EXPERIMENTAL STUDY

Murine Model of Pulmonary Artery Overflow Vasculopathy Revealed Macrophage Accumulation in the Lung

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Summary

Chronic thromboembolic pulmonary hypertension (CTEPH) is usually life-threatening.1,2 The primary manifestation of CTEPH includes intraluminal fibrin clot formation and obstruction of the pulmonary arteries. The obliteration of pulmonary arteries elevates pulmonary artery pressure, leading to right ventricular hypertrophy and heart failure. The pathological processes underlying CTEPH are not simply explained by the fibrin-mediated obliteration of the pulmonary arteries.3 The redistribution of the cardiac output to unobstructed arteries induces vascular remodeling, known as pulmonary arterial overflow vasculopathy (PAOV). Both thrombotic obstruction and subsequent PAOV in small vessels chiefly account for the pathological processes in CTEPH. In treating patients with CTEPH, pulmonary endarterectomy (PEA) or balloon pulmonary angioplasty is performed to restore blood flow in the obliterated arteries.4-5 While these therapeutic approaches are effective in alleviating the severity of CTEPH,6,7 they do not always normalize hemodynamic parameters safely, including pulmonary vascular resistance.7,8 The refractoriness to PEA, therefore, illuminates the importance of the need to understand the pathological processes in PAOV.9

Animal models of PAOV have previously been developed using pigs or sheep.10,11 In these models, a conduit was placed between the innominate artery and the main pulmonary artery, resulting in the development of vascular remodeling in the anastomosed vessels.11-13 These animal models of PAOV have elucidated the roles of endothelin-1 signaling in vascular remodeling.14 However, the extent of shunt flow between the aorta and pulmonary artery can be influenced by several hemodynamic parameters including systemic or pulmonary pressure, pulmonary vascular resistance, and ventricular function. In addition, the oxygenation status of the anastomosed pulmonary arteries is strikingly affected. The development of a murine model of PAOV in which vascular remodeling could be induced in a reproducible manner would greatly aid in understanding...

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the underlying pathological processes.

The aim of this study is to establish a murine PAOV model in which we could elicit vascular remodeling in a reproducible manner. For that purpose, we resected the left lung and elicited redistribution of cardiac output to the spared arteries. Since the expression of chemokine (C-C motif) ligand 2 (CCL2) is increased in the lung tissue of CTEPH patients,\(^{19}\) we examined whether monocytes/macrophages accumulated to the lung in our PAOV model.

Methods

Mice: The study was approved by the University of Tokyo Ethics Committee for Animal Experiments and strictly adhered to animal care guidelines. C57BL/6J mice were housed in a specific pathogen-free facility with a 12-hour light/12-hour dark cycle. Female mice 8-10 weeks of age were used to create the PAOV model.

PAOV model creation: We anesthetized mice with an intraperitoneal injection of pentobarbital (100 μg/10 g body weight). The mice were intubated with the outer cylinder of an indwelling needle (22-G) (Terumo, Tokyo, Japan) and connected to a respirator (SN-480-7 × 2: Shinano Corporation, Tokyo, Japan). We made an incision in the left 5th intercostal space to expose the left hilum. Following this, we grasped the proximal site of the left hilum with forceps and ligated the left hilum with a 5-0 silk tie (Ethicon, Somerville, NJ, USA). We then excised the entire left lung distal to the hilar ligature, removed the lung en bloc, and closed the thoracic cavity with interrupted 5-0 silk sutures. Thoracic incision and closure without manipulation of the left lung was performed in sham pneumonectomy mice. Lungs were harvested 21 days after the operation. A blood gas test was performed using GASTAT navi (Techno Medica Co., Ltd, Kanagawa, Japan).

Histological study: We performed hematoxylin-eosin (HE) and elastica van Gieson (EVG) staining as described previously\(^ {16}\) using residual right lung tissue. Antibody agents (α-smooth muscle actin [A2547, 1:2000; Sigma, St. Louis, USA] and F4/80 [MCA497, 1:400; AbD Serotec, Kidlington, UK]) were used for immunohistological staining. The number of F4/80-positive cells was counted in a blinded manner with × 200 magnification. The average number of F4/80-positive cells within 8 fields was calculated in each mouse.

Vascular media area: The external (internal) elastic lamina of the pulmonary artery were identified with EVG staining. The area surrounded by the external or internal elastic lamina was designated either the external or internal media area. The vascular media area was calculated as follows, using two fields per mouse: (external media area - internal media area) / external media area × 100.\(^ {17}\) The average of the vascular media area within two fields was calculated in each mouse. The external or internal media area was measured using Image J software (Rasband, W. S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012.).

Quantitative RT-PCR: To examine the gene expression, total RNA was collected from lung tissues using RNeasy kits, according to the manufacturer’s instructions (Qiagen, Tokyo, Japan). Complementary DNA was synthesized using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific K.K., Tokyo, Japan). Quantitative real-time PCR (RT-PCR) analyses were performed using the LightCycler system (Roche Diagnostic, Tokyo, Japan). The following primers were used: 5'-cacccgagctgtaagc-3' and 5'-gcctactcattggatcatctg-3' for Klf2, 5'-ggaagggagaagacact-3' and 5'-gattctcagcaacagc-3' for Klf4, 5'-cgaagaaccttgccaaagat-3' and 5'-agcggcatctgtatgctg-3' for I85 rRNA. The abundance of I85 rRNA was calculated as the internal control.

Statistical analysis: Data are shown as a bar graph or a scatter dot plot. Lines depict mean with standard deviation. A comparison between the two groups was performed using an unpaired two-tailed t-test with Welch’s correction. A P value less than 0.05 was considered statistically significant. All statistical analyses were performed using Prism 7 for Windows (version 7.03. GraphPad, San Diego, CA, USA).

Results

PAOV induced pulmonary arterial remodeling and right ventricular hypertrophy: To induce a redistribution of the blood flow, we resected the left lung, which could impair respiratory function and decrease blood oxygen levels. We therefore measured arterial oxygen saturation levels. The oxygen saturation level remained unchanged in the PAOV model (Figure 1A), indicating that the PAOV model did not impair ventilatory function. To examine the pulmonary lesion in the murine PAOV model, we performed histological studies using lung tissue 21 days after the operation. We performed HE and EVG staining and visualized the internal and external elastic laminae (Figure 1B). We then calculated the media thickness using EVG staining and found that the vascular media area was significantly increased in the lung in PAOV compared with that in sham-operated mice (Figure 1C). An immunohistochemical study using α-smooth muscle actin antibody showed positive staining consistent with the vascular media (Figure 1D). While we detected a thickening of the medial layer,plexiform lesions were not detected in our PAOV model. Kluppel-like family of transcription factors (KLFs) are involved in vascular flow response.\(^ {18,19}\) Therefore, we measured their expression in our PAOV model. Findings showed that the transcript level of Klf2 was decreased, whereas that of Klf4 was increased in the lung tissue (Figure 1E). To examine the extent of hypertrophy, we measured right ventricular weight in murine hearts and discovered that the ratio of right ventricular to left ventricular plus septal weight in the PAOV model was significantly greater than that in the sham-operated mice (Figure 1F).

Monocytes/macrophages accumulate in residual pulmonary arteries in the PAOV model: Immunohistological studies to investigate the accumulation of monocytes/macrophages in the residual right lung 21 days after pneumonectomy showed the accumulation of F4/80-positive cells around pulmonary arteries in PAOV mice (Figure 2A). Moreover, the number of F4/80-positive cells was sig-
Figure 1. Pulmonary artery overflow vasculopathy (PAOV) model induces pulmonary artery remodeling and right ventricular hypertrophy. A: Arterial blood gas test was performed in PAOV model and control mice (PAOV, n = 6; sham, n = 3). B: Hematoxylin-Eosin (top) and elastica van Gieson staining (bottom) were performed using lung tissue 21 days after the operation (left: sham, right: PAOV model). Scale bar = 50 μm. Lower magnification image was shown in left panel. Higher magnification of the area indicated by a square was demonstrated in right panel. C: Media vascular area was calculated using residual lung tissue 21 days after the operation (PAOV, n = 8; sham, n = 4). D: Immunohistological study (α-smooth muscle actin: brown, nucleus: blue) was performed using lung tissue 21 days after the operation (left: sham, right: PAOV model). Scale bar = 50 μm. E: The transcript level of Klf2 or Klf4 in each mouse was analyzed 21 days after PAOV operation (PAOV, n = 8; sham, n = 4). F: The ratio of right ventricular to left ventricular plus septal weight was calculated using hearts 21 days after the operation (PAOV, n = 8; sham n = 4). A two-tailed t-test with Welch’s correction was used for statistical analysis, * P < 0.05. NS indicates not significant.
Figure 2. Macrophages accumulate in residual pulmonary arteries in the PAOV model. A: Immunohistological study (F4/80: brown, arrow, nucleus: blue) was performed using residual lung tissue 21 days after the operation. Scale bar = 50 μm. Representative images were shown (× 400 magnification). B: The number of F4/80-positive cells were counted using lung tissue 21 days after the operation (× 200 magnification) (PAOV, n = 8; sham, n = 4). A two-tailed t-test with Welch's correction was used for statistical analysis, * P < 0.05. C: The transcript level of Ccl2 in each mouse was analyzed 21 days after PAOV operation (PAOV, n = 8; sham, n = 4). NS indicates not significant.

In this study, we developed a murine model of PAOV significantly greater in the PAOV model compared with the sham-operated mice (Figure 2B). The abundance of Ccl2 in the lung was unchanged between sham and PAOV model mice at day 21 (Figure 2C), suggesting that macrophages are recruited to the lung through a manner independent of CCL2.

Discussion

In this study, we developed a murine model of PAOV
by decreasing the area of the pulmonary vascular bed. Assuming that cardiac output remains unchanged, redistribution of blood flow to spared arteries is expected to elevate pulmonary artery pressure. In keeping with our hypothesis, a transcript level of Klf2, a flow sensitive transcription factor, was decreased in our PAOV model. Right ventricular hypertrophy was consistently observed in our PAOV model. We performed EVG staining and quantitatively measured the area of the vascular media. While thickening of the vascular media was detected, the vasculopathy seemed to be modest. No plexiform lesion was observed in our PAOV model, as is commonly found in lung specimens of patients with CTEPH. Vascular thrombus has a potential to activate inflammatory pathways, thus the absence of the thrombus may explain the modest vasculopathy in our PAOV model. Further intervention may be required to induce advanced vascular lesions of CTEPH, such as hypoxia exposure, chemical endothelial injury, or an increase in the size of lung resection.

Inflammatory processes are thought to play a role in the pathogenesis of CTEPH. The murine PAOV model in this study revealed that F4/80-positive cells accumulate 21 days after left lung resection. The accumulation of monocytes/macrophages seems to be elicited by the increased blood flow to the pulmonary arteries, since accumulation was not observed in sham-operated mice. CCL2 is one of the chemokines that recruits monocytes/macrophages to inflamed tissues. Notably, the expression of CCL2 is upregulated in the lung tissue of CTEPH patients. The transcript level of Ccl2 in the lung, however, remained unchanged in our PAOV model. The molecular processes by which monocytes/macrophages accumulate in overflow pulmonary arteries still remain undetermined. Endothelin-1 has a potential to activate macrophages in the vessels, thus endothelin receptor antagonists will be useful in elucidating the roles of endothelin system in vascular inflammation.

It is also unclear whether inflammatory cells act to promote vascular remodeling or simply accumulate as a consequence of tissue remodeling. The chemical depletion of monocytes/macrophage using clodronate liposomes may aid in understanding the roles of inflammatory cells in the PAOV model. Alternatively, dichloroacetate, which was recently found to inhibit monocyte/macrophage accumulation in hypoxic areas, may also be useful in elucidating the role of sterile inflammation in OV.

In addition to the vascular remodeling in CTEPH, the murine PAOV model will provide further information on the pathological processes in several disorders, including atrial septal defect or coronary pulmonary artery fistula, where volume and/or pressure overload develop in the right ventricle and pulmonary arteries. The murine PAOV model will also contribute to the prevention or treatment of postoperative pulmonary hypertension in lung cancer patients. Surgical resection of lung cancer decreases both ventilation capacity and the vascular bed; thus, the spared lung volume must be carefully determined. A unilateral pulmonary artery occlusion test is sometimes effective in evaluating pulmonary vascular capacitance. This model might be very useful in the assessment of post-pneumonectomy treatment or optimizing the maximum size of lung resection.

In conclusion, we developed a murine model of PAOV that revealed an accumulation of inflammatory cells in the overflow pulmonary arteries. By performing flow cytometric analysis or using genetically engineered mice, this model will provide further information on the roles of inflammatory cells in the development of PAOV.

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Disclosures

Conflicts of interest: None.

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