Original Article

Establishment of a mouse model for pulmonary inflammation and fibrosis by intratracheal instillation of polyhexamethyleneguanidine phosphate

Sang Jin Lee[1, 3†], Jong-Hwan Park[2†], Jun-Young Lee[1], Yu-Jin Jeong[1], Jeong Ah Song[3], Kyuhong Lee[3, 4*], and Dong-Jae Kim[5*]

[1] Department of Biochemistry, College of Medicine, Konyang University, 158 Gwanjeodong-ro, Seo-gu, Daejeon 302-718, Republic of Korea

[2] Laboratory Animal Medicine, College of Veterinary Medicine, Chonnam National University, 77 Yongbong-ro, Buk-gu , Gwangju 61186, Republic of Korea

[3] Inhalation Toxicology Center, Korea Institute of Toxicology, 30 Baekhak 1-Gil, Jeongeup-si, Jeollabuk-do 580-185, Republic of Korea

[4] Toxicology and Pharmacology, University of Science and Technology, 217 Gajeong-ro, Yuseong-gu, Daejeon 305-350, Republic of Korea

[5] Laboratory Animal Resource Center, Daegu Gyeongbuk Institute of Science & Technology (DGIST), 333 Techno Jungang Daero, Hyeonpung-myeon, Dalseong-gun, Daegu 42988, Republic of Korea

Abstract: Although several animal models have been developed to study human pulmonary fibrosis, lack of a perfect model has raised the need for various animal models of pulmonary fibrosis. In this study, we evaluated the pulmonary effect of polyhexamethyleneguanidine phosphate instillation into the lungs of mice to determine the potential of these mice as a murine model of pulmonary fibrosis. Intratracheal instillation of polyhexamethyleneguanidine phosphate induced severe lung inflammation manifested by the infiltration of mononuclear cells and neutrophils and increased production of IL-6, TNF-α, CCL2 and CXCL1. The lung inflammation gradually increased until 28 days after polyhexamethyleneguanidine phosphate exposure, and increases of collagen deposition and TGF-β production, which are indicators of pulmonary fibrosis, were seen. Our study showed that intratracheal instillation of polyhexamethyleneguanidine phosphate induces pulmonary inflammation and fibrosis in mice. (DOI: 10.1293/tox.2015-0067; J Toxicol Pathol 2016; 29: 95–102)

Key words: lung inflammation, mice, polyhexamethyleneguanidine phosphate, pulmonary fibrosis

Introduction

Idiopathic pulmonary fibrosis (IPF), the most common type of interstitial lung disease (ILD) with unknown etiology, affects 132,000–200,000 people in the USA[1]. Approximately 50,000 new cases are diagnosed and as many as 40,000 Americans die from IPF each year[1]. Pulmonary fibrosis (PF) is the formation of excessive fibrous connective tissue in the lung[2]. A wide range of causes, including viral infections and exposure to radiotherapy, chemotherapeutic drugs and aerosolized environmental toxins, may induce PF

as a secondary effect[3–5]. Although the relative importance of inflammation in the progression of pulmonary fibrosis has been debated, many forms of PF are believed to be induced by inflammatory responses[6]. In the early stage after tissue damage, epithelial cells release inflammatory mediators that allow recruitment of inflammatory cells such as neutrophils and macrophages to the site of injury. The recruited inflammatory cells produce a variety of cytokines and chemokines that trigger fibroblast proliferation and recruitment. Once fibroblasts become activated, they transform into α-smooth muscle actin–expressing myofibroblasts that secrete extracellular matrix (ECM) components leading to fibrosis when the wound is severe, the tissue-damaging irritant persists, or the repair process becomes deregulated[2].

Although anti-inflammatory medicine has been suggested as a potential therapy for PF based on evidence showing involvement of inflammation in PF, it is often ineffective[7]. Moreover, the limited knowledge of PF pathogenesis leads to lack of effective treatment for PF[8]. To understand the pathogenesis of PF and develop a successful therapy, an

animal model that can produce the characteristic features of human PF is necessary. To date, several animal models have been developed for PF, and these models have been used to identify cells and mediators involved in the process of PF. However, these animal models cannot represent human PF appropriately, and the need for various animal models of PF has been raised[9]. Recent case reports showed that lung injury and fibrosis can occur as a result of inhaling a humidifier disinfectant, polyhexamethyleneguanidine phosphate (PHMG-P)[10–13]. PHMG-P, a member of the polymeric guanidine family, is widely used as an antimicrobial additive in paper and plastics and as a disinfectant for sanitation in food processing plants[14]. It has broad-spectrum activity against Gram-positive and Gram-negative bacteria, fungi, yeasts and human immunodeficiency virus by disrupting the cell membrane[15–18]. Although PHMG-P is known as a disinfectant that is noncorrosive and nontoxic to humans and animals[16, 17, 19], a recent report showed that more than 12,500 patients were admitted to hospital owing to drinking illegal cheap "vodka" that was mixed with PHMG, and 9.4% of the patients died[20].

In the present study, we evaluated the pulmonary effect of PHMG-P instillation into the mouse lung to determine potential of these mice as a murine model of PF.

Materials and Methods

Mice

Six-week-old female wild-type C57BL/6 mice were purchased from Koatech (Pyeongtaek, Republic of Korea). All animal experiments were approved and followed the regulations of the Institutional Animal Care and Use Committee (IACUC) of Konyang University (Daejeon, Republic of Korea).

PHMG-P inhalation

Intratracheal instillation of PHMG-P (1.5 mg/kg, SK Chemicals, Seoul, Republic of Korea) into mice was performed as described previously[21]. Mice were euthanized with excessive anesthesia 7, 14 or 28 days after instillation, and lung tissues were collected for further analysis (Fig. 1A).

Histopathology and Masson's trichrome stain

The left lung tissues from each mouse were fixed with 10% neutral-buffered formalin for 24 hours and embedded in paraffin. Tissue sections (2 μm thick) were prepared and stained with hematoxylin and eosin (HE) or Masson's trichrome and then examined under a light microscope.

Measurement of cytokines and chemokines

The right lung tissues from each mouse were homogenized, and the supernatant was obtained after centrifugation. The concentrations of IL-6, TNF-α, CCL2, CXCL1, and TGF-β in the supernatant were determined using commercial ELISA kits (IL-6, TNF-α, CCL2 and CXCL1, R&D System, Minneapolis, MN, USA; TGF-β, eBioscience Inc., San Diego, CA, USA) according to the manufacturers' manuals.
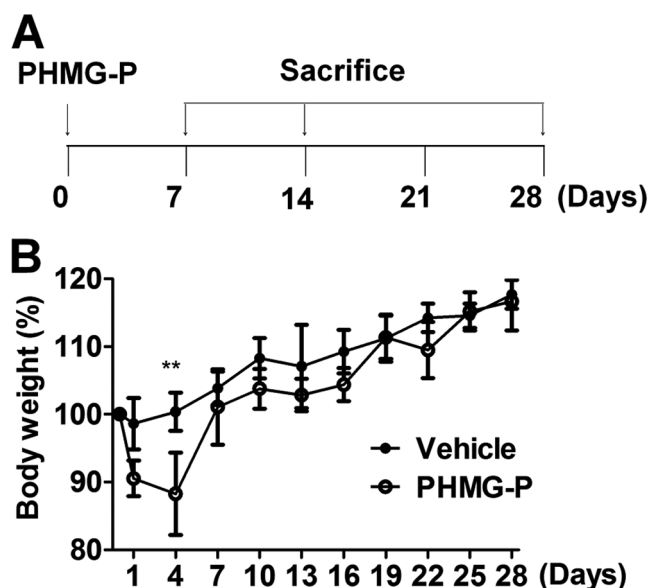

Fig. 1. Body weight changes caused by PHMG-P instillation in mice. A schematic diagram of the experimental design described in Materials and Methods (A). Mice were intratracheally instilled with PHMG-P (1.5 mg/kg) or PBS, and body weight changes were monitored daily (B). N = 5 mice per group. *P<0.05; **P<0.01.

Statistical analysis

The differences in mean values among different groups were assessed, and all data are expressed as the mean ± SD. All statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or by the unpaired Student's test using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Values of P<0.05 were considered statistically significant.

Results

Clinical signs and body weight changes caused by PHMG-P exposure in mice

To determine the optimal dosages for the experiment, mice were exposed to various dosages (0, 1.5, 3 and 6 mg/kg) of PHMG-P, and body weight loss and survival rate were monitored up to 30 days after PHMG-P exposure. All mice (N ≥ 5 per group) except for mice exposed to 6 mg/kg of PHMG-P (survival rate of about 40%) survived to the end of the 30-day period (data not shown). However, exposure to 3 mg/kg of PHMG-P induced nearly 30% body weight loss in mice (data not shown). According to the recommendation of our IACUC, we exposed mice to 1.5 mg/kg of PHMG-P in the subsequent. Mild physical signs of ill health such as body weight loss, decreased movement and ruffled fur were seen in PHMG-P-exposed mice until 4 days after exposure. These clinical signs began to disappear 4 days after exposure (data not shown). The loss of body weight prominent 1 day after exposure and continued until 4 days after ex-
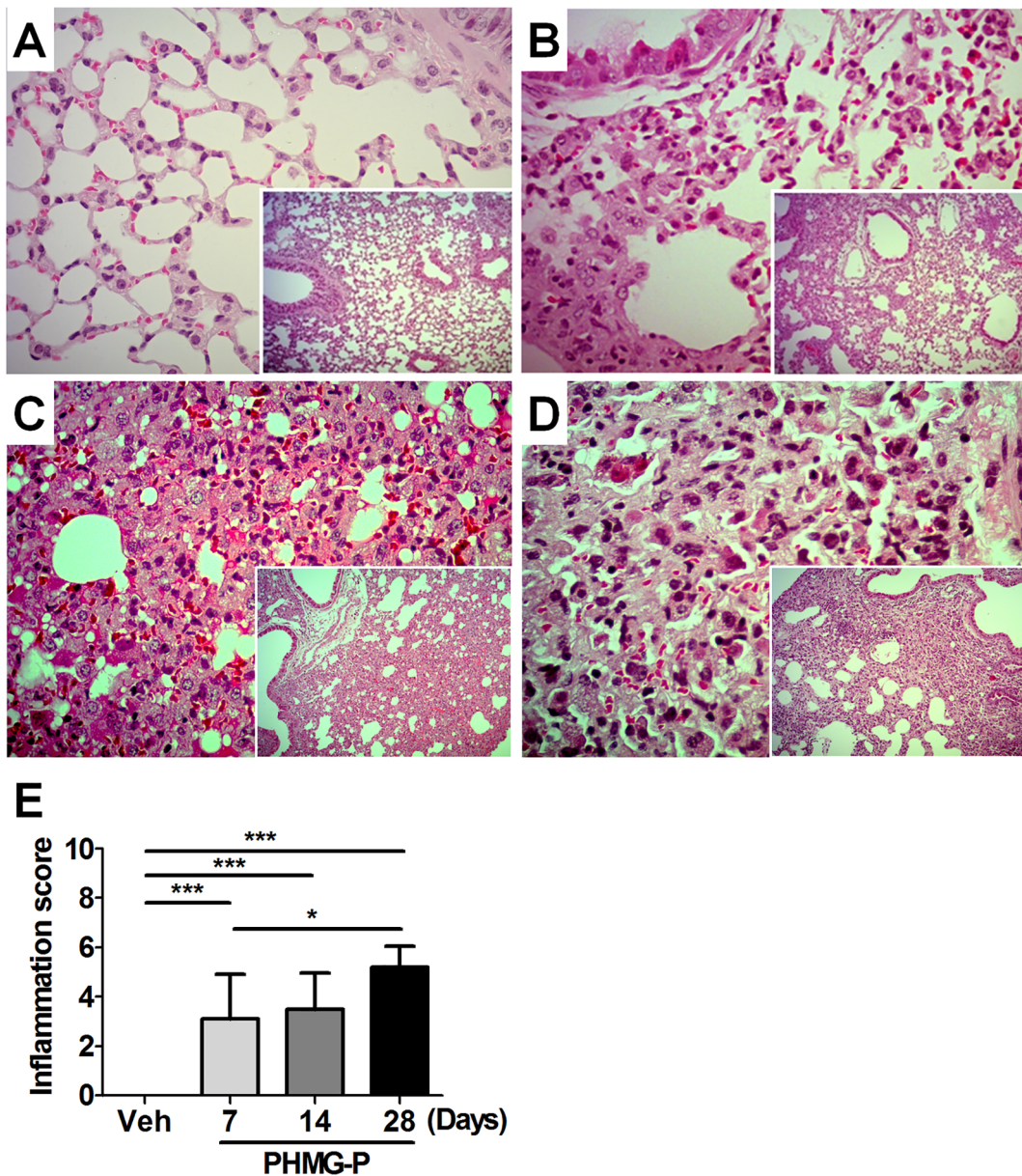
Fig. 2. Histopathology of lung caused by PHMG-P instillation in mice. At 7, 14 and 28 days after PHMG-P exposure, lung tissues were processed and stained with hematoxylin and eosin for histopathologic examination. Representative images of each group are shown at a magnification of 400×. Small quadrangles within each image are at low magnification (40×). (A) Vehicle, (B) 7 days, (C) 14 days and (D) 28 days after PHMG-P exposure. (E) The severity of the inflammation in the lung is presented as the mean ± SD. (*P<0.05; ***P<0.001).

posure, and then most mice gradually regained their body weight until it was in a range comparable to that of vehicle-exposed mice (vehicle = phosphate buffered saline [PBS]) at 7 days after exposure (Fig. 1B).

Histopathology of lung tissue from PHMG-P-exposed mice

Histopathologic examination of lung tissue was performed at 7, 14 and 28 days after PHMG-P exposure. PHMG-P exposure induced the infiltration of polymor-

phonuclear (PMN) cells and macrophages into the alveolar sac and interstitium of the peribronchiolar area and activation of pneumocytes from 7 days after PHMG-P exposure with 100% incidence (Fig. 2B). The severity of lung lesion gradually increased until 28 days after PHMG-P exposure (Fig. 2C-E). However, neither the infiltration of inflammatory cells nor the activation of pneumocytes was seen in lung tissue from vehicle-treated mice (Fig. 2A).
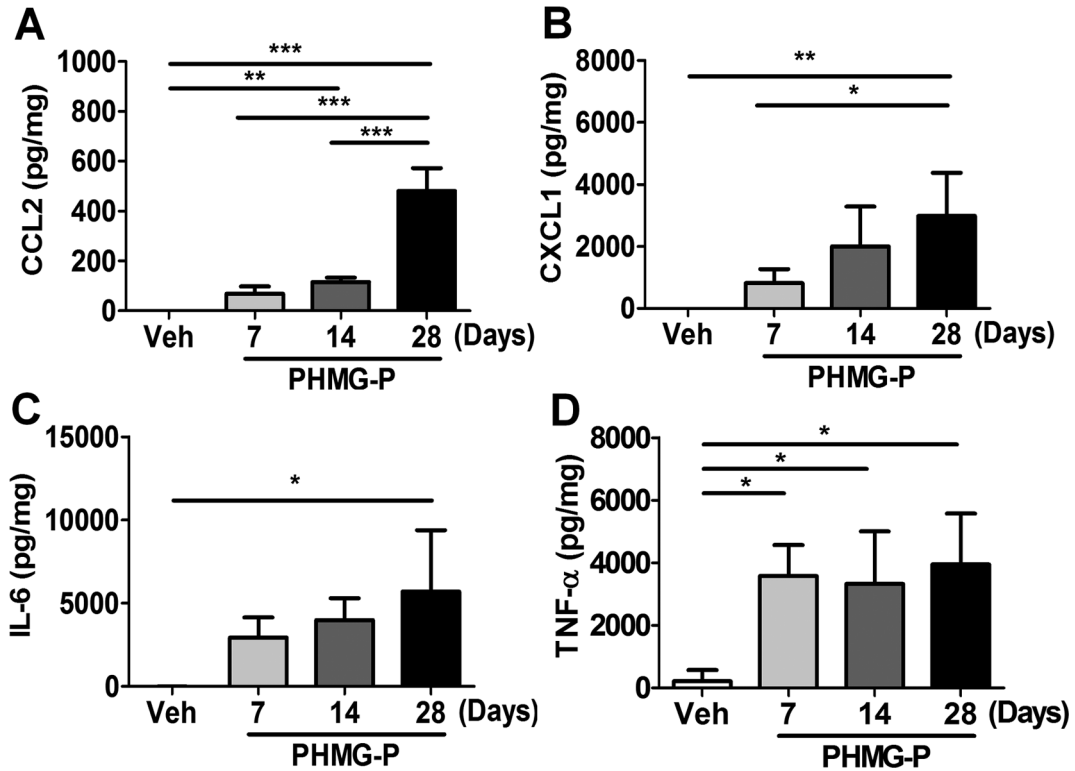
Fig. 3. Production of cytokines and chemokines caused by PHMG-P instillation in the mouse lung. Lung homogenates from vehicle-exposed mice or PHMG-P-treated mice at 7, 14 and 28 days after exposure were collected, and the concentrations of IL-6, TNF-α, CCL2 and CXCL1 in supernatants were determined by ELISA. Vehicle-treated (N = 3), PHMG-P-treated groups (N = 5) mice. (*P<0.05; **P<0.01)

Production of cytokines and chemokines in lung tissue caused by PMHG-P exposure

The levels of IL-6, TNF-α, CCL1 and CCL2 in the supernatants of lung homogenates were measured 7, 14 and 28 days after PHMG-P exposure. All cytokines and chemokines measured gradually increased until 28 days after PHMG-P exposure (Fig. 3). Compared with vehicle exposure, PHMG-P exposure significantly increased the levels of TNF-α and CCL2 at 7 and 14 days after treatment respectively (Fig. 3A-D), and the levels of CXCL1 and IL-6 were significantly higher at 28 days after PHMG-P exposure (Fig. 3B-C).

Pulmonary fibrosis caused by PHMG-P inhalation

We evaluated the level of collagen deposition in the lung, a hall marker of lung fibrosis, by Masson's trichrome staining. The level of Masson's trichrome positivity was increased from 14 days after PHMG-P exposure and became stronger at 28 days after PHMG-P exposure (Fig. 4A-E). Only 2 of 5 mice showed slight Masson's trichrome positivity at 7 days after PHMG-P exposure (Fig. 4B, E). Moreover, the level of TGF-β, which is known to play an important role in lung fibrosis[22], in lung homogenates was gradually increased after exposure to PHMG-P (Fig. 5).
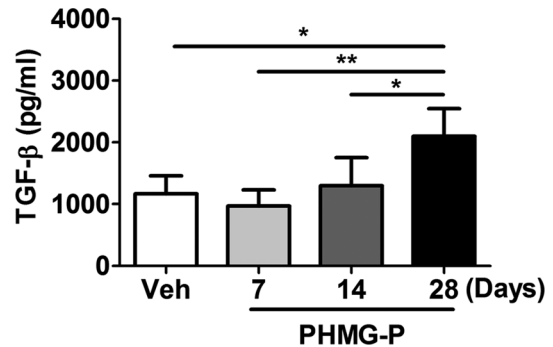

Fig. 5. Level of TGF-β in the lung after PHMG-P instillation. At 7, 14 and 28 days after PHMG-P exposure, lung tissues were collected. The level of TGF-β were assessed by ELISA. (A) Vehicle and (B) 7 days, (C) 14 days and (D) 28 days after PHMG-P exposure.

Discussion

Recently, the toxic effects of PHMG-P, including pulmonary complications, were reported in epidemiological studies[23]. However, the mechanism of toxicity caused by
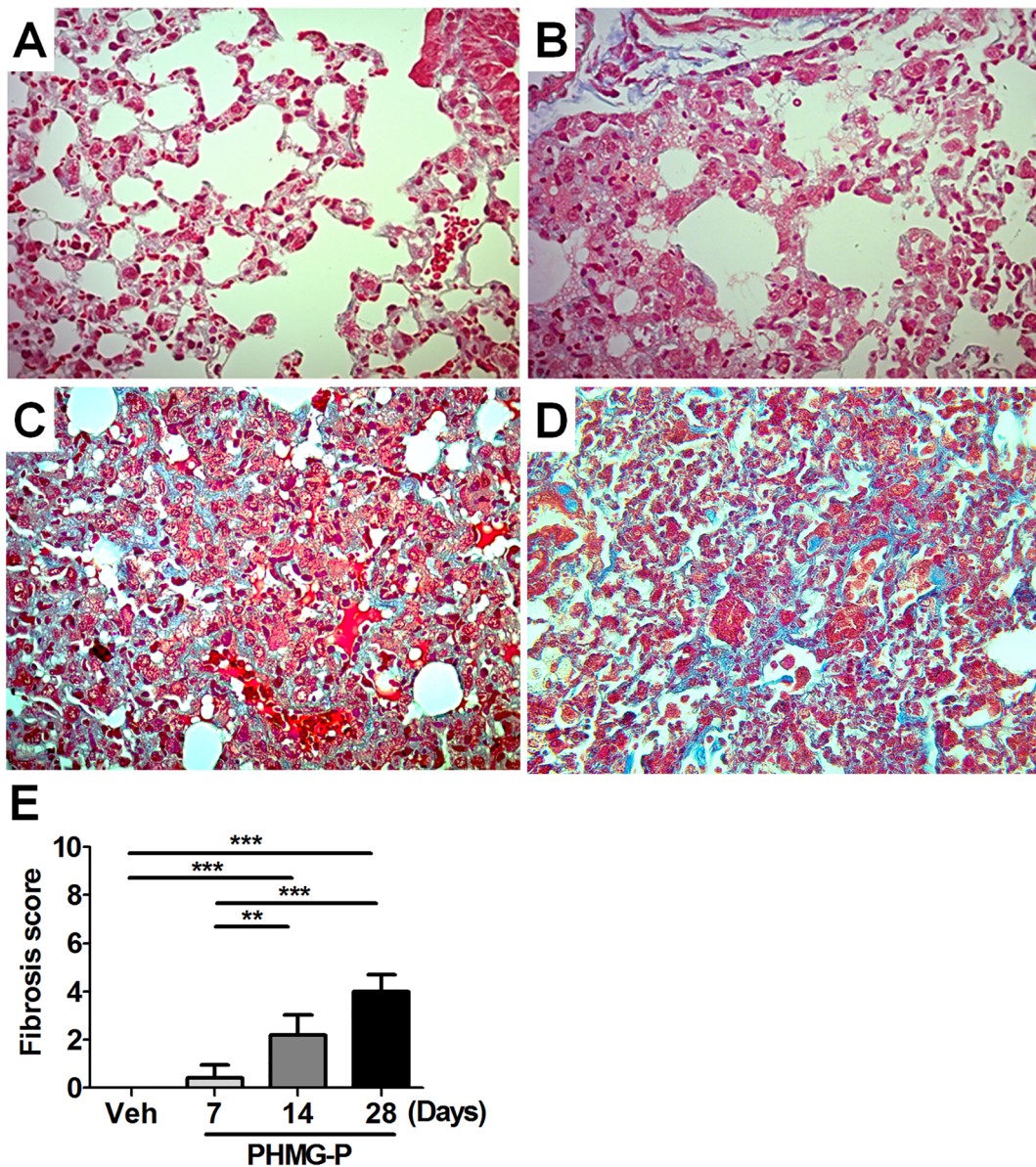
Fig. 4. The degree of collagen deposition in the lung after PHMG-P instillation. At 7, 14 and 28 days after PHMG-P exposure, lung tissues were collected. The degree of collagen deposition was assessed by Masson's trichrome staining. Representative images of each group are shown at a magnification of 400×. (A) Vehicle and (B) 7 days, (C) 14 days and (D) 28 days after PHMG-P exposure. (E) The severity of fibrosis in the lung is presented as the mean ± SD. (**P<0.01; ***P<0.001)

PHMG-P was not fully elucidated, although one study using human cells and zebrafish embryos reported that PHMG-P showed acute cardiovascular toxicity by inducing severe inflammation, atherogenesis and aging, with embryo toxicity[24]. In our mouse study, we observed severe lung inflammation manifested by the infiltration of mononuclear cells and neutrophils and the elevated production of IL-6, TNF-α, CCL2 and CXCL1 after intratracheal inhalation of PHMG-P. The lung inflammation gradually increased up to 28 days after PHMG-P treatment, and increased collagen deposition and TGF-β production were seen. We did not clarify the elaborate mechanism of PHMG-P-induced lung inflammation in this study. However, the production of reactive

oxygen species caused by PHMG-P treatment demonstrated in previous studies with zebrafish embryos[11, 24] could be key, even in the case of lung inflammation caused by PHMG-P inhalation resulting in pulmonary fibrosis.

Inflammatory cytokines and chemokines have been considered to play an important role in both the initiation and progression of some forms of pulmonary fibrosis[23]. Clinical samples from patients with pulmonary fibrosis displayed elevated levels of TNF-α, and mice genetically modified to overexpress TNF-α in the lung developed progressive pulmonary fibrosis[25–27]. IL-1β also contributes to the progression of pulmonary fibrosis by crosstalking with TNF-α or inducing neutrophil chemoattractants[16, 28]. More-

over, T helper cell-associated cytokines also play important role in pulmonary fibrosis[29–31]. Whereas a Th1-associated cytokine, IFN-γ, has been known to inhibit fibrosis, Th2- and Th17-associated cytokines, IL-4, IL-5 and IL13 (Th2) and IL-17A (Th17), respectively, are linked to pulmonary fibrosis[30, 31]. Both IL-4 and IL-13 have a profibrotic function by activating myofibroblasts[32, 33], and IL-5 promotes pulmonary fibrosis by recruiting eosinophils that produce profibrotic mediators[34, 35]. An experiment using mice with bleomycin-induced fibrosis showed that IL-17A and IL-23, an IL-17A inducing cytokine, are important for the development of pulmonary fibrosis[30]. In addition, chemokines and their signaling receptors, including CCL2, CXCL12, CCL12 and CXCR2, promote pulmonary fibrosis by recruiting mononuclear cells, neutrophils or collagen-secreting fibrocytes to the lung[36–38]. The increased production of IL-6, TNF-α, CCL2 and CXCL1 after PHMG-P treatment in our study might function as an initiator of pulmonary fibrosis.

Intratracheal inhalation of PHMG-P induced the production of TGF-β, which has been known to play a central role in the pathogenesis of pulmonary fibrosis by promoting the activation, proliferation and differentiation of collagen-producing myofibroblasts[39, 40]. TGF-β stimulates production of collagens and other extracellular matrix proteins in mesenchymal cells through a classic or nonclassic pathway[41]. In the classic TGF-β pathway, binding of TGF-β to the TGF-β type II receptor (TGF-βRII) recruits a TGF-β type I receptor (TGF-βRI), activating its phosphorylation. Activated TGF-βRI phosphorylates Smad2 and Smad3, which then form a complex with Smad4, resulting in their transport to the nucleus. The Smad complex binds to specific DNA-binding sites in the promoter regions of target genes such as the 1 and 2 chains of type I collagen or extracellular matrix regulatory proteins and modulates their transcriptional activity[39, 42]. In the non-classic TGF-β pathway, TGF-βRI phosphorylates cellular Abelson nonreceptor kinase (c-Abl), resulting in activation of Smad1, early growth response gene (EgR), or protein kinase C-δ (PKC-δ), all of which contribute to the fibrotic response[43]. Although collagen deposition was significantly increased from 14 days after PHMG-P exposure, the level of TGF-β was comparable with the vehicle-exposed group 14 days after exposure and significantly increased 28 days after exposure. This could be explained by the difference in sensitivity between the two methods of measurement used.

Several agents, including bleomycin, asbestos, silica, FITC and radiation, have been known to induce pulmonary fibrosis in mouse models[36]. Among them, bleomycin is the best characterized one in studying murine pulmonary fibrosis[44]. Intratracheal treatment of bleomycin results in direct damage of alveolar epithelial cells followed by alveolar infiltration of neutrophils[19]. DNA strand breakage and oxidant injury[45] are thought to be a main mechanism of bleomycin-induced alveolar cell damage. Subsequently, fibroblast proliferation and synthesis of extracellular matrix appear[46]. During development of pulmonary fibrosis caused by bleomycin, CCL2 recruits inflammatory cells, including mono-

cytes, lymphocytes and fibrocytes, and TGF-β stimulates the production of ECM[47, 48]. The progression of pulmonary fibrosis in our PHMG-P model was very similar to that in the bleomycin model.

In the current study, we characterized PHMG-P-induced lung inflammation and pulmonary fibrosis in mice, and our observations should provide basic data concerning the toxicity of PHMG-P. As we did not clarify the mechanism of PHMG-P-induced lung inflammation in detail, further study is necessary to clarify it in detail.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no financial or commercial conflicts of interest.

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