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Changes in expression of cytokines in polyhexamethylene guanidine-induced lung fibrosis in mice: Comparison of bleomycin-induced lung fibrosis

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ABSTRACT

Inhalation of polyhexamethylene guanidine (PHMG) causes irreversible pulmonary injury, such as pulmonary fibrosis. However, the mechanism underlying PHMG-induced lung injury is unclear. In this study, we compared the difference in time-dependent lung injury between PHMG- and bleomycin (BLM)-treated mice and determined cytokines involved in inducing lung injury by performing cytokine antibody array analysis. Mice were treated once with 1.8 mg/kg BLM or 1.2 mg/kg PHMG through intratracheal instillation and were sacrificed on days 7 and 28. Bronchoalveolar lavage fluid (BALF) analysis showed that the number of neutrophils was significantly higher in PHMG-treated mice than in BLM-treated mice on day 7. Histopathological analysis showed inflammatory cell infiltration and fibrosis mainly in the terminal bronchioles and alveoli in the lungs of PHMG- and BLM-treated mice. However, continuous macrophage infiltration in the alveolar space and bronchioloalveolar epithelial hyperplasia (BEH) were only observed in PHMG-treated mice. Cytokine antibody array analysis showed that 15 and eight cytokines were upregulated in PHMG- and BLM-treated mice, respectively, on day 7. On day 28, 13 and five cytokines were upregulated in PHMG and BLM-treated mice, respectively. In addition, the expressed cytokines between days 7 and 28 in BLM-treated mice were clearly different, but were similar in PHMG-treated mice. Consequently, between PHMG- and BLM-treated mice, we observed differences in the expression patterns and types of cytokines. These differences are considered to be a result of the inflammatory processes induced by both substances, which may mainly involve macrophage infiltration. Therefore, continuous induction of the inflammatory response by PHMG may play an important role in the development of pulmonary fibrosis.

1. Introduction

Polyhexamethylene guanidine (PHMG) is a member of the polymeric guanidine family which is widely used as a biocide in the medicine, agriculture, and food industries because of its broad-spectrum bactericidal activity and low toxicity for humans (Muller and Kramer, 2005; Rosin et al., 2001). However, inhalation of PHMG causes fatal pulmonary injury, such as pulmonary fibrosis in humans (Hong et al., 2014; Korea Centers for Disease Control and Prevention, 2011; Kim et al., 2014a). In 2011, exposure to PHMG-containing humidifier disinfectants was identified as a potential cause of pulmonary damage in Korea (Korea Centers for Disease Control and Prevention, 2011). According to a survey conducted by the Korean government, more than 530 people have been affected by PHMG-containing humidifier disinfectants as of 2015 (Park, 2016). However, the mechanism underlying PHMG-induced lung injury has not yet been determined.

Cytokines are secreted by all cell types and act as fundamental mediators between cells. Cytokines coordinate immune response to infection, tissue damage, tissue repair, and self-tolerance. Furthermore, cytokines play a critical role in inflammatory responses underlying fibrosis induction in injured tissues (Borthwick et al., 2013). Several studies have suggested that the PHMG-induced inflammatory response is significantly associated with the initiation and progression of lung injury. PHMG aerosol particles induce pulmonary inflammation and fibrosis and upregulate the expression of inflammatory and fibrotic cytokines both *in vitro* and *in vivo* (Kim et al., 2016a, 2016b). PHMG exposure increases inflammatory cytokine levels by activating the Nuclear Factor Kappa B (NF-κB) signaling pathway to induce its inflammatory response (Kim et al., 2015). Our previous study showed that intratracheal instillation of PHMG in mice increased the levels of

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inflammatory and fibrotic cytokines (Song et al., 2014). DNA microarray analysis showed that expression levels of genes encoding inflammation and fibrosis-related cytokines increased in the rat lungs exposed to PHMG (Kim et al., 2017). These findings suggest that a cytokine-mediated response may play a crucial role in PHMG-induced lung injury.

Bleomycin (BLM)-treated mice are the best characterized and commonly used animal model to study pulmonary fibrosis because they clearly show BLM-induced inflammatory and fibrotic changes in the lungs (Huang et al., 2013; Moore and Hogaboam, 2008). Intratracheal instillation of BLM triggers an initial inflammatory response in the lungs from days 1 to 7, followed by a fibrotic response after day 7, and increases profibrotic marker expression by day 14. However, after day 28 the BLM-induced inflammatory response becomes variable (Antje et al., 2008; Bethany and Cory, 2008). Cytokines play an important role in the pathogenesis of BLM-induced lung injury. Several inflammation- and fibrosis-associated cytokines, such as IL-6, TNF-α, and MMP-9, have been reported. However, many inflammation- and fibrosis-associated cytokines are still unknown (Arizmendi et al., 2014; Kim et al., 2009; Smith et al., 1998). Cytokine profiling allows for the determination of lung injury progression and may provide a clue about the development of the underlying lung injury. However, no information is available on cytokine profiles of PHMG- and BLM-exposed lungs.

In this study, we compared differences in time-dependent damage responses in the lungs of PHMG- and BLM-treated mice and determined cytokines involved in lung injury induction by performing cytokine antibody array analysis.

2. Materials and methods

2.1. Animals

Seven-week-old male C57BL/7 mice were purchased from Orient Bio Inc. (Seongnam, Korea). The mice were housed in an environmentally controlled animal facility. The animal room was maintained at a temperature of 22 °C ± 3 °C, relative humidity of 50% ± 10%, light intensity of 150–300 lx, a light/dark cycle of 12/12 h, and air ventilation in the animal room was refreshed 10–20 times/h. The mice were fed pelleted food for experimental animals (PMI Nutrition International, Richmond, VA, USA) and were given UV-irradiated (Steritron SX-1; Daeyoung, Inc., Seoul, Korea) and filtered (pore size, 1 μm) tap water *ad libitum*. The mice were used in experiments after 6 days of acclimation. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology.

2.2. Experimental design

Mice (n = 36) were randomly assigned to one of the following six weight-matched experimental groups by using the Pristima System (Version 6.4; Xybion Medical System Co., USA), (six mice per group): mice treated with saline for (1) 7 or (2) 28 days (control groups), mice treated with BLM for (3) 7 or (4) 28 days (BLM groups), or mice treated with PHMG for (5) 7 or (6) 28 days (PHMG groups). Mice in the BLM (Nippon Kayaku, Tokyo, Japan) and PHMG (25%; SK Chemicals, Seoul, Korea) groups received a single intratracheal instillation of 1.8 mg/kg BLM and 1.2 mg/kg PHMG, respectively, in 50 μL saline solution using an automatic video instillator (Kim et al., 2010). Mice in the control groups were instilled with saline through the same route.

Body weight was measured on days 0 (instillation) 1, 3, 5, 7, 9, 13, 17, 21, 25, and 27. Clinical signs of mice in each group were monitored every day. The mice were euthanized at 1 (day 7) and 4 (day 28) weeks after instillation by administering an overdose of isoflurane and necropsy was performed. Bronchoalveolar lavage (BAL) was performed for the right lung lobes and the lungs were removed. The left lung lobe was weighed and fixed in 10% neutral-buffered formalin and the right lung lobes were snap-frozen to evaluate cytokine expression.

2.3. BAL and cell differentiation

The lungs were prepared for lavage by cannulating the trachea with a polypropylene tube attached to a syringe. Lung lavage was obtained by washing three times with 0.7 mL phosphate buffered saline (PBS). Samples were centrifuged at 800 × *g* for 10 min, cell pellets were resuspended in PBS, and total immune cell counts were determined using an automated cell viability analyzer (Vi-CELL™; Beckman Coulter, Carlsbad, CA, USA). The resuspended cell pellets were then centrifuged (Shandon Cytospin 4; Thermo Scientific, Cheshire, UK). Differential cell counts were determined using a light microscope (BX51; Olympus, Tokyo, Japan) at 1000 × magnification by counting 200 cells stained with Diff-Quick.

2.4. Histopathological analysis of the lung tissues

The left lung tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. Next, the paraffin-embedded tissue blocks were cut into 4-μm-thick sections and were stained with hematoxylin and eosin (H&E) for histological analysis and with Masson's trichrome (MT) stain for examination of fibrotic changes. Histological analysis of the paraffin-embedded lung tissue sections was performed using a light microscope at 100 × magnification. The degree of lung injury was estimated by assigning a semi-quantitative score (0–5) for separate categories representing inflammatory alterations, proliferative, and fibrotic change.

2.5. Cytokine profiling by performing cytokine antibody array analysis

Lung lysates obtained from mice in each the control, BLM, and PHMG groups were pooled and their cytokine profiles were analyzed using a mouse cytokine antibody array (Proteome Profiler™ Mouse XL Cytokine Array Kit; R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Briefly, the mouse cytokine antibody array included a membrane spotted with 111 different cytokine antibodies in duplicate. Array membranes, each placed in separate wells of a provided multi-well plate, were blocked with array buffer 6 for 60 min at room temperature (RT) and were incubated overnight with 300 μg pooled protein samples in array buffer 4/6 on a rocking platform at 4 °C. After washing, the membranes were incubated with a reconstituted detection antibody cocktail for 60 min at RT. Next, the membranes were washed and were incubated with 1 × streptavidin–HRP for 60 min at RT. After washing, dots formed on the membranes were detected using an enhanced chemiluminescence reagent, as directed by the manufacturer. Chemiluminescent signal was detected using ImageQuant LAS 500 mini (GE Healthcare Life Sciences, Little Chalfont, UK) and densitometric analysis of the intensities of cytokine dots was performed using ImageQuant TL 8.1 software (GE Healthcare Life Sciences). For every spot, net density gray level was determined by subtracting the average gray levels of four negative controls from the measured density gray levels. The density gray levels of six positive controls were used to normalize the results obtained from different membranes. Next, the density for each cytokine was averaged between the duplicate spot signals.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad InStat v. 3.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as mean ± standard deviation (SD) and all statistical comparisons were performed using one-way analysis of variance followed by Tukey–Kramer post hoc or Dunnett's multiple comparison test. The number of samples or animals in each group is indicated in figure legends or text. A p-value of < 0.05 was considered statistically