Development and Characterization of an Animal Model of Severe Pulmonary Arterial Hypertension

Yoshitaka Morimatsu a–c   Naomi Sakashita a   Yoshihiro Komohara a   Koji Ohnishi a  
Hiroshi Masuda d   Diana Dahan b, c   Motohiro Takeya a   Christelle Guibert b, c 
Roger Marthan b, c

aDepartment of Cell Pathology, Graduate School of Medical Science, Kumamoto University, Kumamoto, Japan;  
bCentre de Recherche Cardio-Thoracique de Bordeaux, Université de Bordeaux, cINSERM, U 1045, Bordeaux, and  
dLaboratoire IMS-Site ENSCBP, Université de Bordeaux, Pessac, France

Key Words
Pulmonary hypertension  ·  Pulmonary vascular remodeling  ·  Monocrotaline  ·  Hypobaric chronic hypoxia  ·  Medial hypertrophy

Abstract
Pulmonary arterial hypertension (PAH) is a serious pathological phenomenon with poor prognosis, which is associated with morphological as well as hemodynamic alteration of the pulmonary circulation. To establish an animal model mimicking severe human PAH, we combined 2 well-described procedures, i.e. exposure to hypobaric chronic hypoxia and administration of monocrotaline hydrochloride in rats. Compared to a single procedure, the combined procedure induced more severe right ventricle hypertrophy and an increase in right ventricle systolic pressure. Histological examination on the combined procedure model revealed a severe medial hypertrophy as well as occlusive vascular changes of the intra-acinar pulmonary arteries with endothelial lesions. It is noteworthy that severe alterations including concentric neointimal thickening, abnormal endothelial proliferation, plexiform lesions and vascular occlusion with fibrin thrombi were observed in the combined pulmonary hypertension model when exposed to a long period of hypoxia. The present data indicate that a combined treatment of monocrotaline injection and hypobaric chronic hypoxia exposure produces more severe hemodynamic changes and histological alterations. Since human PAH diagnosed in clinical practice is often severe, this combined treatment animal model could be useful to identify relevant therapeutic targets acting on both hemodynamic and structural alterations of the pulmonary circulation.

Introduction

Pulmonary arterial hypertension (PAH) is a hemodynamic disorder with pulmonary vasculature remodeling, causing progressive right heart insufficiency leading to right heart failure and ultimately to death [1]. A recent clinical classification of PAH groups has been adopted with PAH of unknown etiology, heritable PAH, drug-in-
duced PAH, disease-associated PAH, newborn persistent PAH and another related category, pulmonary veno-occlusive disease and pulmonary capillary hemangiomatis [2]. Regardless of the etiology, PAH is commonly associated with pulmonary vasculature remodeling. A key event of this process is initial endothelial damage and sequential transdifferentiation, resulting in deregulated vascular smooth muscle cell proliferation and abnormal neointima formation [3]. Moreover, overproduction of vasoactive molecules and altered signal transduction networks are also present [1, 3, 4].

Because of a limited supply of human samples, relevant animal models are needed to investigate the molecular mechanisms of pulmonary vascular remodeling processes in detail. Various animal models of pulmonary hypertension have been designed but most of them mimic human PAH pathophysiology unsatisfactorily [5], while others require complicated surgical techniques that limit their use [6, 7]. Recently, Abe et al. [8] reported a new rat model of PAH with severe pulmonary arteriopathy including concentric neointimal thickening and complex plexiform lesions. These lesions were produced by a single subcutaneous injection of the vascular growth factor receptor blocker Sugen 5416 with subsequent exposure to hypoxia for 3 weeks followed by normoxic observation for 10–11 weeks. These lesions mimicked the severe pulmonary arteriopathy of human PAH; however, thrombotic lesions were not observed.

In this study, we developed and characterized an animal model of severe pulmonary hypertension based on the combination of 2 conventional procedures, namely hypobaric chronic hypoxia (HCH) exposure and monocrotaline hydrochloride (MCT) administration. The model exhibits hemodynamic alteration as well as histological remodeling of pulmonary vasculature, mimicking human PAH. It may thus prove useful to identify relevant therapeutic targets based on both phenomena.

Materials and Methods

Animals
Male Wistar rats were maintained at the animal center of the Université de Bordeaux according to the guidelines issued by the Local Animal Care Ethics Committee (Comité d’Éthique Régional d’Aquitaine – referenced AP 2/11/2005) under conditions of unlimited access to food and water. Rats weighing 360–420 g were randomly assigned into the following 4 groups with 12–15 rats in each group: those exposed to HCH, those treated with MCT, those exposed to HCH and treated with MCT (HCH+MCT), and a control group (CTRL). Rats exposed to a hypobaric atmosphere were placed in a hypobaric chamber (380 mm Hg, i.e. approximately half of the sea-level barometric pressure) for 3 weeks (Minerve, Eternay, France) [9]. In the MCT group, rats were intraperitoneally injected with 60 mg/kg of MCT (Sigma, St. Quentin Fallavier, France) and maintained in a normobaric/normoxic atmosphere for 3 weeks. Rats with the combined treatment were injected first with 60 mg/kg of MCT, then immediately exposed to the hypobaric atmosphere for 3 weeks. To induce more severe pulmonary arteriopathy, 4 additional rats were injected with 60 mg/kg of MCT and exposed to a hypobaric atmosphere for 4 weeks and were then histologically evaluated. Finally, CTRL rats were injected with vehicle and maintained in a normobaric/normoxic atmosphere for 3 weeks. All rats were anesthetized by intraperitoneal injection with 50 mg/kg of ketamine and 10 mg/kg of xylazine before they were subjected to the hemodynamic study, and then sacrificed by exsanguination for histological examination. Three rats in the HCH+MCT group (with 3 weeks’ hypoxic treatment) and 2 rats in the HCH group died. No rats in the CTRL and MCT groups died, and neither did any in the HCH+MCT group with 4 weeks’ hypoxic treatment.

Hemodynamic Studies
Hemodynamic studies were performed as previously described [10]. Briefly, the right jugular vein was isolated, cannulated, and the tip of the 2.5-Fr polyethylene catheter (PE-5, Biotrol) was placed into the right atrium and connected to a Baxter Uniflow gauge pressure transducer. Pressure was displayed on an HP 78342A strip-chart recorder (Hewlett-Packard, Palo Alto, Calif., USA). After recording the right atrium pressure pattern, it was placed into the right ventricle and the right ventricle pressure pattern was monitored.

Evaluation of Right Ventricular Hypertrophy
After completion of the hemodynamic evaluation, the rats were sacrificed by bleeding from the inferior vena cava. The atria, main pulmonary artery and aorta were dissected from the heart. The right ventricle was separated from the left ventricular wall and septum and both were weighed. Right ventricular hypertrophy was evaluated as the ratio of the weight of the right ventricle to that of the left ventricle plus the septum (Fulton Index).

Preparation of Lung Tissue Sample
The rat lungs were fixed for histology by tracheal instillation of 10% buffered formalin (pH 7.2) under a constant pressure (15 cm H2O); the trachea was then ligated for 1 h to sustain the inflation. The lungs were excised and fixed again with 10% neutralized buffered formalin for 4 h. The lungs were rinsed with a series of 1 M phosphate-buffered saline (pH 7.2), and prepared as routine paraffin-embedded tissue samples. All tissue samples were cut into 3-μm-thin sections, deparaffinized and stained with hematoxylin and eosin. Some sections were stained with the elastica-van Gieson and picro-Mallory stains to detect elastic fibers and fibrin, respectively. Selected samples were subjected to immunohistochemical analysis.

Immunohistochemical Staining
For the blockade of endogenous peroxidase activity, deparaffinized paraffin sections with 3-μm thickness were incubated with 1% H2O2 for 30 min. After rinsing, sections were incubated with normal goat serum for 20 min, and incubated overnight at 4°C with antibodies against human factor VIII (Dako, Glostrup,
Denmark), human vascular smooth muscle actin (HHF35; Dako) and Ki67 (NeoMarkers, Fremont, Calif., USA). To detect macrophages, the tissues were reacted overnight at 4°C with anti-CD68 (ED1; Serotec, Oxford, UK), anti-CD163 (ED2; Serotec) and anti-CD204 (SRA-E5; Transgenic, Kumamoto, Japan). The samples were then washed intensively, and further incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After the removal of nonreacted secondary antibodies, the samples were incubated with 3,3'-diaminobenzidine-H$_2$O$_2$ solution to visualize immunolabelling. Some sections were then counterstained with hematoxylin and eosin, and mounted with a coverslip.

**Evaluation of Macrophage Infiltration around Vessels**

We evaluated macrophage infiltration around intra-acinar pulmonary arteries from lung sections immunostained for macrophages as described above. The number of macrophages was calculated in the area from the edge of the adventitia to the outermost distance of macrophage infiltration, namely within the radial distance of 150 μm outward from the external elastic lamina as previously described [11]. We randomly selected 9 vessels per single tissue section and 9 such vessels were considered as 1 case. Six tissue sections were examined from 6 rats per group (CTRL, MCT, HCH and HCH+MCT).

**Semi-Quantitative Evaluation of Arterial Luminal Occlusion and Medial Thickness**

To evaluate intimal changes in pulmonary arteries, elastivon Gieson-stained pulmonary tissue samples were semi-quantitatively evaluated as described elsewhere [12]. In brief, cross-sectioned intra-acinar pulmonary arteries were scored from grade 0 to grade 3 according to their luminal occlusion as follows: grade 0 = normal pulmonary artery, grade 1 = mild intimal proliferation with less than 25% of luminal occlusion, grade 2 = mild intimal proliferation with 25–50% luminal occlusion and grade 3 = severe intimal proliferation with more than 50% luminal occlusion. For the evaluation of medial thickness, immunostaining with HHF35 was performed. Percent medial thickness was calculated by dividing longitudinal thickness of the HHF35-positive area by the diameter, and percent HHF35-positive area was calculated by dividing the HHF35-positive area by the total area of the cross section as previously reported [13, 14]. For both evaluations, we randomly selected 10 intra-acinar arteries per single tissue section obtained from a rat, and 10 tissue samples from 10 rats per group were examined.

**Semi-Quantitative Evaluation of Endothelial Damage**

To evaluate endothelial damage, tissue samples were immunostained with the antibody against the factor VIII-related antigen. We measured the length of the labeled endothelium and divided it by the vessel perimeter. Results were then expressed as a percentage. Again, we evaluated 10 intra-acinar arteries per single tissue section obtained from a rat, and 10 tissue samples from 10 rats per group were examined.

**Statistical Analysis**

All data are expressed as means ± standard error of mean (SEM). The 2-way repeated ANOVA followed by the Mann-Whitney U test was performed to evaluate the differences in rat body weights between the groups. The score of histological data was statistically analyzed among the groups using the Kruskal-Wallis test and the Mann-Whitney U test. A value of p < 0.05 was considered statistically significant.

**Results**

**Hemodynamic Studies**

To compare the severity of pulmonary hypertension between the 3 PAH animal models, hemodynamic studies were carried out. Compared to the CTRL, both single treatments with HCH exposure or MCT administration limited increase in body weight, and the former resulted in a more significant limitation of weight increase than the latter. In contrast, the HCH+MCT treatment caused weight loss (fig. 1a). Compared to the CTRL group, the right ventricle systolic pressure was not significantly increased in the MCT group, but was significantly increased in the HCH group (fig. 1b). In the HCH+MCT model, the right ventricle systolic pressure was significantly higher than in the CTRL, MCT or HCH groups (fig. 1b). Right ventricle hypertrophy, an index of pulmonary hypertension-related cardiac remodeling, was significantly increased in the HCH and MCT groups compared to the CTRL group, and even more significantly increased in the HCH+MCT model (fig. 1c), as expected from the pressure data. Indeed, pressure and right ventricular hypertrophy parameters were significantly correlated (fig. 1d).

**Histopathological Findings**

Compared to the CTRL (fig. 2a), MCT administration induced an accumulation of inflammatory cells including ED1-positive macrophages around intra-acinar pulmonary arteries with a mildly thickened arterial wall (fig. 2b, c). HCH also induced thickening of the intra-acinar arterial wall; however, inflammatory changes were less prominent (fig. 2d). Combined HCH+MCT treatment induced severe medial-wall thickening with perivascular inflammatory infiltrates (fig. 2e, f). Although the infiltration of CD68- (anti-pan macrophage) positive macrophages was less prominent in the HCH+MCT group than in the MCT group, the number of CD163- (ED2) and CD204- (SRA-E5) positive anti-inflammatory macrophages was highest in the HCH+MCT group (fig. 2f, g).

**Semi-Quantitative Evaluation of Arterial Luminal Occlusion and Medial Thickness**

Since the histological changes in PAH lesions are mainly neointima formation and medial hypertrophy [3],
we evaluated the intimal/medial changes semi-quantitatively. Compared to the CTRL (fig. 3a), mild intimal proliferation and mild luminal occlusion were observed in the MCT group (fig. 3b) by elastica-van Gieson staining. HCH induced moderate intimal/medial change (fig. 3c). By contrast, severe luminal occlusion of more than 50% was occasionally observed in the HCH+MCT group (fig. 3d). Figure 3e shows the mean intra-acinar pulmonary artery occlusion score of each group. All 3 PAH groups showed a significantly higher score than the CTRL. The highest score was in the HCH+MCT group compared to the HCH or MCT groups. The occlusion score was also correlated with right ventricle hypertrophy (fig. 3f).

Next, we evaluated medial changes in the pulmonary artery by immunostaining for vascular smooth muscle actin. Compared to the CTRL (fig. 4a), the thickness of the arterial media was mildly increased in the MCT group (fig. 4b), and moderately in the HCH group (fig. 4c). Thickening of the arterial media was more prominent in the HCH+MCT group (fig. 4d). As shown in figure 4, the medial thickness and the HHF35-positive area were significantly increased in the 3 PAH groups in ascending order: MCT, HCH, HCH+MCT.

**Semi-Quantitative Evaluation of Endothelial Damage**

Previous studies revealed that the initial step of PAH pathogenesis is often endothelial damage in the small artery of the pulmonary vasculature [3]. Compared to the CTRL (fig. 5a), endothelial damage was not significant in the HCH group (fig. 5c). In contrast, the MCT (fig. 5b) and HCH+MCT (fig. 5d) groups showed mild-to-moderate endothelial damage, and severe damage was occasionally observed in the combined group (fig. 5d). Figure 5e summarizes the averaged percentage of endothelium positively stained with factor VIII for each group. A significant difference between the CTRL, MCT and HCH+MCT groups was observed.
Severe Pulmonary Arteriopathy in the HCH+MCT Group

The HCH+MCT group, especially those treated with hypoxia for 4 weeks, revealed severe pulmonary arteriopathy. Complete or partial luminal obstructions with formation of fibrin thrombi were found randomly only in the HCH+MCT group (fig. 6a–c). Narrowing of the arterial lumen with abnormal endothelial proliferation was also found in the HCH+MCT group with 4 weeks’ hypoxic exposure (fig. 6d, g, h), and the abnormal lesions were constituted by factor VIII- and Ki67-positive endothelial cells (fig. 6e, f, i). It is noteworthy that early plexiform lesions showing slit-like channels in the lumen were occasionally observed in the HCH+MCT group with 4 weeks' hypoxic exposure (fig. 6a–c). Hematoxylin and eosin coloration was performed (a, b, d, e). Immunostaining with anti-macrophage antibodies for CD68 (c) and CD163 (f) is shown (brown in online version). g Comparison of the mean ± SEM number of macrophages infiltrated around vessels for CD68, CD163 and CD204. The quantity of CD163-positive and CD204-positive macrophages is expressed as a percentage of CD68-positive macrophages for each model. Scale bar is 50 μm (a–e) and 25 μm (f).
weeks’ hypoxic exposure (fig. 6g–i), though such lesions were not observed in the HCH+MCT group with 3 weeks’ hypoxic exposure.

**Discussion**

Our findings underline a new model of severe PAH in rats. The HCH+MCT model revealed strong changes in the hemodynamic parameters (right ventricle pressure) and in the vascular remodeling (endothelial damage and increase in the vascular media). Severe arteriopathy including intimal thickening, abnormal endothelial proliferation and early plexiform lesion, and luminal occlusion with fibrin thrombi was observed in the HCH+MCT group with 4 weeks’ hypoxic exposure.

To explore the molecular mechanism of PAH, various animal models of pulmonary hypertension have been designed but most of them mimic human PAH pathophysiology unsatisfactorily [5], while others require compli-
Via conversion of MCT to monocrotaline pyrrole in the liver [17]. This model shows sequential vascular remodeling with endothelial damage similar to human PAH cases [18]; however, as shown in figure 1, hemodynamic alterations are not severe and do not mimic human PAH cases.

In contrast, our HCH+MCT model exhibits remarkably severe hemodynamic alterations as well as histological remodeling of pulmonary vasculature with prominent macrophage infiltration, mimicking human PAH.

Fig. 4. Histological evaluation of pulmonary arterial medial hypertrophy for the PAH models. Lung sections were labeled with the smooth muscle-specific HHF35 antibody (brown in online version) to evaluate the thickness of the pulmonary arterial wall. 

a Pulmonary artery scored grade 0 in CTRL rats. 
b Pulmonary artery scored grade 1 in MCT-treated rats. 
c Pulmonary artery scored grade 2 in HCH-exposed rats. 
d Pulmonary artery scored grade 3 in HCH+MCT-treated rats. Scale bar is 25 μm. The percentages of the pulmonary arterial wall thickness (e) and smooth muscle-positive area stained with the HHF35 antibody (f) of the PAH models are compared. N.S. = Not significant.
It is interesting to note that the population of the anti-inflammatory macrophage subset predominated in the HCH+MCT group. Since this subset produced angiogenic factors including vascular endothelial growth factor and angiopoietin 1 [19], these factors may induce abnormal endothelial proliferation. Further studies are needed to disclose such a relationship between macrophage infiltration and vascular remodeling in PAH. A protocol combining treatment with MCT and HCH was previously described by Caslin et al. [13]. These authors exposed rats to HCH for 1 month during the neonatal period, allowed them to recover in room air for a period of 3 months and then injected them with MCT. Whereas this rat model did develop more severe remodeling of pulmonary arteries upon MCT treatment, the fact that chronic hypoxia was administered during the neonatal

**Fig. 5.** Histological evaluation of pulmonary arterial endothelial damage for the PAH rat models. To detect the presence or absence of the endothelium, immunostaining experiments with the antibody versus factor VIII were performed in CTRL rats (a), MCT-treated rats (b), HCH-exposed rats (c) and HCH+MCT-treated rats (d). Scale bar is 25 μm. e Comparison of the mean ± SEM percentage of the labeled endothelium for the CTRL, MCT, HCH and HCH+MCT models.
period when the lung was still immature [20, 21] may have introduced some bias with respect to adult PAH. In contrast with this previous report, we demonstrated that HCH+MCT treatment induced severe pulmonary hypertension and intimal thickening in adult rats. Moreover, it is worth noting that severe arteriopathy including intimal thickening, abnormal endothelial proliferation shown by Ki67 staining, early plexiform lesion and luminal occlusion with fibrin thrombi were observed only in the 4-week model of HCH+MCT treatment, and not in the HCH or MCT models. Recently, Abe et al. [8] reported the formation of plexiform lesions mimicking human PAH in a rat PAH model produced by a single subcutaneous injection of the vascular growth factor receptor blocker Sugen 5416, with subsequent exposure to hypoxia for 3 weeks followed by normoxic observation for 10–
11 weeks. The early plexiform lesions observed in our model were less prominent than in Abe’s model. They were, however, produced over a shorter period compared to Abe’s model where it took 13–14 weeks after Sugen 5416 injection to produce severe vascular lesions. In future studies, our model could develop more complex arterial lesions if the rats were monitored for longer than 4 weeks. The thrombotic lesion which was not observed in Abe’s model was observed in our HCH+MCT model; this is worth noting since it was one of the well-established lesions of human PAH resulting from endothelial dysfunction [18].

In summary, we established a new animal model of severe PAH by combining the exposure to HCH and the administration of MCT. In this model, as in the human situation, both hemodynamic and histopathological alterations with inflammation were present. With respect to translational research, a common finding in therapeutic trials in animal models of PAH is that drugs that are highly effective under such conditions (e.g. dehydroepiandrosterone, sildenafil, calcium channel antagonists, endothelin receptor blockers, etc.) [9, 22, 23] are more disappointing in clinical trials in humans [24]. Our animal model that combined marked pulmonary hypertension and vascular remodeling may thus prove useful to identify relevant therapeutic targets involving both phenomena for application in clinical situations.

References


