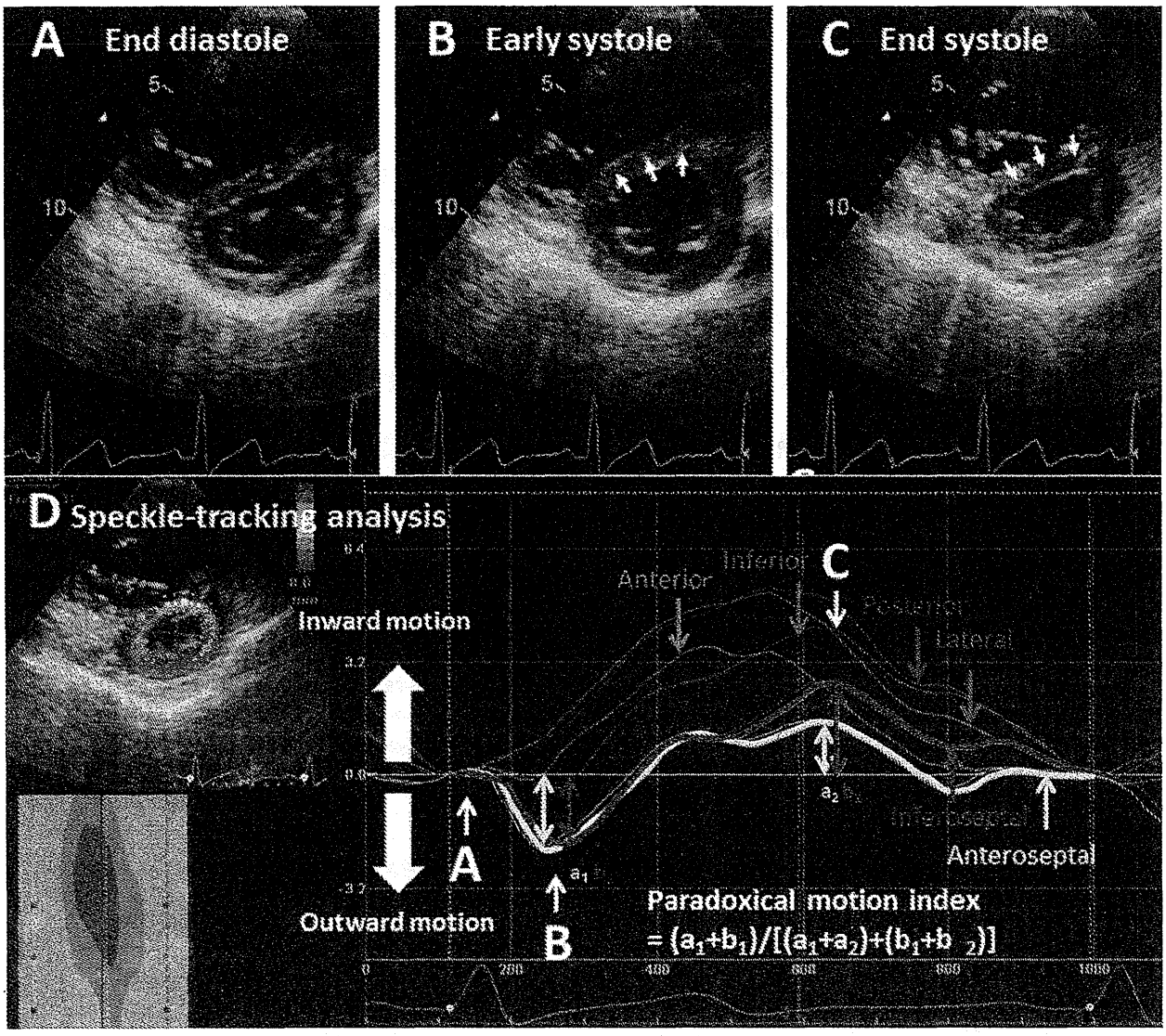

Figure 2. Quantitative assessment of paradoxical motion of the interventricular septum. Panels A, B and C show short axis images of the left ventricle at three cardiac phases of a patient with PH. From the end-diastolic phase (A) to the early systolic phase (B), the interventricular septum moves outward (toward right ventricle) in a paradoxical manner (B, arrows). Subsequently, from early systole (B) to end-systole (C), the interventricular septum moves inward in a manner similar to that of the other segments of the LV wall (panel C, arrows). However, the maximum inward motion was less than that of the other LV wall segments (D). To quantify the paradoxical interventricular septal motion indicated by arrows in panel B, we used speckle-tracking echocardiography by which the left ventricular free walls and interventricular septum were automatically divided into six segments, and each motion pattern was visualized by six lines (D). Yellow and red lines indicate the motion of the anterior and inferior interventricular septum, respectively, during a single cardiac cycle. The dips in the early systolic phase (a1 and b1) indicate their paradoxical motion. For quantitative assessment, the maximum depths of the yellow and red lines (a1+ b1) were added. Next, the entire distance of the two interventricular septal segments [early systole (a1 and b1) and end-systole (a2 and b2)] were added. Then, (a1+ b1) was divided by (a1+ a2+ b1+ b2), and the result was used as the paradoxical motion index of the interventricular septum.
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index, smoking history and the prevalence of cardiovascular/respiratory diseases. Table 1 shows the characteristics of the PH patients.

CMR Measurements in Controls and PH Patients

CMR images were acquired from all participants, and the image quality was sufficient for the subsequent analysis. When compared with control subjects, PH patients exhibited a greater RV EDV index (PH 98 [82–134] mL/m^2, control 66 [52–77] mL/m^2; $p<0.001$) and RV mass index (PH 36 [28–48] g/m^2, control 19 [17–22] g/m^2; $p<0.001$), and a smaller RV EF (PH 40 [32–47]%, control 52 [47–54]%; $p<0.001$) and LV EF (PH 59 [54–67]%, control 63 [61–69]%; $p=0.036$). LGE at VIPs was found in 41 of 46 PH patients. Median LGE volume of PH patients was 2.02 [0.47–2.99] mL. LGE volume in VIPs of PH patients who were receiving vasodilators was 2.19 [0.49–3.02] mL, which was not significantly different from that of PH patients who were not on such treatment (1.83 [0.6–3.28] mL, $p=0.804$). Also, no significant difference in LGE volume at VIPs were observed when comparing among the three different treatment regimens used in PH patients ($p=0.976$) (beraprost alone, 0.65 [0.18–4.23]

Table 1. Characteristics of patients with pulmonary hypertension.

Number of patients	**46**
Diagnosis	
Pulmonary arterial hypertension/pulmonary veno-occlusive disease	23/1 (50/2%)
Pulmonary hypertension due to left heart disease	0 (0%)
Pulmonary hypertension due to lung diseases and/or hypoxia	6 (13%)
Chronic thromboembolic pulmonary hypertension	14 (30%)
Other	2 (4%)
World health organization -functional class	
II	22 (48%)
III	20 (46%)
IV	4 (7%)
Use of pulmonary hypertension-specific vasodilators	
Beraprost	17 (37%)
Sildenafil/tadalafil	11/1 (24%/2%)
Bosentan/ambrisentan	14/1 (30%/2%)
Intravenous epoprostenol	4 (9%)
Combination therapy	17 (37%)
None	19 (41%)
Pulmonary hemodynamics	
Systolic pulmonary artery pressure (mmHg)	57 (46–75)
Diastolic pulmonary artery pressure (mmHg)	24 (18–28)
Mean pulmonary artery pressure (mmHg)	39 (30–43)
Pulmonary capillary wedge pressure (mmHg)	8±2
Right ventricular end-diastolic pressure (mmHg)	8±3
Mean right atrial pressure (mmHg)	6±2
Cardiac index (L/min/m^2)	2.6 (2.4–3.1)
Pulmonary vascular resistance (dyne·s·cm^{-5})	513 (386–785)

Mean ± standard deviation for those normally distributed or medians and interquartile ranges.
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mL (n = 5); phosphodiesterase 5 inhibitor with or without beraprost, 2.27 [0.34–2.75] mL (n = 5); endothelin receptor antagonists with or without beraprost, 2.33 [1.19–2.44] mL (n = 9).

Echocardiography Measurements in Controls and PH Patients

Echocardiography was completed in all subjects, and the image quality obtained was sufficient for all image analysis. The end-diastolic and end-systolic eccentricity indices were significantly greater in PH patients (end-diastole: 1.27 [1.10–1.39], end-systole: 1.45 [1.30 - 1.73]) than in controls (end-diastole: 1.00 [1.00–1.00], end-systole: 1.00 [1.00–1.01]) ($p<0.001$ for both indices). Paradoxical IVS motion index was also significantly greater in PH patients (0.23±0.14) than in controls (0.04±0.03) ($p<0.001$).

Associations of LGE Volume at VIPs with Clinical Parameters of PH

LGE volume at VIPs significantly correlated with mean PAP ($r = 0.50$, $p<0.001$), RV EDV index ($r = 0.53$, $p<0.001$), RV ESV index ($r = 0.59$, $p<0.0001$), RV mass index ($r = 0.53$, $p<0.001$), RV EF ($r = -0.56$, $p<0.001$), end-diastolic eccentricity index ($r = 0.60$, $p<0.001$), end-systolic eccentricity index ($r = 0.55$, $p<0.001$), and paradoxical IVS motion index ($r = 0.77$, $p<0.001$), but not with cardiac index (CI) ($r = -0.06$, $p = 0.657$), pulmonary vascular resistance (PVR) ($r = 0.28$, $p = 0.06$), LV EDV index ($r = -0.07$, $p = 0.63$), LV ESV index ($r = 0.09$, $p = 0.56$), LV mass index ($r = 0.10$, $p = 0.543$), LV EF ($r = -0.26$, $p = 0.078$), and the disease duration of PH ($r = 0.17$, $p = 0.25$).

Figure 3 shows associations between LGE volume at VIPs and six prespecified explanatory variables chosen for multivariate analysis. Among these six variables, paradoxical IVS motion index indicated the highest regression coefficient (6.27). Also, in the multiple regression analysis, paradoxical IVS motion index significantly predicted LGE volume at VIPs ($p<0.001$) but other five variables did not (mean PAP, $p = 0.463$; RV EDV index, $p = 0.64$; RV mass index, $p = 0.933$; RV EF, $p = 0.264$; and diastolic eccentricity index, $p = 0.626$).

Reproducibility and Reliability of the Measurements of LGE Volume and Paradoxical IVS Motion Index

Bland-Altman analysis of the intraobserver variability of LGE volume at VIPs showed low mean differences and limits of agreement (0.02±0.30 ml). The ICC was 0.99. Regarding interobserver variability, Bland-Altman analysis showed similarly small mean differences and limits (0.2±6.1 ml). The ICC was 0.97.

Bland-Altman analysis of the intraobserver variability of the paradoxical IVS motion index showed low mean differences and

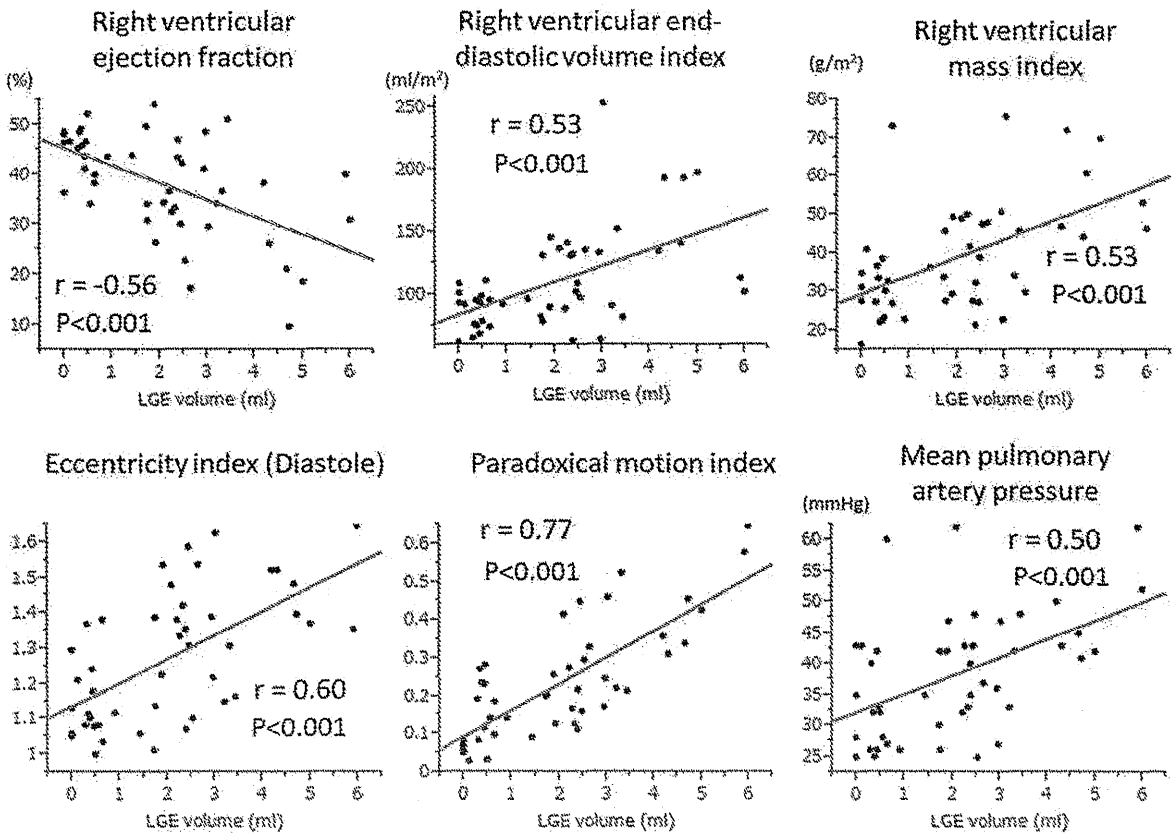

Figure 3. Relationships between late gadolinium enhancement volume at ventricular insertion points and other clinical parameters. Among the six clinical indices of pulmonary hypertension, the paradoxical motion index of the interventricular septum exhibited the highest correlation coefficient ($r = 0.77$, $p < 0.001$) with late gadolinium enhancement volume.
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limits of agreement (0.8% ±3.5%). The ICC was 0.98. Regarding interobserver variability, Bland-Altman analysis revealed similarly small mean differences and limits (−1.0% ±4.4%). The ICC was 0.96.

Discussion

In the present prospective observational case-control study, 46 PH patients and 21 matched controls were examined. Results suggested the following: (1) LGE at VIPs is a common CMR finding (positive in 42 (91%) of 46 PH patients); (2) early systolic paradoxical motion of VIPs is also common; (3) the volume of LGE correlates with the pulmonary hemodynamics, and with CMR and echocardiographic parameters of the right ventricle; and (4) the paradoxical IVS motion index is the only independent explanatory variable of LGE volume at VIPs in PH.

Recent studies have indicated significant associations between LGE at VIPs and various parameters of PH. For example, mean PAP, RV volume and RV mass index have been reported to positively correlate with LGE volume. [4,6,10] Also, functional parameters such as RV EF and CI have also been shown to inversely correlate with LGE volume at VIPs. [4–6,10] Indeed, the results of univariate analysis in the present study were consistent with observations from these previous publications. Importantly, however, no prior studies have examined the possible link between LGE at VIPs and the pattern of IVS motion during a cardiac cycle. In the present study, we indexed the degree of paradoxical IVS motion using speckle tracking echocardiography and found that the degree of such IVS motion is an independent explanatory variable of LGE at VIPs in PH. Conversely, MPAP and other RV indices did not predict LGE volume at VIPs in multivariate regression analysis.

Abnormal IVS motion has been documented by M-mode [14,15] and by speckle tracking echocardiography in PH. [19] Previous reports have focused on the underlying mechanism or the impact of this IVS motion on the overall cardiac performance. In line with these reports, the present study demonstrated significant associations of the paradoxical IVS motion index with the pulmonary hemodynamic measurements and RV EF. However, the main focus of the present study was the possible regional impact of paradoxical IVS motion on VIPs. Regarding this issue, two prior animal studies (dog PH model) demonstrated that myocardial tissue at VIPs is prone to encounter pull and increased tension. [20,21] Also, Spottiswoode et al. reported that paradoxical IVS motion can generate high stresses and strains at VIPs in a non-PH patient. [22] These prior publications, along with the results of the present study, suggest that LGE at VIPs might develop due to the mechanical impact of paradoxical IVS motion on VIPs irrespective of PH.

Contrast pooling at VIPs was suggested as the primary mechanism of LGE in PH in a recent autopsy report. [8] Notably, this report demonstrated disarrayed myocardium but no abnormal fibrosis or damaged myocardium at VIPs. Accordingly, the authors speculated that contrast pooling in the widened intermyocardial fibers caused LGE at VIPs in their case. The present

study partly supports this notion, because paradoxical IVS motion is likely to affect the architecture of the myocardial tissue at VIPs, as was reported in prior studies. [20,22]

Freed et al. recently reported that the presence of myocardial LGE at VIPs is a marker of poor prognosis in PH [9]. In this regard, the present study showed that LGE at VIPs was independently associated with paradoxical IVS motion but not with established predictors such as RV mass and EF [23]. Indeed, LGE at VIPs may be a sole reflection of paradoxical IVS motion and resultant contrast pooling [8] and thus whether LGE at VIPs can be a better prognostic marker over previously reported indices needs to be investigated in future prospective studies.

One limitation of the present study is the inclusion of PH patients with diverse etiologies. Some underlying diseases of PH are known to affect the myocardium; thus, the experimental results might have been affected by the different underlying illnesses among the patient population. Also, in the subgroup analysis, the number of PH patients on different treatment regimens was small. Thus, any confounding influence of the specific treatment on the experimental results cannot be excluded. Also, the present study analyzed patients with relatively less advanced PH, which makes it difficult to extrapolate the findings of this study to those with advanced PH. Further, IVS motion was evaluated using a methodology that is not commonly used. However, speckle-tracking echocardiography is an established method of regional analysis of IVS motion. Indeed, the calculated intra- and inter-observer reproducibility of the paradoxical IVS motion index were favorably high. Lastly, we did not conduct pathological observations in the present study. Thus, further clinicopathological studies are warranted to clarify the true mechanisms of LGE at VIPs in PH.

In conclusion, the present CMR study of PH demonstrated an independent association of LGE at VIPs with early systolic paradoxical IVS motion, but not with indices of pulmonary hemodynamics and RV morphology. This suggests that LGE at VIPs is a hallmark of altered IVS motion but not of PH per se. The clinical relevance of this distinct CMR finding must be clarified in future long-term studies.

Author Contributions

Conceived and designed the experiments: TS IT HO TN AY DI TW. Performed the experiments: TS NO-M. Analyzed the data: TS YMI. Contributed reagents/materials/analysis tools: YMI. Wrote the paper: TS IT MN.

References

1. Galie N, Hoeper MM, Humbert M, Torbicki A, Vachiery JL, et al. (2009) Guidelines for the diagnosis and treatment of pulmonary hypertension: the Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT). Eur Heart J 30: 2493–2537.
2. McLure LE, Peacock AJ (2009) Cardiac magnetic resonance imaging for the assessment of the heart and pulmonary circulation in pulmonary hypertension. Eur Respir J 33: 1454–1466.
3. McCann GP, Beek AM, Vonk-Noordegraaf A, van Rossum AC (2005) Delayed contrast-enhanced magnetic resonance imaging in pulmonary arterial hypertension. Circulation 112: e268.
4. Blyth KG, Groenning BA, Martin TN, Foster JE, Mark PB, et al. (2005) Contrast enhanced-cardiovascular magnetic resonance imaging in patients with pulmonary hypertension. Eur Heart J 26: 1993–1999.
5. McCann GP, Gan CT, Beek AM, Niessen HW, Vonk Noordegraaf A, et al. (2007) Extent of MRI delayed enhancement of myocardial mass is related to right ventricular dysfunction in pulmonary artery hypertension. AJR Am J Roentgenol 188: 349–355.
6. Sanz J, Dellegrottaglie S, Kariisa M, Sulica R, Poon M, et al. (2007) Prevalence and correlates of septal delayed contrast enhancement in patients with pulmonary hypertension. Am J Cardiol 100: 731–735.
7. Junqueira FP, Macedo R, Coutinho AC, Loureiro R, De Pontes PV, et al. (2009) Myocardial delayed enhancement in patients with pulmonary hypertension and right ventricular failure: evaluation by cardiac MRI. Br J Radiol 82: 821–826.
8. Bradlow WM, Assomull R, Kilner PJ, Gibbs JS, Sheppard MN, et al. (2010) Understanding late gadolinium enhancement in pulmonary hypertension. Circ Cardiovasc Imaging 3: 501–503.
9. Freed BH, Gomberg-Maitland M, Chandra S, Mor-Avi V, Rich S, et al. (2012) Late gadolinium enhancement cardiovascular magnetic resonance predicts clinical worsening in patients with pulmonary hypertension. J Cardiovasc Magn Reson 14: 11.
10. Shehata ML, Lossnitzer D, Skrok J, Boyce D, Lechtzin N, et al. (2011) Myocardial delayed enhancement in pulmonary hypertension: pulmonary hemodynamics, right ventricular function, and remodeling. AJR Am J Roentgenol 196: 87–94.
11. Gaynor SL, Maniar HS, Prasad SM, Steendijk P, Moon MR (2005) Reservoir and conduit function of right atrium: impact on right ventricular filling and cardiac output. Am J Physiol Heart Circ Physiol 288: H2140–2145.
12. Sechtem U, Mahrholdt H, Vogelsberg H (2007) Cardiac magnetic resonance in myocardial disease. Heart 93: 1520–1527.
13. Sato T, Tsujino I, Ohira H, Oyama-Manabe N, Nishimura M (2012) Paradoxical motion of the interventricular septum as a primary mechanism of late gadolinium enhancement at ventricular insertion points. Int J Cardiol 158: 156–157.
14. Tanaka H, Tei C, Nakao S, Tahara M, Sakurai S, et al. (1980) Diastolic bulging of the interventricular septum toward the left ventricle. An echocardiographic manifestation of negative interventricular pressure gradient between left and right ventricles during diastole. Circulation 62: 558–563.
15. Jessup M, Sutton MS, Weber KT, Janicki JS (1987) The effect of chronic pulmonary hypertension on left ventricular size, function, and interventricular septal motion. Am Heart J 113: 1114–1122.
16. Dohi K, Onishi K, Gorcsan J, 3rd, Lopez-Candales A, Takamura T, et al. (2008) Role of radial strain and displacement imaging to quantify wall motion dyssynchrony in patients with left ventricular mechanical dyssynchrony and chronic right ventricular pressure overload. Am J Cardiol 101: 1206–1212.
17. Ryan T, Petrovic O, Dillon JC, Feigenbaum H, Conley MJ, et al. (1985) An echocardiographic index for separation of right ventricular volume and pressure overload. J Am Coll Cardiol 5: 918–927.
18. Machin D CM, Fayers PM, Pinol A. (1997: 168–173) Sample Size Tables for Clinical Studies. Malden, MA: Blackwell Science Ltd.
19. Lindqvist P, Calcutteea A, Henein M (2008) Echocardiography in the assessment of right heart function. Eur J Echocardiogr 9: 225–234.
20. Gibbons Kroeker CA, Adeeb S, Tyberg JV, Shrive NG (2006) A 2D FE model of the heart demonstrates the role of the pericardium in ventricular deformation. Am J Physiol Heart Circ Physiol 291: H2229–2236.
21. Gibbons Kroeker CA, Adeeb S, Shrive NG, Tyberg JV (2006) Compression induced by RV pressure overload decreases regional coronary blood flow in anesthetized dogs. Am J Physiol Heart Circ Physiol 290: H2432–2438.
22. Spottiswoode B, Russell JB, Moosa S, Meintjes EM, Epstein FH, et al. (2008) Abnormal diastolic and systolic septal motion following pericardiectomy demonstrated by cine DENSE MRI. Cardiovasc J Afr 19: 208–209.
23. van Wolferen SA, Marcus JT, Boonstra A, Marques KM, Bronzwaer JG, et al. (2007) Prognostic value of right ventricular mass, volume, and function in idiopathic pulmonary arterial hypertension. Eur Heart J 28: 1250–1257.

CHEST Original Research

Duration of Benefit in Patients With Autoimmune Pulmonary Alveolar Proteinosis After Inhaled Granulocyte-Macrophage Colony-Stimulating Factor Therapy [AQ1]

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Background: **Treatment of autoimmune pulmonary alveolar proteinosis (aPAP) by subcutaneous injection or inhaled therapy of granulocyte-macrophage colony-stimulating factor (GM-CSF) has been demonstrated to be safe and efficacious in several reports. However, some reports of subcutaneous injection described transient benefit in most instances. The durability of response to inhaled GM-CSF therapy is not well characterized.**

Methods: **To elucidate the risk factors for recurrence of aPAP after GM-CSF inhalation, 35 patients were followed up, monitoring for the use of any additional PAP therapies and disease severity score every 6 months. Physiologic, serological, and radiologic features of the patients were analyzed for the findings of 30-month observation after the end of inhalation therapy.**

Results: **During the observation, 23 patients remained free from additional treatments, and twelve patients required additional treatments. There were no significant differences in age, sex, symptoms, oxygenation indices, or anti-GM-CSF antibody levels at the beginning of treatment between the two groups. Baseline vital capacity (% predicted, %VC) were higher among those who required additional treatment ($P < .01$). Those patients not requiring additional treatment maintained the improved disease severity score initially achieved. A significant difference in the time to additional treatment between the high %VC group (%VC ≥ 80.5) and the low %VC group was seen by a Kaplan-Meier analysis and a log-rank test ($P < .0005$).**

Conclusions: **These results demonstrate that inhaled GM-CSF therapy sustained remission of aPAP in more than one-half of cases, and baseline %VC might be a prognostic factor for disease recurrence.**

Trial registry: **ISRCTN Register; No.: ISRCTN18931678; URL: http://www.isrctn.org/**

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Abbreviations: A-aDO_2 = alveolar-arterial oxygen difference; Ab = antibody; aPAP = autoimmune pulmonary alveolar proteinosis; AT = additional treatments; BALF = BAL fluid; DLCO = diffusing capacity of the lung for carbon monoxide; DSS = disease severity score; FR = free from additional treatments; GM-CSF = granulocyte-macrophage colony-stimulating factor; IQR = interquartile range; PAP = pulmonary alveolar proteinosis; ROC = receiver operating characteristics curve; SP-A = surfactant protein A; SP-D = surfactant protein D; VC = vital capacity; WLL = whole-lung lavage

Autoimmune pulmonary alveolar proteinosis (aPAP) is a rare lung disease characterized by the accumulation of surfactant protein, which causes progressive respiratory insufficiency.[1-3] The pathogenesis has been attributed to the excessive production of a neutralizing autoantibody against granulocyte-macrophage colony-stimulating factor (GM-CSF) that impairs GM-CSF-dependent surfactant clearance mediated by alveolar macrophages.[4-8] On pulmonary function testing, the most common

pattern seen is that of a restrictive defect, with a disproportionate reduction in diffusing capacity of the lung for carbon monoxide (DLCO) relative to a modest impairment of vital capacity (VC).[2] The disease is usually treated by whole-lung lavage (WLL), which remains the standard therapy to date.

The first patient successfully treated with subcutaneously administered GM-CSF was reported in 1996.[9] In an international multicenter phase 2 trial study, 14 patients were treated with GM-CSF by subcutaneous injection in escalating doses over a 3-month period, with an overall response rate of 43%.[10,11] A subsequent single-center study of 21 patients with aPAP treated with GM-CSF by subcutaneous administration in escalating doses for 6 to 12 months reported an overall response rate of 48%.[12] Several single cases of subcutaneous GM-CSF therapy have reported similar outcomes.[13,14] However, local reaction at sites of injection and other minor toxicities occurred in 85% of patients receiving subcutaneous GM-CSF.[2]

[AQ2] Manuscript received March 12, 2013; revision accepted October 1, 2013.
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GM-CSF inhalation is a promising alternative therapy for aPAP that has been demonstrated to lead to functional, biologic, and radiologic improvement.[15-18] Our national, multicenter phase 2 study revealed that the therapy reduced alveolar-arterial oxygen difference (A-aDO_2) by 12.3 mm Hg in 35 patients who completed the therapy, resulting in 24 responders. No treatment-related side effects were noted. Of importance, our previous phase 2 study showed that there was no significant difference in serological, physiologic, and CT scan testing, except for serum KL-6 levels, between the responders and the [AQ9] nonresponders.[18]

There is limited information regarding the duration of benefit after various treatments of aPAP. In the literature analysis of 55 cases with a therapeutic response to WLL, the median duration of clinical benefit from lavage was 15 months.[2] A phase 2 study of subcutaneous GM-CSF administration demonstrated that 45% of patients required WLL during follow-up observation of 39 ± 17.3 months.[12] In a retrospective analysis of inhaled GM-CSF therapy (250 μg bid), five of 12 patients manifest progressive disease during observation.[17] As the disease progresses very slowly and can fluctuate in some cases, it is necessary to evaluate the prognosis by monitoring prospectively at the same time points after the treatment and by disease severity score as well as the need for additional treatment. The aim of this study was to define the duration of benefit among patients who underwent GM-CSF inhalation therapy.

Materials and Methods

Patients and Protocols

The present study prospectively observed patients who participated in a multicenter phase 2 trial (35 patients, registered as ISRCTN18931678, JMAIIA00013) of GM-CSF inhalation therapy described previously. In brief, patients who had lung biopsy or cytologic findings diagnostic for PAP, including elevated serum anti-GM-CSF antibody (Ab) levels and no improvement during a 12-week observation period, entered the treatment phase. Recombinant human GM-CSF dissolved in 2 mL of sterile saline was inhaled using an LC-PLUS nebulizer (PARI International). The treatment consisted of high-dose GM-CSF administration (125 μg bid on days 1-8, none on days 9-14; Leukine; Bayer HealthCare Pharmaceuticals) for six repetitions of 2-week cycles, then low-dose administration (125 μg once daily on days 1-4, none on days 5-14) for six repetitions of two-week cycles (for a total dose of 15 mg). The clinical information including physiologic, serologic, and radiologic features obtained[18] was compared with the results of the following 30-month observation. [AQ10]

Patients were regularly evaluated by their physicians at the network hospitals after the GM-CSF inhalation therapy. The worsening dyspnea was evaluated with pulse oximetry and/or arterial blood gas analysis in outpatient settings. Disease severity in patients was evaluated using PAP disease severity score (DSS) described previously.[19] Patients underwent additional treatments

based on either of the following criteria: (1) DSS is 3 or 4 and symptoms are worsening; or (2) DSS 5, as shown in Figure 1. The consortium office of Niigata University contacted the network hospitals every 6 months with a questionnaire regarding additional treatment and disease severity score of the patient. The follow-up clinical information obtained at each network hospital was entered into a database to be compared with the results of the baseline clinical evaluation of each patient. The data were collected from nine clinical research centers in Japan (Hokkaido University, Tohoku University, Chiba University, Kitasato University, Niigata University, Aichi Medical University, National Hospital Organization Kinki-Chuo Chest Medical Center, National Hospital Organization Yamaguchi-Ube Medical Center, and Nagasaki University Institute of Tropical Medicine).

[AQ11] The study was approved by Institutional Review Board of Niigata University (approval No. NH17-006) and the institutional review boards at all participating centers. Informed consent was obtained from all control subjects. The clinical information obtained by the clinical studies was entered into a database to be compared with the results of the 30-month observation. The study was designed and monitored for data quality and safety by a steering committee composed of the principal investigator at each participating site. The steering committee held a conference twice a year, where the findings of the observation were monitored.

BAL Procedures and GM-CSF Autoantibodies

The steering committee edited a standard operational procedure for BAL, which was followed by all participating institutes and described previously.[18,20] The concentration of GM-CSF autoantibodies in BAL fluid (BALF) or in serum were measured using a sandwich enzyme-linked immunosorbent assay as described previously.[4,21]

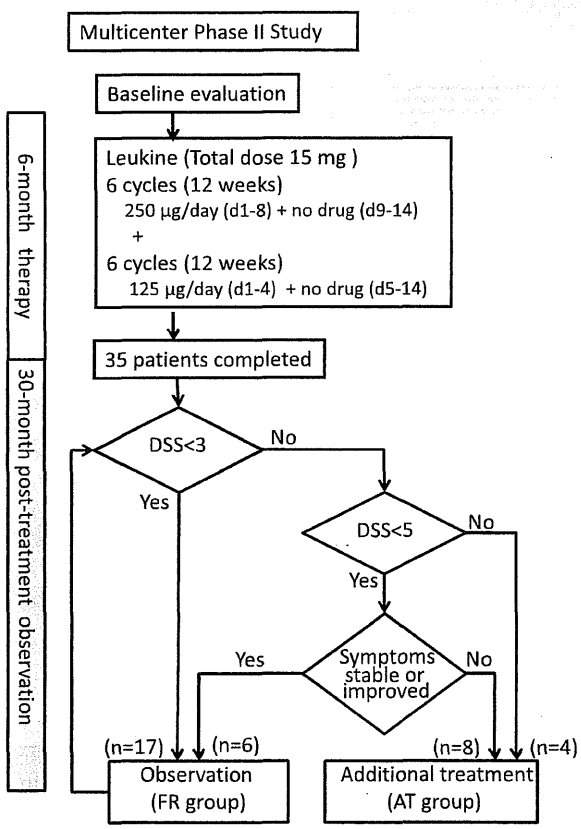

FIGURE 1. Profile of the study cohort. AT = additional treatment; DSS = disease severity score; FR = free from additional treatments.

Statistical Analysis

Numerical results are presented as the mean ± SE or the median and interquartile range (IQR). The χ^2 test was used to evaluate proportions for variables between high and low responders. The paired *t* test was used for comparisons between normally distributed data and the treatment periods. Comparisons of nonparametric data were made using the Wilcoxon signed-rank test. For group comparisons, unpaired *t* tests and Wilcoxon rank-sum tests were used. All *P* values were reported as two-sided. Analysis was performed using JMP software version 8.0.2.

RESULTS

Patient Characteristics and Requirements for Additional Treatments as an Indicator of Recurrence

Demographic data of patients are shown in Table 1. During the 30 months of observation after the end of GM-CSF inhalation, the need for treatments was monitored as an indicator of disease recurrence in each patient. Twenty-three patients were free from additional treatments during 30 months of observation and were designated as FR. Twelve patients who required additional treatments, including six patients with recurrence described in our previous study,[18] were designated as AT. Of those, two patients maintained most severe disease (DSS 5) even after the GM-CSF treatment and underwent subsequent WLL. One patient who had dyspnea, cough, and sputum production did not respond to the GM-CSF treatment and underwent subsequent WLL. One patient with cough and dyspnea showed worsening in PaO_2 and cough and had WLL 12 months after the GM-CSF inhalation. The other eight patients with dyspnea showed worsening in PaO_2/oxygen saturation by pulse oximetry (two patients worsened to DSS 5) and underwent additional therapy (e-Figure 1); five underwent additional GM-CSF inhalation treatments, two had WLL, and one patient, a nonresponder, declined WLL and underwent acetylcysteine inhalation, showing much improvement in hypoxia. Median time to additional treatment of the 12 patients was 50.5 weeks, with a range of 8.5 to 117.5 weeks. There was no significant difference in age, sex, symptoms, smoking status, history of dust exposure, arterial blood gas analysis, numbers of responders to GM-CSF inhalation, history of previous lung lavage, and anti-GM-CSF-Ab titer between the FR and AT groups (Table 1). There was no significant difference in disease markers, including baseline levels of PaO_2, A-aDO_2, %VC, %DLCO, CT scan scores, LDH, and [AQ12] KL-6 between the patients who underwent WLL (n = 6, AT-WLL) and those treated with GM-CSF

Table 1—*Baseline Clinical Characteristics of Patients Free From Additional Treatment and Those Who Required Additional Treatment After GM-CSF Inhalation*

[AQ23] Characteristic	FR (n = 23) No.	%	Median (IQR) or Mean ± SE	AT (n = 12) No.	%	Median (IQR) or Mean ± SE	*P* Value
Age, y	23		52.5 (48-61)	12		52.5 (41.75-58)	.33[a]
Sex							.54[b]
Female	9	39		6	50		
Male	14	61		6	50		
Responders	17	74		7	58		.35[b]
Duration of symptoms, mo	23		20 (11-61)	12		18 (7.75-72)	.78[a]
Symptoms							
Dyspnea	22	96		12	100		.36[b]
Cough	10	43		7	58		.65[b]
Sputum	8	35		4	33		.71[b]
Smoking status							.39[b]
Current smoker	8	35		2	17		
Ex-smoker	5	22		2	17		
Never smoked	10	43		8	67		
Dust exposure	22			11			.27[b]
Yes	8	36		3	18		
No	14	64		8	82		
Arterial blood gas analysis							
$PaCO_2$, torr[c]	23		38.0 ± 0.7	12		39.0 ± 0.9	.40[d]
PaO_2, torr[c]	23		60.6 ± 2.1	12		56.3 ± 3.0	.25[d]
A-aDO_2, torr[e]	23		43.5 ± 2.4	12		46.2 ± 3.3	.51[d]
Disease severity score	23		3 (3-4)	12		3.5 (3-5)	.58[a]
GM-CSF autoantibody, μg/ml	23		22.8 (8.5-33.2)	12		23.1 (16.9-34.2)	.94[a]
Previous lung lavage (>6 mo prior to study)							.22[b]
Yes	5	22		5	42		
No	18	78		7	58		

Thirty-five patients completed both the high-dose and low-dose period of GM-CSF inhalation therapy. A-aDO_2 = alveolar-arterial oxygen difference; AT = patients requiring additional treatment; FR = patients free from additional treatment; GM-CSF = granulocyte-macrophage colony-stimulating factor; IQR = interquartile range (range from the 25th to the 75th percentiles of the distribution).

[a]Calculated using the Wilcoxon rank sum test.

[b]Calculated using the χ^2 test.

[c]Measured with patient in a supine position and breathing room air.

[d]Calculated using Student *t* test.

[e]Calculated using the following equation: A-aDO_2 = (PB − PH_2O) × FIO_2 − $PaCO_2$/R + {$PaCO_2$ × FIO_2 × (1 − R)/R} − PaO_2, where PB = barometric pressure measured by local observatories; PH2O = partial pressure of water vapor in inspired air (assumed to be 47 mm Hg); FIO_2 = fractional concentration of oxygen in dry gas (assumed to be 0.21); R = respiratory quotient (assumed to be 0.8).

inhalation (n = 5, AT-GM) (e-Table 1). However, changes in A-aDO_2 during the GM-CSF treatment were significantly higher in the AT-GM group,

Association of Clinical Parameters With Requirement for Additional Treatment

There was no significant difference in baseline findings in terms of PaO_2, $PaCO_2$, FEV_1, and DLCO between AT and FR groups. Both percent VC (% predicted value; %VC) and percent FVC (%FVC) were higher in the FR group ($P < .01$) (Fig 2A, Table 2, e-Figure 2). There was no correlation between baseline %VC and age ($P = .97$), sex ($P = .41$), baseline PaO_2 ($P = .18$), or baseline %DLCO ($P = .34$). There was no significant difference in high-resolution computed tomography scan scores and serum markers, including LDH, KL-6, CEA, surfactant protein A (SP-A), and surfactant protein D (SP-D) (Table 2). [AQ13] As for differential blood cell counts, no significant difference was observed between FR and AT groups, except for numbers of basophils and platelets. The cell density of macrophages in BALF was lower in the FR group than those in the AT group ($P < .05$), whereas lymphocytes were lower in the AT group as compared with the FR group.

Next, clinical parameters at the end of treatment were evaluated. The %DLCO was lower in the AT group than that in the FR group, and serum markers (eg, LDH, KL-6, CEA, SP-D, SP-A) and CT scan scores were higher in the AT group than those in the FR group at the end of treatment ($P < .05$). However, there was no significant difference in A-aDO_2, blood cell counts, and cell differentials in BALF (Table 3).

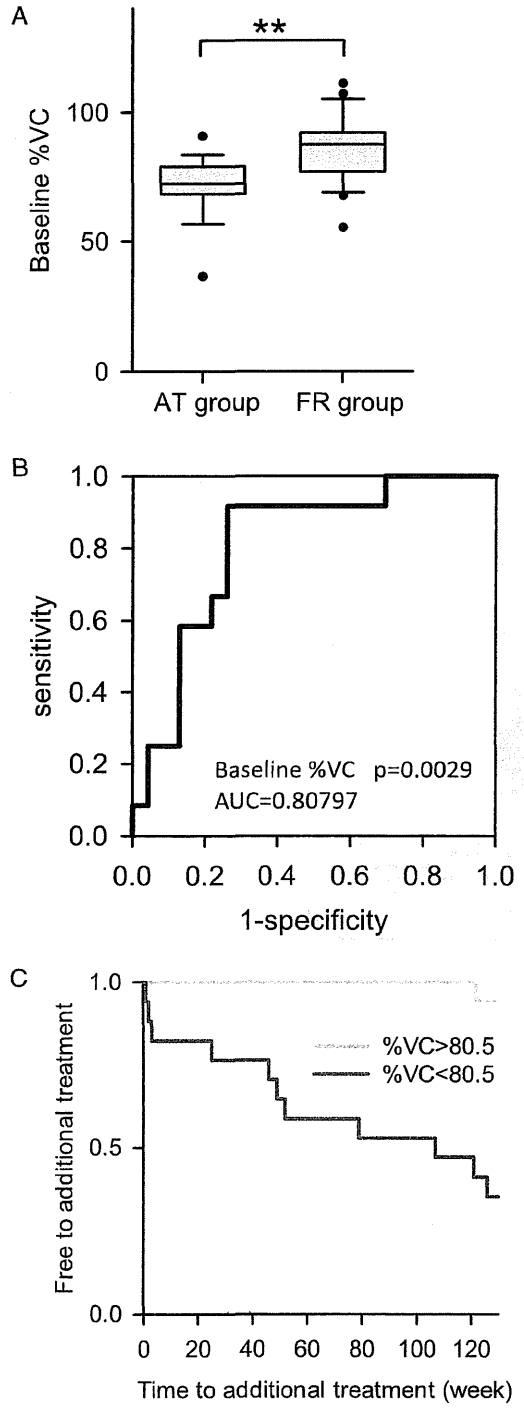

FIGURE 2. The association between VC (% predicted, %VC) and additional treatments during the 30-month observation period (**$P < .01$). A, Baseline levels of %VC in FR and AT patient groups. B, Receiver operating curve of %VC. C, Kaplan-Meier plot showing patients of the high %VC group (%VC ≥ 80.5) and those of the low %VC group (%VC < 80.5). AUC = area under the receiver operating curve; VC = vital capacity

The patients free from additional treatment maintained the improved disease severity score initially achieved (e-Figure 3).

Predictive Value of VC for Prognosis After GM-CSF Inhalation

Because only %VC and %FVC differed between FR and AT groups among treatment-related pretreatment factors, the predictive value of parameters for recurrence after GM-CSF inhalation was evaluated using receiver operating characteristics curve (ROC) analysis and Kaplan-Meier analysis of time to additional treatment.

For ROC analysis, the area under the ROC curve was calculated nonparametrically, as proposed by Hanley and McNeil.[22] An additional therapy was defined as a positive indicator for disease recurrence. When the cutoff level of 80.5% was set for %VC, the baseline %VC predicted the additional therapy with a sensitivity of 92% and a specificity of 74% (Fig 2b).

For Kaplan-Meier analysis of time to additional treatment, we divided the patients into two groups, namely the high %VC group (%VC ≥ 80.5) and the low %VC group (%VC < 80.5). A significant difference in the time to additional treatment between the two groups was seen when the whole period of follow-up was compared ($P = .0001$) (Fig 2c). In the univariate Cox proportional analysis of baseline markers, %VC < 80.5% (hazard ratio, 18.42; 95% CI, 3.55-337.68; $P < .0001$) was associated with additional treatment, whereas no correlations were found between additional treatment and age, sex, baseline Pao_2, changes in A-aDO_2, and baseline levels of LDH, KL-6, SP-A, CEA, and anti-GM-CSF-Ab.

Subgroup Analysis: To test whether VC is an independent predictive factor for the time to additional therapy, we did subgroup analyses because of the small number of the AT patients. The patients were divided into two groups of an upper half and a lower half regarding age; sex; baseline Pao_2; change in A-aDO_2; baseline levels of LDH, KL-6, SP-A, CEA; and anti-GM-CSF-Ab. In these subgroups, a significant difference in the time to additional treatment between the high %VC group (%VC ≥ 80.5) and the low %VC group (%VC < 80.5) was still evident, suggesting that VC might be an independent factor predicting the time to additional therapy (e-Figure 4).

Time Course of Autoantibody Levels: In our previous reports, serum levels of anti-GM-CSF-Ab levels did not change during treatment.[16] To study longitudinal changes of serum levels of anti-GM-CSF-Ab after the inhaled GM-CSF therapy, serum samples were collected for anti-GM-CSF-Ab testing as an optional evaluation after the 30-month observation pe-

Table 2—*Baseline Pulmonary Function, Radiologic Appearance, Serum Biomarkers, Hematologic Indices, and BALF Cell Findings in Patients With PAP in FR and AT Groups Before GM-CSF Inhalation Treatment*

[AQ24] Measure	FR		AT		*P* Value
	No.	Mean ± SE or Median (IQR)	No.	Mean ± SE or Median (IQR)	
Pulmonary function					
VC, % predicted	23	85.9 ± 2.7	12	71.6 ± 3.8	.0045[a]
FVC, % predicted	23	85.3 ± 2.8	12	71.4 ± 3.9	.0064[a]
FEV_1/FVC	23	87.1 ± 2.0	12	84.9 ± 2.7	.51[a]
DLCO, % predicted	23	57.0 ± 3.4	10	46.0 ± 5.1	.082[a]
HRCT scores[b]					
Upper lung region	23	3 (2-5)	12	4.5 (2-5)	.12[c]
Middle lung region	23	4 (3-5)	11	4 (3-5)	.38[c]
Lower lung region	23	4 (3-5)	12	5 (4-5)	.36[c]
Serum biomarkers of PAP					
LDH, IU/L	23	287 ± 19	12	325 ± 26	.26[a]
CEA, ng/mL	23	6.2 ± 1.0	12	8.0 ± 1.4	.30[a]
KL-6, U/L	23	10,038 ± 1,531	12	9,434 ± 2,120	.81[a]
SP-A, ng/mL	23	127 ± 15	12	153 ± 20	.29[a]
SP-D, ng/mL	23	227 ± 25	12	290 ± 34	.14[a]
Hematologic indices					
WBC count, cells/μL	23	5,608 ± 267	12	6,358 ± 370	.11[a]
Neutrophils, cells/μL	22	3,428 ± 200	12	3,596 ± 271	.62[a]
Monocytes, cells/μL	22	344 ± 21	12	396 ± 28	.15[a]
Lymphocytes, cells/μL	22	1,730 ± 147	12	2,122 ± 198	.12[a]
Eosinophils, cells/μL	22	107 ± 28	12	199 ± 38	.058[a]
Basophils, cells/μL	22	18.3 ± 4.3	12	45.3 ± 5.9	.0008[a]
Hemoglobin, g/dL	23	15.4 ± 0.3	12	14.4 ± 0.4	.058[a]
Platelets, × 10^3 cells/μL	23	224 ± 9.1	11	271 ± 13	.0046[a]
BALF cell classification, %					
Alveolar macrophages	17	63 ± 3.6	5	38 ± 6.7	.0036[a]
Neutrophils	17	5.2 ± 1.5	5	10.8 ± 2.7	.082[a]
Eosinophils	17	0.84 ± 0.32	5	0.40 ± 0.60	.52[a]
Lymphocytes	17	31.2 ± 3.8	5	50.4 ± 7.1	.027[a]

BALF = BAL fluid; DLCO = diffusing capacity of the lung for carbon monoxide; HRCT = high-resolution CT; PAP = pulmonary alveolar proteinosis; SP-A = surfactant protein A; SP-D = surfactant protein D; VC = vital capacity. See Table 1 legend for expansion of other abbreviations. [AQ25]

[a]Calculated using Student *t* test.

[c]Calculated using the Wilcoxon rank sum test.

[b]Described previously.[16]

riod. The serum levels were unchanged during the observation period except for three cases (e-Figure 5). In two cases, the serum levels increased by > 100 μg/mL, and one case required an additional treatment, whereas the others did not. In the third case, the serum levels decreased to 0.47 μg/mL, and additional treatments were not required.

Discussion

In the present study we have prospectively ana- [AQ14] lyzed, for the time to our knowledge, the requirements of additional therapy and disease severity scores in 35 patients who completed GM-CSF inhalation therapy. The results demonstrate that 23 patients were free from administration of additional treatment during the 30-month observation period, indicating the enduring nature of the therapy. VC could be a useful predictive parameter for the recurrence of disease after GM-CSF therapy. This study contributes to the promotion of GM-CSF inhalation for initial therapy of aPAP.

WLL remains the standard of care today. A retrospective analysis of 231 cases found clinically significant improvement in Pao_2, FEV_1, VC, and DLCO and reported that the median duration of clinical benefit from lavage was 15 months.[2] In a report of 21 patients with PAP who underwent WLL in an experienced center, > 70% of patients remained free from recurrent PAP during 7-year observation.[23] In our study, the median time to application of additional therapy was 30 months after GM-CSF therapy, suggesting the effects of GM-CSF inhalation may be comparable to those of WLL. Notably, the difference in changes in A-aDO_2 during the GM-CSF treatment between the AT-WLL patients and the AT-GM patients suggests that nonresponders to the first GM-CSF treatment might be likely to undergo WLL when disease recurred.

Table 3—*Pulmonary Function, Radiologic Appearance, Serum Biomarkers, Hematologic Indices, and BALF Cell Findings in Patients With PAP in FR and AT Groups at the End of GM-CSF Inhalation Treatment and Before the 30-mo Observation*

Measure	FR		AT		*P* Value
	No.	Mean ± SE or Median (IQR)	No.	Mean ± SE or Median (IQR)	
Pulmonary function					
VC, % predicted	23	93.4 ± 3.0	12	74.2 ± 4.2	.0007[a]
FVC, % predicted	23	80.5 ± 3.3	12	72.2 ± 4.5	.0025[a]
FEV_1/FVC	23	85.6 ± 1.6	12	84.7 ± 2.2	.73[a]
DLCO, % predicted	23	68.4 ± 3.4	11	46.8 ± 4.7	.0006[a]
HRCT scores[†]					
Upper lung region	23	2 (2-3)	12	3.5 (2-4)	.036[b]
Middle lung region	23	3 (2-3)	12	4 (2.25-4.75)	.023[b]
Lower lung region	23	2 (2-3)	12	4 (2.25-5)	.0039[b]
Serum biomarkers of PAP					
LDH, IU/L	23	242 ± 13	12	308 ± 18	.0064[a]
CEA, ng/mL	23	2.7 ± 0.6	12	5.7 ± 0.8	.0075[a]
KL-6, U/L	23	3,675 ± 735	12	6,565 ± 1,017	.028[a]
SP-A, ng/mL	23	80 ± 12	12	131 ± 16	.015[a]
SP-D, ng/mL	23	170 ± 34	12	304 ± 47	.027[a]
Hematologic indices					
WBC count, cells/μL	23	5,213 ± 306	12	5,797 ± 424	.27[a]
Neutrophils, cells/μL	22	2,961 ± 205	12	3,026 ± 277	.85[a]
Monocytes, cells/μL	22	320 ± 30	12	338 ± 41	.74[a]
Lymphocytes, cells/μL	22	1,755 ± 131	12	2,153 ± 177	.080[a]
Eosinophils, cells/μL	22	145 ± 40	12	233 ± 55	.20[a]
Basophils, cells/μL	22	27.4 ± 5.9	12	43.7 ± 8.4	.12[a]
Hemoglobin, g/dL	23	14.8 ± 1.3	12	14.4 ± 1.4	.52[a]
Platelets, × 10^3 cells/μL	23	214 ± 9.0	12	235 ± 12	.17[a]
BALF cell classification, %					
Alveolar macrophages	13	67 ± 4.1	5	58 ± 6.7	.28[a]
Neutrophils	13	6.6 ± 2.2	5	7.4 ± 3.5	.86[a]
Eosinophils	13	0.90 ± 0.46	5	0.82 ± 0.75	.93[a]
Lymphocytes	13	25.6 ± 4.8	5	33.2 ± 7.7	.41[a]

See Table 1 and 2 legends for expansion of abbreviations.
[a]Calculated using Student *t* test.
[b]Described previously.[16]
[c]Calculated using the Wilcoxon's rank sum test.

In a single-center, phase 2 study for subcutaneous administration of GM-CSF for PAP, Venkateshiah et al[11] reported that nine of 21 patients (43%) required WLL. In a retrospective study of 12 patients who underwent aerosolized GM-CSF therapy, Wylamn et al reported that five of 11 responders had recurrence of disease. In four of five patients, the mean time to relapse was 6.3 months and ranged from 5.5 to 12 months.[15] It is notable that the dose of GM-CSF used in their study was twice that used in our study, although the prognosis of our cases was comparable to that of their study.

PAP is often described as a lung disorder with restrictive physiology. In the present study, 18 of 35 patients were in the normal range (≤ 80) in %FVC, whereas the other 17 patients were mildly to moderately restricted, which was comparable to previous studies.[24] Seymour et al[25] investigated 14 patients who underwent subcutaneous GM-CSF administration and suggested that higher VC before treatment was one marker to define responsiveness to GM-CSF therapy. In the present study, VC did not correlate with responsiveness to GM-CSF therapy, but it showed significant association with the requirement for additional treatment. Although limited by the small number of cases, the subgroup analyses suggested that VC is an independent factor from age, sex, baseline PaO_2, change in A-aDO_2, and baseline levels of serum markers, including anti-GM-CSF-Ab. However, there is a possibility that some clinical variables might be intrinsically related to VC. The physicians' decision for retreatment might be influenced by such clinical markers. Notably, a recent study of a series of patients with PAP followed in a reference center reported that the need for lavage was significantly associated with FVC.[26]

Reduction of VC might be due to two different factors: accumulation of surfactant-derived materials in the alveolar space and fibrotic changes of lung tissue. In a study of a quantitative CT scan analysis of patients

with PAP who underwent WLL and showed improvements in %DLCO and %FVC, Perez et al[27] demonstrated that there was a reduction in lung weight following lavage, which correlated with the dry weight of the lavage effluent. The study demonstrated a shift in the regional lung inflation toward more inflated lung with a corresponding increase in the mean lung inflation. Surfactant accumulation might be associated with an elevated ventilation-perfusion mismatch and disproportionately impaired DLCO in patients with aPAP.[2] Seymour et al[23] demonstrated serum levels of SP-A correlated with VC in 14 patients at baseline. The present study also showed that serum levels of SP-A correlated with VC at baseline as well as after treatment. However, requirement of additional therapy was not significantly associated with SP-A at baseline. Surfactant materials might be easily redistributed in alveolar spaces and may not be related to the impairment of lung tissue that might lead to additional treatment.

The other factor, fibrotic changes of lung tissue, might be maintained even after GM-CSF therapy or WLL. Pulmonary fibrosis has been reported to be associated with PAP, and exposure to oxygen or repeated WLL have been suggested as potential contributors to fibrosis. Although irreversible scarring of the lung is rarely associated with PAP, a small fraction of patients with PAP demonstrated substantially impaired %VC and rather poor prognosis. To investigate this possibility, two radiologists re-evaluated baseline CT scans of 32 of the 35 participants for findings other than PAP without knowing the study results regarding responsiveness and prognosis of the GM-CSF inhalation. They only pointed out traction bronchiectasis in one patient (responder, FR), bronchiectasis in one patient (responder, FR), and multiple bullae in one patient (responder, AT). Thus, we failed to find any significant association between fibrotic change in CT scan and requirement of additional treatments. In the present study, the mean %VC levels of patients in the FR group improved from 85.9% to 93.4%, whereas those of patients in the AT group changed from 71.6% to 74.2%. The difference in improvement between the groups might be associated with the balance of surfactant accumulation and lung fibrosis in the lungs of patients.

For future studies, it would be useful to explore novel treatment regimens for patients with moderately impaired VC. As shown in this study, inhaled GM-CSF therapy did not change serum levels of anti-GM-CSF-Ab. However, the BALF titers of anti-GM-CSF-Ab were reduced in responders, which was likely due to the improved clearance in alveolar spaces. The future treatments might include a combination of GM-CSF inhalation with WLL to improve the environment of airway/alveolar spaces or with administration of rituximab to reduce the systemic production of anti-GM-CSF-Ab.

In conclusion, this study demonstrated that VC might be clinically useful in predicting the need for additional therapy in patients with aPAP who were treated with inhaled GM-CSF therapy. We believe this study contributes to improving the quality of life and treatments for patients with aPAP.

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Author contributions:
Dr Tazawa: contributed to study conception, design, collection and analysis of data, and writing the manuscript.
Dr Inoue: contributed to study design and assisting to write the manuscript.
Dr Arai: contributed to data collection and manuscript preparation.
Dr Takada: contributed to data collection and manuscript preparation.
Dr Kasahara: contributed to manuscript preparation and providing critical patient samples and data.
Dr Hojo: contributed to data collection and manuscript preparation.
Dr Ohkouchi: contributed to study collection and analysis of data, and manuscript preparation.
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Additional information: The e-Figures and e-Table can be found in the "Supplemental Materials" area of the online article.

References

1. Rosen SH, Castleman B, Liebow AA. Pulmonary alveolar proteinosis. *N Engl J Med.* 1958;258(23):1123-1142.
2. Seymour JF, Presneill JJ. Pulmonary alveolar proteinosis: progress in the first 44 years. *Am J Respir Crit Care Med.* 2002;166(2):215-235.
3. Trapnell BC, Whitsett JA, Nakata K. Pulmonary alveolar proteinosis. *N Engl J Med.* 2003;349(26):2527-2539.
4. Kitamura T, Tanaka N, Watanabe J, et al. Idiopathic pulmonary alveolar proteinosis as an autoimmune disease with neutralizing antibody against granulocyte/macrophage colony-stimulating factor. *J Exp Med.* 1999;190(6):875-880.
5. Uchida K, Nakata K, Trapnell BC, et al. High-affinity autoantibodies specifically eliminate granulocyte-macrophage colony-stimulating factor activity in the lungs of patients with idiopathic pulmonary alveolar proteinosis. *Blood.* 2004;103(3):1089-1098.
6. Dranoff G, Crawford AD, Sadelain M, et al. Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. *Science.* 1994;264(5159):713-716.
7. Stanley E, Lieschke GJ, Grail D, et al. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A.* 1994;91(12):5592-5596.
8. Sakagami T, Beck D, Uchida K, et al. Patient-derived granulocyte/macrophage colony-stimulating factor autoantibodies reproduce pulmonary alveolar proteinosis in nonhuman primates. *Am J Respir Crit Care Med.* 2010;182(1):49-61.
9. Seymour JF, Dunn AR, Vincent JM, Presneill JJ, Pain MC. Efficacy of granulocyte-macrophage colony-stimulating factor in acquired alveolar proteinosis. *N Engl J Med.* 1996;335(25):1924-1925.
10. Seymour JF, Presneill JJ, Schoch OD, et al. Therapeutic efficacy of granulocyte-macrophage colony-stimulating factor in patients with idiopathic acquired alveolar proteinosis. *Am J Respir Crit Care Med.* 2001;163(2):524-531.
11. Kavuru MS, Sullivan EJ, Piccin R, Thomassen MJ, Stoller JK. Exogenous granulocyte-macrophage colony-stimulating factor administration for pulmonary alveolar proteinosis. *Am J Respir Crit Care Med.* 2000;161(4 pt 1):1143-1148.
12. Venkateshiah SB, Yan TD, Bonfield TL, et al. An open-label trial of granulocyte macrophage colony stimulating factor therapy for moderate symptomatic pulmonary alveolar proteinosis. *Chest.* 2006;130(1):227-237.
13. Price A, Manson D, Cutz E, Dell S. Pulmonary alveolar proteinosis associated with anti-GM-CSF antibodies in a child: successful treatment with inhaled GM-CSF. *Pediatr Pulmonol.* 2006;41(4):367-370.
14. Schoch OD, Schanz U, Koller M, et al. BAL findings in a patient with pulmonary alveolar proteinosis successfully treated with GM-CSF. *Thorax.* 2002;57(3):277-280.
15. Anderson PM, Markovic SN, Sloan JA, et al. Aerosol granulocyte macrophage-colony stimulating factor: a low toxicity, lung-specific biological therapy in patients with lung metastases. *Clin Cancer Res.* 1999;5(9):2316-2323.
16. Tazawa R, Hamano E, Arai T, et al. Granulocyte-macrophage colony-stimulating factor and lung immunity in pulmonary alveolar proteinosis. *Am J Respir Crit Care Med.* 2005;171(10):1142-1149.
17. Wylam ME, Ten R, Prakash UB, Nadrous HF, Clawson ML, Anderson PM. Aerosol granulocyte-macrophage colony-stimulating factor for pulmonary alveolar proteinosis. *Eur Respir J.* 2006;27(3):585-593.
18. Tazawa R, Trapnell BC, Inoue Y, et al. Inhaled granulocyte/macrophage-colony stimulating factor as therapy for pulmonary alveolar proteinosis. *Am J Respir Crit Care Med.* 2010;181(12):1345-1354.
19. Inoue Y, Trapnell BC, Tazawa R, et al. Characteristics of a large cohort of autoimmune pulmonary alveolar proteinosis in Japan. *Am J Respir Crit Care Med.* 2008;177(7):752-762.
20. Ohashi K, Sato A, Takada T, et al. Direct evidence that GM-CSF inhalation improves lung clearance in pulmonary alveolar proteinosis. *Respir Med.* 2012;106(2):284-293.
21. Uchida K, Beck DC, Yamamoto T, et al. GM-CSF autoantibodies and neutrophil dysfunction in pulmonary alveolar proteinosis. *N Engl J Med.* 2007;356(6):567-579.
22. Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology.* 1982;143(1):29-36.
23. Beccaria M, Luisetti M, Rodi G, et al. Long-term durable benefit after whole lung lavage in pulmonary alveolar proteinosis. *Eur Respir J.* 2004;23(4):526-531.
24. Bonella F, Bauer PC, Griese M, Ohshimo S, Guzman J, Costabel U. Pulmonary alveolar proteinosis: new insights from a single-center cohort of 70 patients. *Respir Med.* 2011;105(12):1908-1916.
25. Seymour JF, Doyle IR, Nakata K, et al. Relationship of anti-GM-CSF antibody concentration, surfactant protein A and B levels, and serum LDH to pulmonary parameters and response to GM-CSF therapy in patients with idiopathic alveolar proteinosis. *Thorax.* 2003;58(3):252-257.
26. Campo I, Mariani F, Rodi G, et al. Assessment and management of pulmonary alveolar proteinosis in a reference center. *Orphanet J Rare Dis.* 2013;8:40.
27. Perez A IV, Coxson HO, Hogg JC, Gibson K, Thompson PF, Rogers RM. Use of CT morphometry to detect changes in lung weight and gas volume. *Chest.* 2005;128(4):2471-2477.

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Review

Crosstalk between endothelial cell and thrombus in chronic thromboembolic pulmonary hypertension: perspective

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Summary. It is generally accepted that chronic thromboembolic pulmonary hypertension (CTEPH) results from pulmonary emboli originating from deep vein thrombosis. However, this consensus opinion has been challenged, and the concept that some aspects of CTEPH exacerbation might result from a small-vessel disease leading to secondary thrombosis has been suggested.

In addition to the effect of recurrent thromboembolism, a number of lines of clinical evidence indicate that progressive worsening is contributed to by remodeling in the small pulmonary arteries. Histopathological studies of the microvascular changes in CTEPH have identified vascular lesions similar to those seen in idiopathic pulmonary arterial hypertension (IPAH). Especially in *in vitro* and *ex vivo* experiments, pulmonary artery endothelial cells (ECs) in pulmonary hypertensive diseases are suggested to exhibit an unusual hyperproliferative potential with decreased susceptibility to apoptosis, indicating that dysfunctional ECs may contribute to the progression of the diseases. Although the degree and mechanisms of EC dysfunction as a contributor to CTEPH are unclear, EC dysfunction may occur in small arteries. Indeed, the cells stimulated by the microenvironment created by the unresolved clot may release substances that induce EC dysfunction. The EC dysfunctions in CTEPH may lead to disorders of the anti-coagulation properties in ECs and may result in additional clots *in situ*. Moreover, these may lead to the progression, not only of distal thrombus, but also of proximal clotting.

This article reviews the pathobiological concepts of CTEPH and explains a crosstalk between EC dysfunction and *in situ* thrombi which may contribute to the vascular lesions of CTEPH.

Key words: Endothelial cell, Thrombus, CTEPH

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Introduction

Chronic thromboembolic pulmonary hypertension (CTEPH) has emerged as one of the leading causes of severe pulmonary hypertension. CTEPH is characterized by intraluminal thrombus formation and fibrous stenosis or complete obliteration of the pulmonary arteries (Klepetko et al., 2004). The consequence is increased pulmonary vascular resistance, resulting in pulmonary hypertension and progressive right heart failure. Pulmonary endarterectomy (PEA) is the current mainstay of therapy for CTEPH (Jamieson et al., 2003). Recently, there has been evidence suggesting that the existing consensus that the pathophysiology of CTEPH results from unresolved pulmonary emboli may have been too simplistic (Hoeper et al., 2006). Although acute pulmonary embolism is generally accepted as the main initiating event in CTEPH, small-vessel disease is believed to appear and worsen later during the course of disease, and to contribute to the progression of hemodynamic and symptomatic decline (Hoeper et al., 2006). Moreover, *in situ* thrombosis and pulmonary arteriopathy have been proposed as potential causes of CTEPH (Shure, 1996; Peacock et al., 2006).

This article reviews the pathobiological concepts of CTEPH, including pulmonary microvascular disease, the endothelial-mesenchymal transition (EnMT), EC dysfunction, and *in situ* thrombosis, which are important pathological features of pulmonary arterial hypertension (PAH) (Eisenberg et al., 1990; Welsh et al., 1996; Wolf et al., 2000; Bauer et al., 2002; Cool et al., 2004; Humbert et al., 2004; Reesink et al., 2004). Furthermore,

it explains a crosstalk between EC dysfunction and *in situ* thrombi which may contribute to the vascular lesions of CTEPH.

Microvascular lesions

In addition to the effect of recurrent thromboembolism, a number of lines of clinical evidence indicate that progressive worsening is contributed to by remodeling in the small distal pulmonary arteries in the open vascular bed (Moser and Bloor, 1993; Azarian et al., 1997; Yi et al., 2000). Indeed, the PH and right ventricular dysfunction are progressive, even in the absence of recurrent thromboemboli (Azarian et al., 1997). Moreover, there is a low degree of correlation between the extent of vascular obstruction visible on pulmonary angiography and the severity of PH (Azarian et al., 1997). There is likely a vascular stealing phenomenon, which means that there is redistribution of the pulmonary blood flow from the nonoccluded to newly endarterectomized vasculature after PEA (Moser and Bloor, 1993). There is often no hemodynamic improvement and persistent PH despite successful PEA in approximately 35% of patients (Condliffe et al., 2008).

Pulmonary microvascular disease, which is an important pathological feature of PAH, leads to increased pulmonary vascular resistance and reduced compliance, with marked proliferation of pulmonary artery smooth muscle cells (SMCs) and endothelial cells (ECs), resulting in the obstruction of blood flow in pulmonary arteries (Humbert et al., 2004). Recently, we reviewed pathogenetic concepts of pulmonary arterial hypertension (PAH) and explained the vascular lesions with EC dysfunction, i.e., apoptosis and proliferation (Sakao et al., 2009, 2010). Taraseviciene-Stewart et al. showed that a vascular endothelial growth factor (VEGF) receptor blocker induced some of the "angioproliferative" features typical of advanced PAH in a rat model, i.e., worsening of the pathological vascular remodeling, and those features were reversed by inhibitors of apoptosis, suggesting that increased apoptosis of ECs in response to loss of survival signaling provided a selection pressure that induced the emergence of actively proliferating ECs without evidence of apoptosis (Taraseviciene-Stewart et al., 2001). Moreover, our *in vitro* experiments have demonstrated that the emergence of apoptosis-resistant proliferating ECs depended on initial EC apoptosis induced by blockade of VEGF receptors and these phenotypically altered ECs expressed the tumor marker survivin and the antiapoptotic protein Bcl-$_{XL}$ (Sakao et al., 2005). Consistent with our results, Masri et al. have shown that pulmonary artery ECs isolated from patients with idiopathic PAH (IPAH) were hyperproliferative and apoptosis-resistant (Masri et al., 2007). However, these results were from an animal model and tissue culture experiments, not from human. It remains unknown whether they actually contribute to pathobiology of human PAH.

The studies of the microvascular changes in CTEPH have identified histopathological characteristics similar to those seen in IPAH and Eisenmenger's syndrome (Moser and Bloor, 1993; Azarian et al., 1997; Yi et al., 2000; Piazza and Goldhaber, 2011). Therefore, dysfunctional ECs may contribute to the progression of the microvascular changes in CTEPH as shown in PAH. Although PEA is the current mainstay of therapy for CTEPH, a recent study showed that specific vasodilative compounds, e.g., prostanoids, endothelin receptor antagonists, phosphodiesterase type 5 inhibitors or a combination, as used for PAH therapy, improved cumulative survival in the patients with inoperable CTEPH, suggesting that there may be vasodilative reactivity in the vasculature of some populations of CTEPH patients as shown in the vasculature of PAH (Seyfarth et al., 2010). Indeed, there exists evidence that patients with CTEPH show similar acute vasoreactivity to inhaled nitric oxide and iloprost (Ulrich et al., 2006; Skoro-Sajer et al., 2009).

The similarities between the microvascular changes in CTEPH and those seen in IPAH suggest that specific vasodilative compounds as used for PAH therapy may be appropriate for some populations of CTEPH, as the patients with no hemodynamic improvement and persistent PH despite successful PEA.

Endothelial-mesenchymal transition (EnMT)

EnMT is a term which has been used to describe the process through which ECs lose their endothelial characteristics and gain the expression of other mesenchymal cell characteristics (Arciniegas et al., 2007). There is the intriguing possibility that intimal SMCs may arise from ECs (Majesky and Schwartz, 1997). In the systemic circulation, Arciniegas et al. demonstrated that mesenchymal cells that existed in the intimal thickening may arise from ECs (Arciniegas et al., 2000). Indeed, the existence of "transitional cells" demonstrating features of both ECs and vascular SMCs in the plexiform lesions in the lungs from patients with IPAH has been identified (Cool et al., 2004). Our *in vitro* studies of human pulmonary microvascular endothelial cells (HPMVECs) showed that blockade of VEGF receptors generated a selection pressure that killed some ECs and expanded resident progenitor-like cells to transdifferentiate into other mesenchymal phenotypes (Sakao et al., 2007). Although there is the limitation of this study based on *in vitro* experiment, this result may support the concept that transdifferentiation of pulmonary ECs to other mesenchymal cells may contribute to the muscularization of the pulmonary arteries. Because of histopathological similarity of the microvascular changes between CTEPH and IPAH (Moser and Bloor, 1993; Azarian et al., 1997; Yi et al., 2000, Piazza and Goldhaber, 2011), EnMT may contribute to the progression of the microvascular changes in CTEPH.

Recently, we have shown the existence of not only myofibroblast-like cells, but also endothelial-like cells in endarterectomized tissues from patients with CTEPH (Maruoka et al., 2012). Our experiments demonstrated that the endothelial-like cells included a few transitional cells (coexpressing both endothelial- and smooth muscle- cell markers). Moreover, experiments using commercially available HPMVECs and myofibroblast-like cells, which were isolated from the PEA tissues of CTEPH patients, demonstrated that substances associated with myofibroblast-like cells might induce the EnMT (Sakao et al., 2011). Indeed, transitional cells which co-expressed both endothelial- and smooth muscle- cell markers were identified in the PEA tissues of patients with CTEPH (Sakao et al., 2011). In support of our findings, Yao et al. showed the presence of CD34 (an endothelial marker) positive cells co-expressing α-smooth muscle actin (a smooth muscle- cell marker) in endarterectomized tissues from patients with CTEPH (Yao et al., 2009).

As shown in our experiment, Firth et al. demonstrated that a myofibroblast cell phenotype was predominant within endarterectomized tissues from patients with CTEPH, contributing extensively to the vascular lesion/clot (Firth et al., 2010). Moreover, the existence of putative endothelial progenitor cells in endarterectomized tissues of patients with CTEPH has been demonstrated (Yao et al., 2009). Firth et al. have reported the presence of multipotent mesenchymal progenitor cells within the tissues of patients with CTEPH (Firth et al., 2010). These studies suggested that the unique microenviroment created by the stabilized clot may promote these progenitor cells to differentiate into myofibloblast-like cells, and the misguided differentiation of these progenitor cells may enhance intimal remodeling (Yao et al., 2009; Firth et al., 2010). Therefore, myofibloblast-like cells may participate directly in vascular remodeling and they may induce EnMT to lead to EC dysfunction.

Indeed, it may be possible that the cells coexpressing both endothelial- and SM- cell markers in endarterectomized tissues are more likely progenitor cells rather than the cells which are differentiated by EnMT. However, in our *in vitro* experiments, there was no bone marrow-derived cell (defined as born marrow cell markers) in the cultured endothelial-like cells because *ex vivo* conditions may allow these cells to differentiate (Sakao et al., 2011).

EnMT may contribute to the development of vascular remodeling in the patients with CTEPH and interrupting this transition may provide a therapeutic target for CTEPH.

EC dysfunction

The degree and mechanisms of EC dysfunction as a contributor to CTEPH in small muscular arteries distal to nonobstructed pulmonary elastic vessels are unclear (Yi et al., 2000; Dartevelle et al., 2004; Hoeper et al., 2006). However, EC dysfunction may play a crucial role in these areas. Indeed, EC related humoral markers that have been linked to CTEPH include anticardiolipin antibodies, a known risk factor for venous thromboembolism (Torbicki et al., 2008), elevated endothelial factor VIII (Wolf et al., 2000; Bonderman et al., 2003), and monocyte chemoattractant protein 1 (Kimura et al., 2001). Moreover, markers of endothelial trauma or dysfunction, such as endothelins, regularly observed in IPAH, are also found in cases of pulmonary embolism (Sofia et al., 1997). In particular, the endothelin-1 levels in CTEPH closely correlated with the hemodynamic and clinical severity of the disease (Reesink et al., 2006). Endothelin-mediated vascular remodeling and impairment of nitric oxide function may play a crucial role in the development of vascular lesions distal to occluded vessels in CTEPH, as well as in severe PH (Bauer et al., 2002; Reesink et al., 2004). It has been observed that PH is more likely to occur following partial vascular occlusions of pulmonary artery segments than following complete occlusions (Robin et al., 1966), thus suggesting that vasoactive substances produced by the turbulent flow in CTEPH may be involved in EC dysfunction. However, it seems to be difficult to define EC dysfunction in patients with CTEPH.

Several lines of evidence indicate that autophagy has an important role in many different pathological conditions. Moreover, fewer mitochondria, the decreased expression of superoxide dismutase and normoxic decreases in reactive oxygen species have been shown to be the characteristics of mitochondrial abnormalities in PAH (Archer et al., 2008). Our recent findings demonstrated that endothelial-like cells lost their ability to form autophagosomes and had defective mitochondrial structure/function (Sakao et al., 2011), indicating that EC dysfunctions occur in the proximal lesions of patients with CTEPH. Moreover, experiments using commercially available HPMVECs and myofibroblast-like cells demonstrated that factors associated with myofibroblast-like cells might induce HPMVEC dysfunction through the inactivation of autophagy, the disruption of the mitochondrial reticulum, and the improper localization of superoxide dismutase-2 (Sakao et al., 2011). The PCR array data analysis showed that substances associated with myofibroblast-like cells induced the alterations in the endothelial cell biology of HPMVECs (Sakao et al., 2011). Although it is uncertain whether EC dysfunctions actually contribute to microvascular remodeling in patients with CTEPH, the myofibroblast-like cells in the proximal lesions may contribute to EC dysfunction in the vasculature of CTEPH. Indeed, it has been demonstrated that ECs in noninvolved pulmonary vascular beds are different from ECs in regions of organized thromboembolic material in patients with CTEPH (Lang et al., 1994a,b). In patients with CTEPH, primary ECs cultured from pulmonary arteries without thrombus had no abnormalities in the expression of fibrinolytic proteins or responses to thrombin stimulation (Lang et al., 1994a,b). However,

ECs within yellowish-white thrombi, i.e., the highly organized tissues, showed elevated type 1 plasminogen activator inhibitor (PAI-1) mRNA levels (Lang et al., 1994a). Therefore, we have to separate them to consider EC dysfunction.

The correlation between endothelins and vascular remodeling in CTEPH seems to support the possibility that pharmacological therapy using endothelin receptor antagonists are effective treatment for the patients with CTEPH.

In situ thrombosis

ECs not only facilitate the thrombotic process, but also actively inhibit thrombosis and promote fibrinolysis. The production and release of nitric oxide and prostacyclin, two potent inhibitors of platelet aggregation, by ECs are important for the prevention of intravascular thrombosis (Moncada et al., 1991). In addition, the expression of thrombomodulin (TM), a high affinity receptor for thrombin, on the surface of ECs prevents the cleavage of fibrinogen to fibrin. ECs are also a source of tissue plasminogen activator (t-PA), a key activator of plasminogen in the *fibrinolytic* cascade. On the other hand, ECs also synthesize and release plasminogen activator inhibitor (PAI)-1, an inhibitor of t-PA, highlighting the role of the endothelium in regulating the fine balance between prothrombotic and antithrombotic processes.

Indeed, the plasma concentration of soluble TM in patients with CTEPH was found to be significantly lower than that in the control group, suggesting that a

Table 1. Clinical and pathobiological features of CTEPH.

Hallmarks	Features	Reference	Tissue Culture/ Clinical
Microvascular lesions	Progressive worsening by remodeling in the small distal pulmonary arteries	Azarian et al. (1997)	Clinical
		Moser and Bloor (1993)	Clinical
		Yi et al. (2000)	Clinical
	No hemodynamic improvement and persistent PH despite successful PEA	Condliffe et al. (2008)	Clinical
	microvascular changes in CTEPH similar to those seen in IPAH	Azarian et al. (1997)	Clinical
		Moser and Bloor (1993)	Clinical
		Yi et al. (2000)	Clinical
		Piazza and Goldhaber (2011)	Clinical
	Vasodilative reactivity in the vasculature of CTEPH	Seyfarth et al. (2010)	Clinical
EnMT	Transitional cells in endarterectomized tissues	Sakao et al. (2011)	Tissue Culture
		Yao et al. (2009)	Tissue Culture
	EnMT induced by substances associated with the cells in endarterectomized tissues	Sakao et al. (2011)	Tissue Culture
	The existence of endothelial and mesenchymal progenitor cells in endarterectomized tissues	Yao et al. (2009)	Tissue Culture
		Firth et al. (2010)	Tissue Culture
EC dysfunction	Humoral markers related with EC in CTEPH: Anticardiolipin antibodies Endothelial factor VIII Monocyte chemoattractant protein 1 Endothelins	Torbicki et al. (2008)	Clinical
		Bonderman et al. (2003)	Clinical
		Kimura et al. (2001)	Clinical
		Sofia et al. (1997)	Clinical
		Reesink et al. (2006)	Clinical
	Endothelin-mediated vascular remodeling	Reesink et al. (2006)	Clinical
	The loss of the ability to form autophagosomes	Sakao et al. (2011)	Tissue Culture
	Structure-function defects of mitochondria	Sakao et al. (2011)	Tissue Culture
	ECs with abnormalities in the expression of fibrinolytic proteins or responses to thrombin stimulation	Lang et al. (1994a,b)	Tissue Culture
		Lang et al. (1994a,b)	Tissue Culture
In situ thrombosis	A decreased plasma TM concentration	Sakamaki et al. (2003)	Clinical
	Elevated PAI-1 mRNA levels	Lang et al. (1994)	Tissue Culture
	The decreased expression of Annexin A and plasminogen activator genes in HPMVECs co-cultured with the cells from the PEA tissues	Sakao et al. (2011)	Tissue Culture

CTEPH: Chronic thromboembolic pulmonary hypertension; PH: Pulmonary arterial hypertension; PEA: Pulmonary endarterectomy; IPAH: Idiopathic pulmonary arterial hypertension; EnMT: Endothelial-mesenchymal transition; EC: endothelial cell; TM: Thrombomodulin; PAI-1: type 1 plasminogen activator inhibitor; HPMVECs: Human pulmonary microvascular endothelial cells.

decreased plasma TM concentration might reflect pulmonary vascular EC dysfunction, leading to altered anticoagulant and fibrinolytic function in CTEPH (Sakamaki et al., 2003). ECs within the highly organized tissues in CTEPH exhibited elevated PAI-1 mRNA levels in comparison to patient pulmonary artery specimens that were free of thrombus, suggesting that the prevalence of PAI-1 expression within pulmonary thromboemboli may play a role in the stabilization of vascular thrombi (Lang et al., 1994a). Moreover, there were decreases in the expression of the Annexin A5 and plasminogen activator, urokinase genes in HPMVECs co-cultured with myofibroblast-like cells from the PEA tissues of CTEPH patients (Sakao et al., 2011). Annexin A5 plays an important role in anticoagulant function and is a protein that has a high affinity for negatively-charged phospholipids (Funakoshi et al., 1987; Tait et al., 1988), over which it forms trimers (Voges et al., 1994) that become an annexin A5 shield. The formation of this shield blocks the phospholipids from phospholipid-dependent coagulation enzyme reactions (Andree et al., 1992). Plasminogen activator, urokinase, is a thrombolytic agent. Its primary physiological substrate is plasminogen, which is an inactive zymogen form of the serine protease plasmin. The activation of plasmin triggers a proteolysis cascade that, depending on the physiological environment, participates in thrombolysis or extracellular matrix degradation (Collen and Lijnen, 2005). The decreased expression of Annexin A and plasminogen activator, urokinase, may contribute to the disorder of the anti-coagulation properties in CTEPH patients. However, there is no validation of these data in an *in vivo* experiment.

There are several lines of evidence indicating that EC dysfunction might interfere with the normal balance between the pro-thrombotic and anti-thrombotic mechanisms, resulting in local thrombosis, and may contribute to the pathophysiology of PAH (Eisenberg et al., 1990; Welsh et al., 1996; Wolf et al., 2000). The EC dysfunction in CTEPH may lead to disorder of the anti-coagulation properties in ECs, i.e., may inactivate a vascular fibrinolytic system, and result in the formation of additional clots *in situ*, like PAH, because the histopathological features in the CTEPH vasculature are similar to those seen in IPAH.

Crosstalk between the unresolved clot, EC dysfunction and *in situ* thrombi

Although the first pulmonary embolism is generally accepted as the main initiating event in CTEPH, we hypothesize that the emergence of the microenvironment created by the unresolved clot may result in the local induction of substances that circulate to cause a more widespread predisposition to vascular remodeling affecting the rest of the pulmonary vascular bed, i.e., beyond the site of initial thrombosis. Our recent study suggested that myofibroblast-like cells stimulated by the microenvironment created by the unresolved clot might release substances that promote ECs to transition to other mesenchymal phenotypes and/or induce EC dysfunction, contributing not only to the proximal vasculature, but also to the distal vasculature (Sakao et al., 2011). The precise reasons for the lung-specific action of these substances in CTEPH remain unknown. One explanation may be that the pulmonary vascular

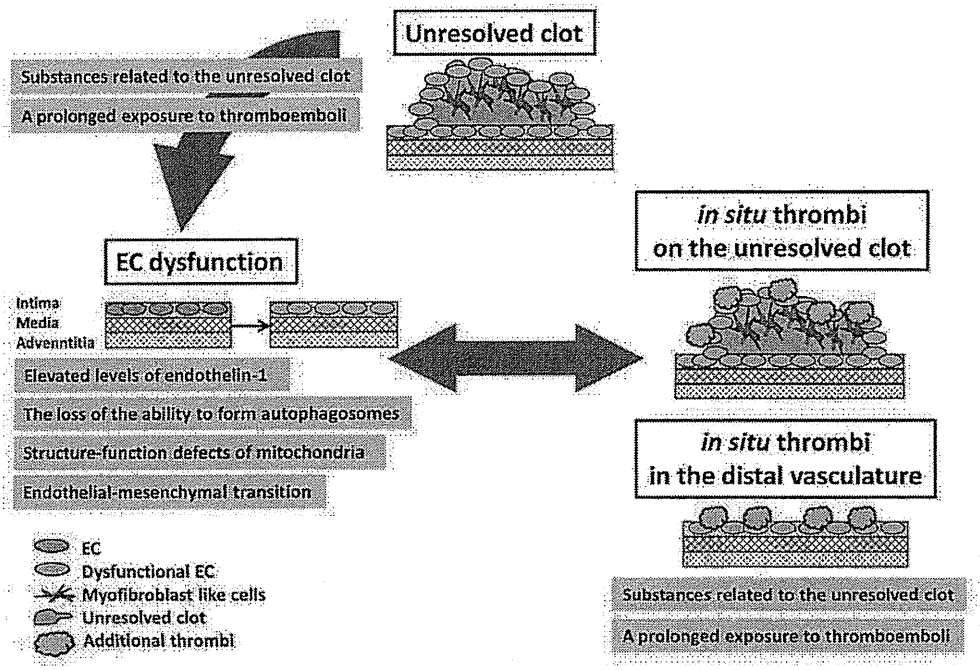

Fig. 1. Crosstalk between EC dysfunction and *in situ* thrombi in CTEPH (a hypothetical mechanism). The cells stimulated by the microenvironment created by an unresolved clot may release substances that induce EC dysfunction. The pulmonary vasculature in patients with CTEPH is subjected to prolonged exposure to thromboemboli. Indeed, thrombin is known to have potent effects on ECs, leading to endothelial barrier dysfunction due to mobilization of Ca^{2+} and rearrangement of the cytoskeleton. An impairment of the EC function in patients with CTEPH may lead to additional thrombi *in situ*, as is seen in patients with PAH, and these may lead to the progression of the proximal clot. A crosstalk between EC dysfunction and *in situ* thrombi may therefore contribute to the vascular lesions of CTEPH. CTEPH: chronic thrombo-embolic pulmonary hypertension, EC: endothelial cells.

beds, i.e., alveolar arteries, are exposed to the highest oxygen tensions in the body, which may induce the different response against substances related by an unresolved clot in comparison to systemic artery ECs. However, this explanation is not sufficient and further explanations are needed.

We fully recognize the limitation of our data interpretation which is based on *in vitro* studies of cultured cells and that this study does not confer any pathological evidence in CTEPH. Indeed, extensive small vessel disease may be a complication of a minority of CTEPH cases. Therefore, besides substances related to the microenviroment created by the stabilized clot, a second factor may be required to induce EC dysfunction which results in extensive disease. However, it remains unknown what is the second factor that is responsible for whether extensive small vessel disease occurs in a given patient.

In the pathogenesis of CTEPH, pulmonary microvascular lesions develop in the distal areas of unoccluded as well as occluded pulmonary arteries (Moser and Bloor, 1993; Azarian et al., 1997; Yi et al., 2000). The development of microvascular lesions distal to totally obstructed pulmonary arteries may be promoted by substances related to the microenvironment created by the unresolved clot. The development of the lesions distal to nonobstructed pulmonary arteries may be promoted not only by substances, but also by increased shear stress caused by hypoxic pulmonary vasoconstriction, because shear stress has been shown to inhibit apoptosis of ECs (Pi et al., 2004) and to stimulate EC growth (Ameshima et al., 2003; Sakao et al., 2005), contributing to vascular remodeling. However, unless the occlusion is enormous, it seems unlikely that vessel occlusion alone increases shear stress in unoccluded arteries because of the large reservoir capacity of the normal pulmonary vasculature. A more likely explanation for the lesions distal to nonobstructed pulmonary arteries may be that the pulmonary arteriopathy could be the initial pathology of the lesions in the patients with IPAH (Peacock et al., 2006). In any case, a persistent clot in the peripheral pulmonary arteries despite successful PEA may continue to create the microenvironment that induces microvascular changes. This may be the reason why there are patients who do not respond to PEA.

In the proximal lesions in patients with CTEPH, the pulmonary vasculature is subjected to a prolonged exposure to thromboemboli, i.e., components in the final common pathway of the coagulation cascade. Indeed, thrombin, a serine protease that catalyzes the conversion of fibrinogen to fibrin, is known to have potent effects on ECs, leading to endothelial barrier dysfunction due to the mobilization of Ca^{2+} and rearrangement of the cytoskeleton (Ellis et al., 1999). Moreover, chronic exposure to fibrinogen, fibrin, and thrombin caused changes in the cytosolic Ca^{2+} in pulmonary artery ECs, suggesting that such changes might contribute to EC dysfunction, thus leading to vascular changes in patients with CTEPH (Firth et al., 2009).

Based on these observations, it has been suggested that many kinds of insults to ECs of the pulmonary arteries may initiate a sequence of events which leads to the EC dysfunctions in CTEPH. Numerous factors such as hypoxia, endogenous vasoconstrictors, and inflammatory cytokines could help to sustain this process (Egermayer et al., 1999). An impairment of the EC function in patients with CTEPH may lead to additional thrombi *in situ* similar to that observed in patients with PAH, and these may also lead to the progression of the proximal clot.

It has been suggested that the core of the pathological process in CTEPH is not only related to thrombus formation, but it is also linked to disturbed thrombus resolution (Morris et al., 2006, 2007; Suntharalingam et al., 2008). An altered coagulation process may account for the pathological features of CTEPH (Wolf et al., 2000). Recently, the fibrinogen A Thr312Ala polymorphism was shown to correspond to significant differences in the genotype and allele frequencies between CTEPH and control subjects. The presence of these polymorphisms may confer resistance to fibrinolysis that subsequently contributes to the development of thrombus organization (Suntharalingam et al., 2008). The other mechanism may be the development of more fibrinolysis-resistant fibrin clots from patients with CTEPH, when compared with the fibrin clots from healthy control subjects (Morris et al., 2006). An abnormally elevated amount of disialylated fibrinogen γ-chain can render a clot resistant to plasmin, which could lead to the subsequent development of CTEPH (Morris et al., 2007). However, these explanations are not sufficient because there are many patients without known coagulation problems who have these factors, and because numerous genetic variants of human fibrinogen have been implicated in thrombotic diseases (Matsuda and Sugo, 2002). Therefore, the resistance could be ascribed to not only fibrinogen genetic polymorphisms, but also variations in the post-translational modifications.

Conclusion

Besides the altered coagulation process, a crosstalk between EC dysfunction and *in situ* thrombi may contribute to the vascular lesions of CTEPH (Fig. 1) (Table 1). Moreover, this may explain why pulmonary thromboemboli in CTEPH patients are stable. Indeed, pulmonary thromboendarterectomy may be the best way to break this crosstalk. Recently, we demonstrated that poor subpleural perfusion on pulmonary angiography might be related to distal vascular remodeling and an inadequate surgical outcome of CTEPH (Tanabe et al., 2012). No satisfactory hemodynamic improvement and persistent PH despite successful PEA in the patients with CTEPH (Condliffe et al., 2008) suggests the existence of distal vascular remodeling. Although it remains uncertain whether vascular remodeling is actually related

to the crosstalk between EC dysfunction and *in situ* thrombi, the care for these patients should be directed toward pharmacologically reducing pulmonary vascular resistance with specific vasodilative compounds as used for PAH therapy. The next step in the future is to find out new ways to define EC dysfunction and vascular remodeling in CTEPH objectively.

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References

Ameshima S., Golpon H., Cool C.D., Chan D., Vandivier R.W., Gardai S.J., Wick M., Nemenoff R.A., Geraci M.W. and Voelkel N.F. (2003). Peroxisome proliferatoractivated receptor gamma (PPARgamma) expression is decreased in pulmonary hypertension and affects endothelial cell growth. Circ. Res. 92, 1162-1169.

Andree H.A., Stuart M.C., Hermens W.T., Reutelingsperger C.P., Hemker H.C., Frederik P.M. and Willems G.M. (1992). Clustering of lipid-bound annexin V may explain its anticoagulant effect. J. Biol. Chem. 267, 17907-17912.

Archer S.L., Gomberg-Maitland M., Maitland M.L., Rich S., Garcia J.G. and Weir E.K. (2008). Mitochondrial metabolism, redox signaling, and fusion: a mitochondria-ROS-HIF-1alpha-Kv1.5 O2-sensing pathway at the intersection of pulmonary hypertension and cancer. Am. J. Physiol. Heart. Circ. Physiol. 294, 570-578.

Arciniegas E., Ponce L., Hartt Y., Graterol A. and Carlini R.G. (2000). Intimal thickening involves transdifferentiation of embryonic endothelial cells. Anat. Rec. 258, 47-57.

Arciniegas E., Frid M.G., Douglas I.S. and Stenmark K.R. (2007). Perspectives on endothelial-to-mesenchymal transition: potential contribution to vascular remodeling in chronic pulmonary hypertension. Am. J. Physiol. Lung. Cell. Mol. Physiol. 293, L1-8.

Azarian R., Wartski M., Collignon M.A., Parent F., Herve P., Sors H. and Simonneau G. (1997). Lung perfusion scans and hemodynamics in acute and chronic pulmonary embolism. J. Nucl. Med. 38, 980-983.

Bauer M., Wilkens H., Langer F., Schneider S.O., Lausberg H. and Schafers H.J. (2002). Selective upregulation of endothelin B receptor gene expression in severe pulmonary hypertension. Circulation 105, 1034-1036.

Bonderman D., Turecek P.L., Jakowitsch J., Weltermann A., Adlbrecht C., Schneider B., Kneussl M., Rubin L.J., Kyrle P.A., Klepetko W., Maurer G. and Lang I.M. (2003). High prevalence of elevated clotting factor VIII in chronic thromboembolic pulmonary hypertension. Thromb. Haemost. 90, 372-376.

Condliffe R., Kiely D.G., Gibbs J.S, Corris P.A., Peacock A.J., Jenkins D.P., Hodgkins D., Goldsmith K., Hughes R.J., Sheares K., Tsui S.S., Armstrong I.J., Torpy C., Crackett R., Carlin C.M., Das C., Coghlan J.G. and Pepke-Zaba J. (2008). Improved outcomes in medically and surgically treated chronic thromboembolic pulmonary hypertension. Am. J. Respir. Crit. Care. Med. 177, 1122-1127.

Cool C.D., Wood K. and Voelkel N.F. (2004). Transdifferentiation of endothelial cells in primary pulmonary hypertension. Am. J. Resp. Crit. Care. Med. 167: A844.

Collen D. and Lijnen H.R. (2005). Thrombolytic agents. Thromb. Haemost. 93, 627-630.

Dartevelle P., Fadel E., Mussot S., Chapelier A., Herve P., de Perrot M., Cerrina J., Ladurie F.L., Lehouerou D., Humbert M., Sitbon O. and Simonneau G. (2004). Chronic thromboembolic pulmonary hypertension. Eur. Respir. J. 23, 637-648.

Egermayer P., Town G.I. and Peacock A.J. (1999). The role of serotonin in the pathophysiology of acute and chronic pulmonary hypertension. Thorax 54, 161-168.

Eisenberg P.R., Lucore C., Kaufman L., Sobel B.E., Jaffe A.S. and Rich S. (1990). Fibrinopeptide A levels indicative of pulmonary vascular thrombosis in patients with primary pulmonary hypertension. Circulation 82, 841-847.

Ellis C.A., Tiruppathi C., Sandoval R., Niles W.D. and Malik A.B. (1999). Time course of recovery of endothelial cell surface thrombin receptor (PAR-1) expression. Am. J. Physiol. Cell. Physiol. 276, C38-C45.

Firth A.L., Yau J., White A., Chiles P.G., Marsh J.J., Morris T.A. and Yuan J.X. (2009). Chronic exposure to fibrin and fibrinogen differentially regulates intracellular Ca2+ in human pulmonary arterial smooth muscle and endothelial cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 296, L979-986.

Firth A.L., Yao W., Ogawa A., Madani M.M., Lin G.Y. and Yuan J.X. (2010). Multipotent mesenchymal progenitor cells are present in endaretectomized tissues from patients with chronic thromboembolic pulmonary hypertension. Am. J. Physiol. Cell. Physiol. 298, C1217-C1225.

Funakoshi T., Heimark R.L., Hendrickson L.E., McMullen B.A. and Fujikawa K. (1987). Human placental anticoagulant protein: isolation and characterization. Biochemistry 26, 5572-5578.

Hoeper M.M., Mayer E., Simonneau G. and Rubin L. (2006). Chronic thromboembolic pulmonary hypertension. Circulation 113, 2011-2020.

Humbert M., Morrell N.W., Archer S.L., Stenmark K.R., MacLean M.R., Lang I.M., Christman B.W., Weir E.K., Eickelberg O., Voelkel N.F. and Rabinovitch M. (2004). Cellular and molecular pathobiology of pulmonary arterial hypertension. J. Am. Coll. Cardiol. 43, 13S-24S.

Jamieson S.W., Kapelanski D.P., Sakakibara N., Manecke G.R., Thistlethwaite P.A., Kerr K.M., Channick R.N., Fedullo P.F. and Auger W.R. (2003). Pulmonary endarterectomy: experience and lessons learned in 1,500 cases. Ann. Thorac. Surg. 76, 1457-1462.

Kimura H., Okada O., Tanabe N., Tanaka Y., Terai M., Takiguchi Y., Masuda M., Nakajima N., Hiroshima K., Inadera H., Matsushima K. and Kuriyama T. (2001). Plasma monocyte chemoattractant protein-1 and pulmonary vascular resistance in chronic thromboembolic pulmonary hypertension. Am. J. Respir. Crit. Care. Med. 164, 319-324.

Klepetko W., Mayer E., Sandoval J., Trulock E.P., Vachiery J.L., Dartevelle P., Pepke-Zaba J., Jamieson S.W., Lang I. and Corris P. (2004). Interventional and surgical modalities of treatment for pulmonary arterial hypertension. J. Am. Coll. Cardiol. 43 (suppl S), 73S-80S.

Lang I.M., Marsh J.J., Olman M.A., Moser K.M., Loskutoff D.J. and