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Lung fibroblasts may play an important role in clearing apoptotic bodies of bronchial epithelial cells generated by exposure to PHMG-P-containing solution

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ABSTRACT

Polyhexamethylene guanidine (PHMG) has been widely used in the industry owing to its excellent biocidal, anti-corrosive, and anti-biofouling properties. In Korea, consumers exposed to PHMG-phosphate (PHMG-P)-containing humidifier disinfectant have begun to suffer from fibrotic lung injury-related symptoms for unknown reasons. However, no appropriate treatment has yet been found because the detail toxic mechanism has not been identified. Herein, we first studied the toxic mechanism of PHMG-P-containing solution using human normal bronchial epithelial cells (BEAS-2B cells). When exposed for 24 h, PHMG-P-containing solution rapidly decreased cell viability from around 6 h after exposure and significantly increased of the phosphatidylserine exposure and the LDH release. At 6 h of exposure, the material contained in the solution was found to be bound to the cell membrane and the inner wall of vacuoles, and damaged the cell membrane and organelles. In addition, a significant increase of IFN-γ was observed among cytokines, the expression of apoptosis-, autophagy-, and membrane and DNA damage-related proteins was also enhanced. Meanwhile, the level of intracellular ROS and the secretion of IL-8 and CXCL-1, which are chemokines for professional phagocytes, decreased. Thus, we treated dead BEAS-2B cells to lung fibroblasts (HFL-1), non-professional phagocytes, and then we observed that the dead cells rapidly attached to HFL-1 cells and were taken up. Additionally, increased secretion of IL-8 and CXCL-1 was observed in the cells. Based on these results, we suggest that pulmonary exposure to PHMG-P induces apoptosis of bronchial epithelial cells and lung fibroblasts might play an important role in the clearance of the apoptotic debris.

1. Introduction

Polyhexamethylene guanidine phosphate (PHMG-P), a water-soluble guanidine derivative, has been known to have excellent antiseptic and sterilizing power against fungi and bacteria with low toxicity (Barkova and Bogachuk, 1995; Vitt et al., 2015). Thus, it has been extensively used in a variety of consumer products including detergents, air conditioners, heaters, humidifiers, and tattoo pigments. Meanwhile, in Korea, the unexplained fibrotic lung injury-related patients consistently occurred since 2002. Epidemiological studies concluded that PHMG-P or oligo(2-(2-ethoxy)-ethoxyethyl)guanidinium-chloride (PGH) contained in humidifier disinfectants is the causative materials (Park et al., 2014; Paek et al., 2015; Park et al., 2015a), and in 2016, their use was banned in Korea. However, owing to its outstanding properties, PHMG-related materials are still considered for application in industrial areas, raising the possible exposure to the workers (https://www.reportsmonitor.com Mashat, 2016; Protasov et al., 2017; Vitt et al., 2015; Walczak et al., 2014).

Pulmonary fibrosis is a respiratory disease which is characterized by the accumulation of excess fibrous connective tissue, leading to serious breathing problem and reduced oxygen supply (Barkauskas and Noble, 2014; Dong et al., 2016; Goodwin and Jenkins, 2016; Knudsen et al.,

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2016). However, the cause and cellular mechanism of some types of fibrosis are still unclear, thus they are categorized as 'idiopathic pulmonary fibrosis (IPF)'. In recent, some researchers suggested that inhaled PHMG-P may result in fibrotic lesion by disturbing host's immune balance *in vivo* (Kim et al., 2016; Song et al., 2014), and that PHMG induces apoptosis and inflammatory response via oxidative stress *in vitro* (Jung et al., 2014; Kim et al., 2015a; Kim et al., 2016). The inflammatory response is a normal defense mechanism induced in the host against foreign bodies. However, if the immune system does not work properly in the early phase, the immune response can become more extensive and severe, leading to tissue damage. Additionally, Zhou et al. (2010) suggested membrane damage (a key indicator for necrosis) occurs by exposure to PHMG-P (Fink and Cookson, 2005). Cell death is largely categorized to programmed (apoptosis, autophagy, paraptosis, pyroptosis, and so on) and non-programmed (necrosis). Programmed cell death slowly proceeded by intracellular signals, whereas non-programmed cell death is caused rapidly by external strong stimulation. Additionally, inflammatory response is closely linked to some cell death pathways including autophagy, pyroptosis, and necrosis, but not apoptosis.

Although both are driven by chronic inflammatory diseases, final pathological feature of cancer and fibrosis is different. Additionally, the prognosis of patients with IPF has not been clearly improved by application of anti-oxidants or anti-inflammatory agents (Buhl and Vogelmeier, 1994; Gharaee-Kermani and Phan, 2005; Gharaee-Kermani et al., 2007; Marinari et al., 2017; Selman et al., 2004). In this regard, all the previous studies used cell lines derived from the host which is carcinogenic, and bronchial epithelial tissue is one of the important sites in the development of pulmonary fibrosis (Camelo et al., 2014; Kim et al., 2015b). Thus, we first aimed to confirm the previous data by exposing PHMG-P-containing solution to BEAS-2B cells, a human normal bronchial epithelial cell line, in this study. Moreover, considering that dead cells can stimulate the immune system as the foreign body, and that both professional and non-professional phagocytes play a key role in the process to remove foreign bodies or dead (or damaged) cells out of the body, we explored the role of phagocytes in the process to remove dead BEAS-2B cells out of the body together with another possible toxic mechanism.

2. Materials and methods

2.1. Test materials

Commercial PHMG-P-containing solution was obtained from Oxy-Reckitt Benckiser. The concentration of PHMG-P, the main component of a test material, was measured by Ultraviolet/Visible Spectrophotometry using SKYBIO1125 (PHMG-P of 25%, SK Chemicals, Seongnam, Korea) as a standard material, and that was 0.128 ± 0.002% (http://eco.ohois.com).

2.2. Cell culture

The human lung bronchial epithelial cells (BEAS-2B) and human lung fibroblast (HFL-1) were purchased from ATCC (VA, USA). BEAS-2B and HFL-1 cells were cultured in F12, and DMEM medium containing FBS (10%), penicillin (100 IU/mL), and streptomycin (100 μg/mL), respectively, and cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

2.3. Cell viability

Cell viability was evaluated using 3-(4–5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA). Cells (2 and 3 × 10^4 cells/well for 24 and 6 h, respectively) were seeded onto 96-well plates and stabilized overnight. A designated dose of PHMG-P-containing solution (0.25, 0.5, 0.75, 1.0 and 1.25% of solution corresponding to 3.2, 6.4, 9.6, 12.8 and 16.0 μg/mL of PHMG-P) was added to the cells and the cells were then incubated for 6 or 24 h. Subsequently, 20 μL of MTT solution (2 mg/mL) per well was added and the plate was incubated for 3 h at 37 °C. Blue formazan crystals were solubilized with dimethyl sulfoxide (200 μL), and the absorbance was quantified at 540 nm using a microplate spectrophotometer system (Molecular Devices, Sunnyvale, CA, USA). The viability of the treatment group was expressed as a percentage of the control group (100%).

2.4. Real-time cell proliferation assay

Real-time cell proliferation was measured using an xCELLigence RTCA DP system (Roche Applied Science, Indianapolis, IN, USA). Briefly, cells (5 × 10^3 cells/100 μL/well) were seeded into an E-Plate 16 and stabilized overnight. 100 μL of fresh culture medium with or without PHMG-P-containing solution was added to each well, and then the cell proliferation was monitored for 96 h.

2.5. TEM image

For TEM image, cells (70–80% confluency) were treated with PHMG-P-containing solution (1.25%, 16.0 μg/mL as PHMG-P) for 6 h. After washing with phosphate buffered saline (PBS), cells were fixed in 2% glutaraldehyde/0.1 M sodium cacodylate buffer (pH 7.2) for 2 h as previously reported (Park et al., 2014b). Then, the cells were stained for 30 min with 0.5% aqueous uranyl acetate, dehydrated in graded ethanol solutions, and embedded in Spurr's resin. Thin sections were cut using an ultramicrotome (MT-X, RMC, Tucson, AZ, USA), stained with 2% uranyl acetate and Reynolds's lead citrate, and imaged with a LIBRA 120 TEM (Carl Zeiss, Oberkochen, Germany) at an accelerating voltage of 80 kV.

2.6. ROS and NO production

Cells (5 × 10^5/well) were incubated in 6-well plates overnight, and PHMG-P-containing solution (0.25, 0.5, 0.75, 1.0 and 1.25% of solution) was added to each well. After exposure for 6 h, the live cells were further incubated in FBS-free culture medium containing 2′,7′-dichlorfluorescin-diacetate (5 μM, Invitrogen, NY, USA) for 30 min at 37 °C. After washing with PBS, cells were resuspended in FBS-free culture medium, and fluorescent intensity in cells was evaluated using the FACSCalibur system and CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA). Also, cells (2 × 10^6 cells/mL) were incubated with or without PHMG-P-containing solution for 6 h, and the supernatant (100 μL/well) was transferred to a new 96-well plate and reacted with a NO detection solution (1:1, iNtRON Biotech, Gyeonggi-do, Korea). Then, the absorbance was measured at 540 nm using a microplate spectrophotometer system (Molecular Devices). The NO level was calculated from the linear portion of a standard curve generated under the same measurement conditions with $NaNO_2$.

2.7. Organelle damage

First, we measured lactate dehydrogenase (LDH) level released into the supernatants according to the manufacturer's instructions (LDH assay kit, Biovision, CA, USA) to evaluate membrane damage. In brief, cells (2 or 1 × 10^5 cells/200 μL/well for 6 or 24 h,) were incubated in a 96-well plate with a culture medium with or without PHMG-P-containing solution. A part of the supernatants (10 μL/well) was transferred to a new 96-well plate and reacted with LDH reaction solution (100 μL/well) for 10 min at room temperature (RT). Absorbance was quantified at 450 nm using a microplate spectrophotometer system (Molecular Devices), and the relative LDH level of the treated group was calculated with the absorbance value of the control group (100%). Additionally, cells (5 × 10^5/well) were seeded in 6-well plates and incubated overnight. After 6 h exposure, mitochondrial mass and active mitochondria