorbance differed among the patients. It has been demonstrated that the maximum absorbance
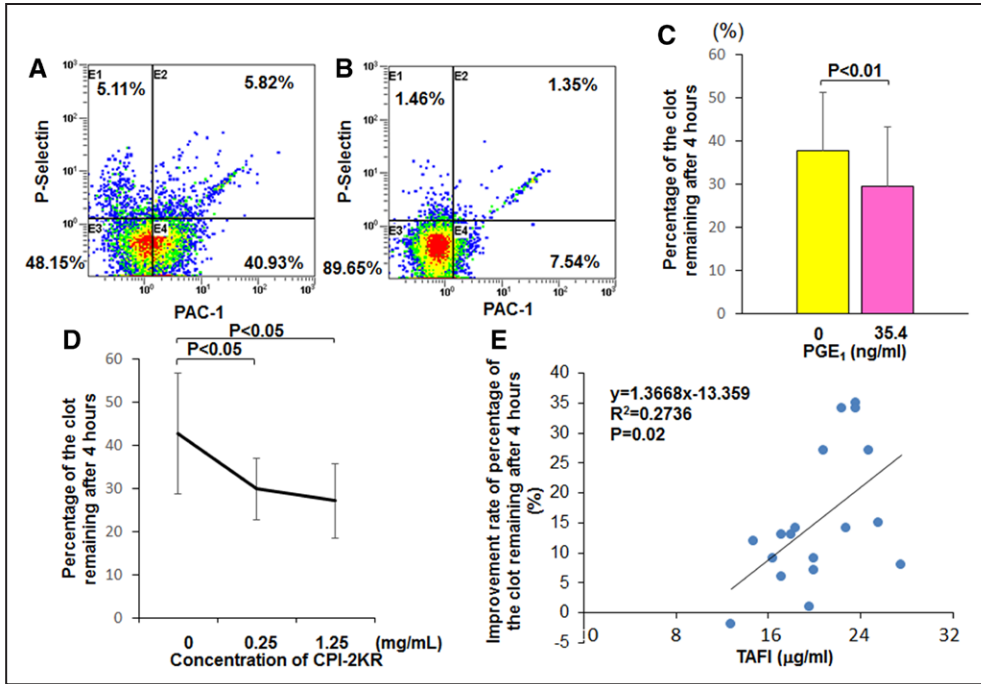
Figure 4. Effects of prostaglandin E$_1$ (PGE$_1$) and activated thrombin–activatable fibrinolysis inhibitor (TAFIa) inhibitor in whole blood clot lysis assay. The response to PGE$_1$ or TAFIa inhibitor of the clots in patients with chronic thromboembolic pulmonary hypertension (CTEPH, n=18) in whole blood clot lysis assay (100 ng/mL monteplase). The flow cytometric analysis of the platelets with stimulation by tapping each 3 minutes for 20 minutes without PGE$_1$ (**A**) or with 35.4 ng/mL of PGE$_1$ (**B**). The improvement of the percentage of the clot remaining after 4 hours (mean±SD) with 100 ng/mL monteplase in CTEPH patients with cotreatment with PGE$_1$ (**C**) or CPI-2KR (an inhibitor of TAFIa, **D**). The correlation between improvement rate of thrombus reduction in response to TAFIa inhibitor and the levels of TAFI (**E**). Statistical significance was determined with Tukey–Kramer test in **C** and **D** and with Spearman rank correlation coefficient in **E**.

is correlated with the plasma levels of fibrinogen.[31] Thus, the difference of maximum absorbance among the patients might be related to the different plasma levels of fibrinogen.

Elevated Plasma Levels of TAFI as a Pathogenesis of CTEPH

Carboxypeptidase N and TAFI are members of a large family of zinc metalloproteases.[32] Carboxypeptidase N is also a liver-derived plasma carboxypeptidase and exists as an active form in plasma. Although carboxypeptidase N has effects on bradykinin or anaphylatoxins[33] and regulates immune system, TAFI regulates not only immune system but also fibrinolysis.[32] TAFIa removes the C-terminal lysines from fibrin, which reduces the binding of tPA and plasmin to fibrin.[11] Although it has been reported that plasma levels of TAFI can be a risk factor of deep vein thrombosis,[13,34] this study clearly demonstrates that plasma levels of TAFI are significantly elevated in patients with CTEPH than those of non-PH and PAH.
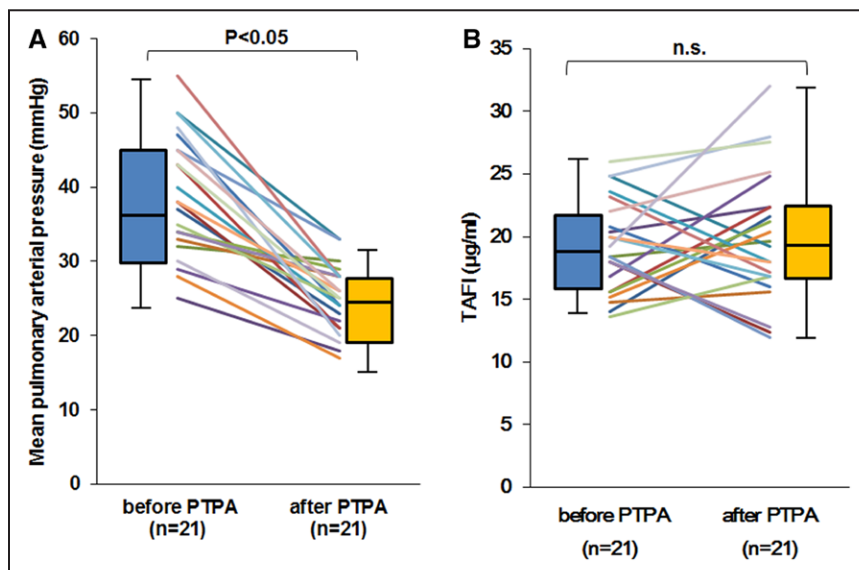

Figure 5. Plasma thrombin-activatable fibrinolysis inhibitor (TAFI) levels before and after percutaneous transluminal pulmonary angioplasty (PTPA). Although mean pulmonary arterial pressure (median [interquartile range]) was significantly improved after PTPA (**A**), plasma levels of TAFI (median [interquartile range]) were unchanged (**B**). Statistical significance was determined with paired *t* test.

However, the levels of other fibrinolysis antigens, such as PAI-1 and plasminogen, were comparable among the 3 groups, which is consistent with the previous reports.[10] Although PAH has the obstruction of small pulmonary arteries with thrombus, CTEPH has the obstruction of large pulmonary arteries with organized thrombus. This suggests that the mechanism of thrombus formation in CTEPH is different from that of PAH.[35] The present results suggest that TAFI is a new risk factor in CTEPH but not in PAH. Although plasma levels of TAFI correlated with plasma clot lysis time, it only had a weak correlation with whole blood clot lysis time. As previously reported,[29] whole blood clot lysis assay has more factors than plasma clot lysis assay, such as red blood cells and platelets. Importantly, plasma levels of TAFI did not change for 3 months after the hemodynamic improvement with PTPA. These results suggest that the increased plasma levels of TAFI are the cause, but not the simple results, of elevated pulmonary arterial pressure. Furthermore, we analyzed the correlation between plasma levels of TAFI and clot lysis time in patients with CTEPH. Interestingly, plasma TAFI levels tended to be positively correlated with clot lysis time. Thus, further increase of the number of patients with CTEPH enrolled may have determined the relationship between them.

The possible correlation between TAFI and platelets was examined previously[12,36] because TAFI can be released from activated platelets and cause vascular damage and pathological thrombus formation.[36] It was also demonstrated that TAFI can be more activated on the surface of aggregating platelets and that activated platelets inhibit fibrinolysis through activation of TAFI and clot retraction.[12] In this study, to evaluate the amount of TAFI released from platelets, we washed platelets twice with buffer (first and second supernatants) and completely removed TAFI from the plasma. It has been demonstrated that TAFI released from platelets was only 0.1% of TAFI in the plasma.[36] Moreover, it remains to be elucidated whether TAFI is produced in platelets or took up from the plasma. However, when the clots are formed in vivo, aggregating platelets secrete TAFI into the clots, which will significantly increase the local levels of TAFI and stabilize the clots. Thus, in spite of the small amount of TAFI released from platelets, it is considered that platelet-derived TAFI plays an important role for clot stabilization in vivo.[36] Consistently, we demonstrated that TAFI was released from activated platelets, and its levels were significantly higher in platelets from CTEPH than those from non-PH or PAH. Importantly, Mosnier et al[37] have reported that TAFI was synthesized in megakaryocyte cell lines. Thus, our data may implicate that the increased TAFI released from platelets in patients with CTEPH was, at least part, not from uptake. In addition, the resistance of the clot to monteplase in patients with CTEPH was significantly improved by cotreatment with TAFIa inhibitor peptide or PGE$_1$ as an inhibitor of activation of platelets.

Genotype of *CPB2* in CTEPH

It has been reported that single-nucleotide polymorphisms of *CPB2* (eg, −1120 G/T, rs799916836 and −1583 A/T, rs108737) were related to the elevation of plasma levels of TAFI.[22–24] Moreover, it has been demonstrated that Thr325/Ile

(rs192644738) is associated with differences in the stability of the TAFIa.[24] Thus, we performed genotyping of *CPB2* in patients with CTEPH.

We found the minor allele of rs7999168 in 4 patients with CTEPH (24%), the minor allele of rs1926447 in 4 patients (24%), and the minor allele of rs1087 in 6 patients (35%). The HapMap with the Asian population found that the prevalence of the minor allele was 0% for rs7999168, 24% for rs1926447, and 42% for rs1087.[38] Thus, it is possible that the prevalence of the minor allele of rs7999168 in patients with CTEPH is higher compared with the standard Asian population. We need to increase the number of patients with CTEPH to elucidate the role of single-nucleotide polymorphism in the pathogenesis of the disorder.

Potential Usefulness of PGE$_1$ and TAFIa inhibitor in CTEPH

As demonstrated in this study, the resistance of thrombus to fibrinolysis in patients with CTEPH was significantly improved by cotreatment with PGE$_1$ or TAFIa inhibitor. Continuous intravenous epoprostenol therapy is also effective for CTEPH, especially in the distal type, probably because of its vasodilator effects.[5,16,39] The present result suggests that antiplatelet effect of epoprostenol may also contribute to the amelioration of the resistance to fibrinolysis in patients with CTEPH.

We used inhibiting peptide of TAFIa, which is called CPI-2KR.[21] This drug specifically inhibits TAFIa with no off-target effects even at high concentration.[21] Indeed, clot lysis time was shorter by the treatment with 253 µmol/L of CPI-2KR in plasma clot lysis assay.[21] Because CPI-2KR is a peptide, CPI-2KR could have been digested in the whole blood.[21] On the basis of this report, we used CPI-2KR at the concentration of 250 µmol/L and 1.25 mmol/L. However, we need to consider potential off-target effects at high concentration.

Nowadays, we use warfarin or novel oral anticoagulants as anticoagulant therapy to prevent the progression of CTEPH. However, this study suggests that TAFIa inhibitor could be a novel therapeutic agent that may be safer than anticoagulants in terms of bleeding side effects, which frequency (≤3% per year) cannot be ignored.

Study Limitations

Several limitations should be mentioned for this study. First, the number of the study subjects was relatively small. Thus, there was a wide spread of the PAI-1 levels because of the small number of patients. Because it is known that the PAI-1 levels do not change in patients with CTEPH,[10] our main message as to the plasma levels of TAFI may not be influenced by the levels of PAI-1. Second, we had only 1 chance to get the blood from pulmonary artery at the catheterization and we had to experiment whole blood clot lysis assay, plasma clot-lysis assay, the TAFI derived from platelets, and whole blood clot lysis assay with inhibitors using fresh blood. Therefore, as it took a lot of time and blood samples to do all experiments, we were unable to perform all assays at one time in all patients. Thus, we randomly selected patients to perform each assay, which could have caused selection bias in this study.

Conclusions

In this study, we were able to demonstrate that TAFI is substantially involved in the pathogenesis of CTEPH and that TAFIa inhibitor could be a new therapeutic agent of the disorder.

Disclosures

None.

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Highlights

- The capacity of fibrinolysis was impaired in patients with chronic thromboembolic pulmonary hypertension.
- The serum levels of thrombin-activatable fibrinolysis inhibitor were high in patients with chronic thromboembolic pulmonary hypertension and were correlated with the impairment of fibrinolysis.
- The levels of thrombin-activatable fibrinolysis inhibitor released from platelets were also high in patients with chronic thromboembolic pulmonary hypertension.
- The impairment of fibrinolysis in patients with chronic thromboembolic pulmonary hypertension was improved with the inhibition of activated thrombin-activatable fibrinolysis inhibitor.

Thrombin-Activatable Fibrinolysis Inhibitor in Chronic Thromboembolic Pulmonary Hypertension

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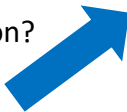
Graphic Abstract

Single nuculeotide polymorphisms?

The increase of the plasma levels of thrombin-activatable fibrinolysis inhibitor

The increase of thrombin-activatable fibrinolysis inhibitor from platelets

Activation?

Activation of platelets

Impairment of fibronolysis

Incompletely dissolving blood clot

Occlusion of pulmonary artery

Chronic thromboembolic pulmonary hypertension
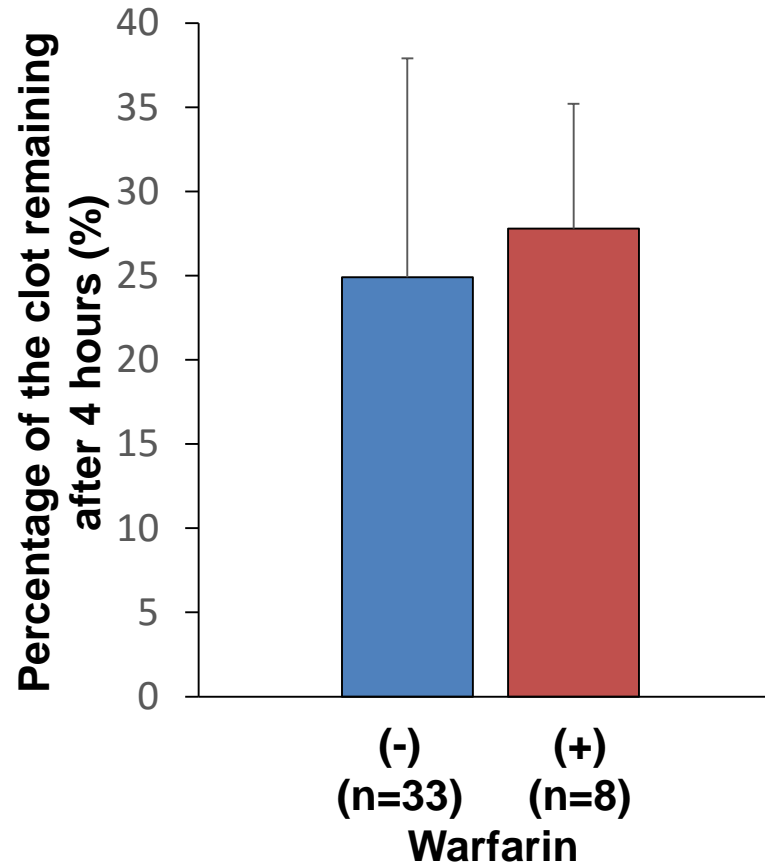
Supplemental Figure I. Effects of warfarin to percentage of the clot remaining after 4 hours
The percentage of the clot remaining after 4 hours with or without treatment of warfarin in non-CTEPH patients. Statistical significance was determined with Mann-Whitney U test.
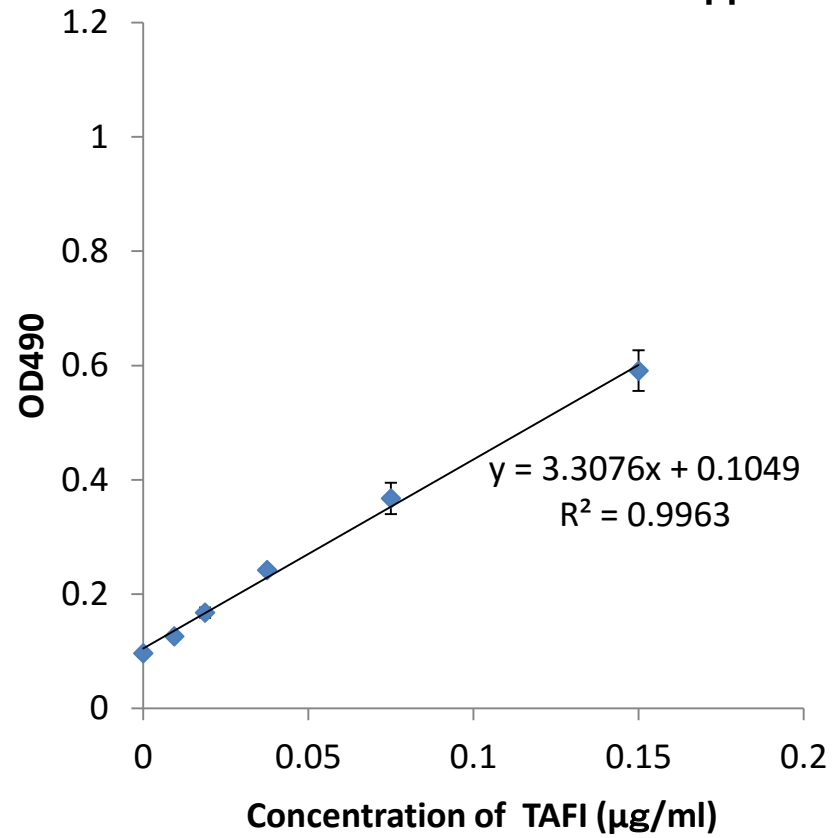
Supplemental Figure II. Standard curve for the ELISA for TAFI
Standard curve for ELISA for TAFI with the solution of commercial product TAFI.

Supplemental Figure III. The correlation between the levels of TAFI and the clot-lysis time in CTEPH
Plasma TAFI levels tended to be positively correlated with CLT in CTEPH patients (p=0.07). Statistical significance was determined with Spearman's rank correlation coefficient.
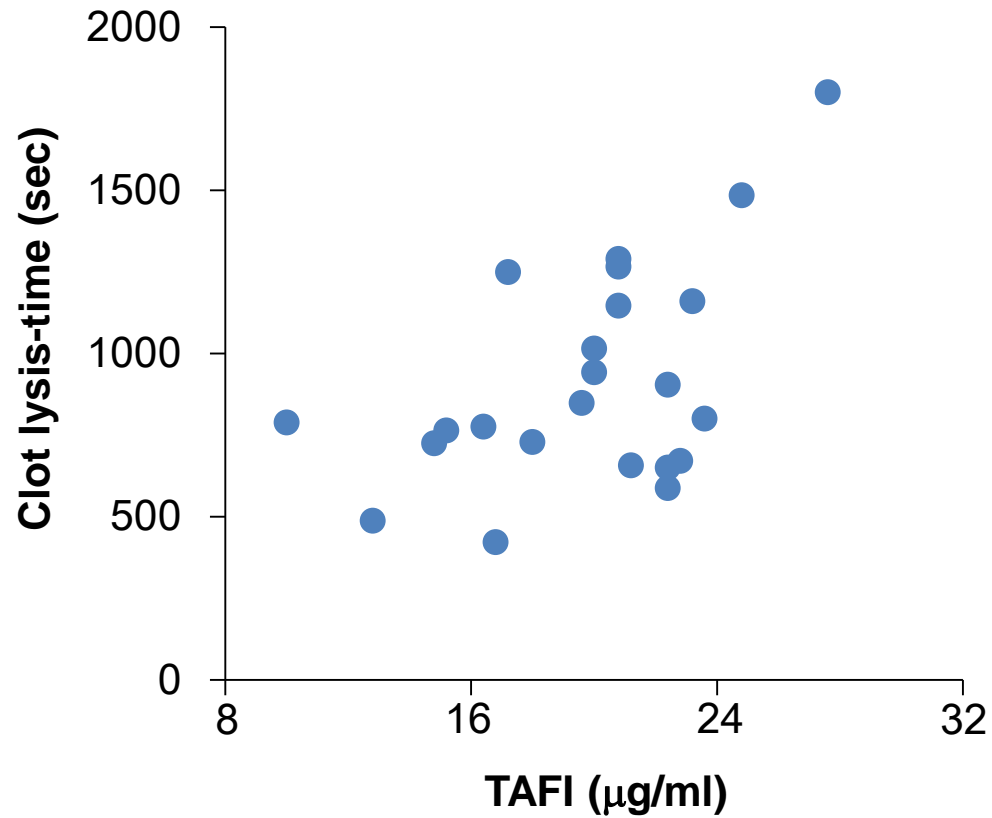
Supplemental Figure IV. The change of percentage of the clot remaining after 4 hours with CPI-2KR
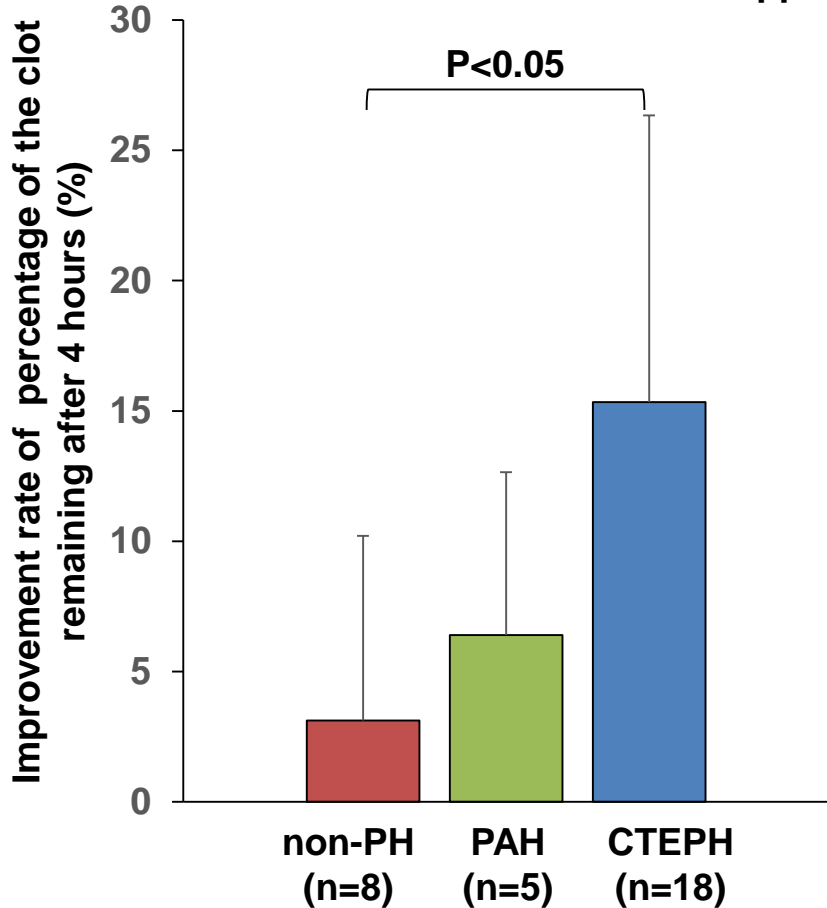
We evaluated percentage of the clot remaining with whole blood clot-lysis assay with or without CPI-2KR(1.25 mg/ml). Then, In CTEPH patients the percentage of the clot remaining was significantly improved than non-PH patients. Statistical significance was determined with Tukey-Kramer test.

Thrombin-activatable Fibrinolysis Inhibitor in Chronic Thromboembolic Pulmonary Hypertension

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Running title: TAFI in CTEPH

TOC category : Clinical study

TOC subcategory : Thrombosis

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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 ≤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_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.[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),[12] 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.[13,14]   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.[15]   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 α_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).[16,17] Ninety-six-well microtiter plates were coated with 6 µg/ml of a monoclonal antibody (2A16) against TAFI (Protein Science Co. Ltd., Nagoya, Japan). After washing with PBS in 0.05% tween20, 50 µl 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 µl 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 µl of *o*-phenylenediamine-H_2O_2 and 100 µl of 3N H_2SO_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).[18]   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 marker[19] (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

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), PGE_1 (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.

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