主論文の要旨

Synergistic Effect of Bolus Exposure to Zinc Oxide Nanoparticles on Bleomycin-Induced Secretion of Pro-Fibrotic Cytokines without Lasting Fibrotic Changes in Murine Lungs

（酸化亜鉛ナノ粒子大量瞬時曝露のマウス肺における持続的線維化変化を伴わないブレオマイシン誘導性線維化促進サイトカイン分泌に対する相乗作用）

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[Introduction]

Zinc oxide (ZnO) nanoparticles are widely used in various products, but biological effects of this manufactured nanomaterial remain elusive. Several in vitro studies showed adverse effects of exposure to ZnO nanoparticles on alveolar macrophages and lung epithelial cells. Moreover inflammatory infiltration, cytotoxicity, oxidative stress and fibrotic changes were observed in the lungs of rats after inhalation or intratracheal instillation of ZnO nanoparticles. The present study investigated how exposure to ZnO nanoparticles accelerates or enhances bleomycin (BLM)-induced fibrosis in mice.

[Materials and Methods]

ZnO nanoparticles (MKN-ZnO-020; mkNano) with a primary diameter of 20 nm and a surface area of 50.72 m^2/g were used. For the animal experiments, suspensions of ZnO nanoparticles were prepared in a biocompatible dispersion medium (DM) developed by US National Institute for Occupational Safety and Health. Dynamic light scattering (DLS) showed aggregation of the nanoparticles in the DM with an average hydrodynamic size of 153.3 ± 1.0 nm.

9-week-old female C57BL/6J mice were exposed to 10, 20 or 30 μg ZnO nanoparticles in suspension or only vehicle medium by pharyngeal aspiration. After pharyngeal aspiration, mice were divided into 2 groups: mice treated with BLM (the BLM groups) and non BLM-treated mice (the SALINE groups). In the BLM groups, lung injury was induced by 7-day constant subcutaneous infusion of bleomycin sulfate at 100 mg/kg body weight, dissolved in sterile saline using osmotic minipumps. Minipumps only loaded with saline were placed in the SALINE groups. At 10 and 14 days after administration, mice were euthanized by collecting blood through the inferior vena cava under deep pentobarbital anesthesia. Bronchoalveolar lavage fluid (BALF) was collected and the perfused lungs were then dissected out. The total and differential cell counts, the levels of interleukin (IL)-1β, monocyte chemotactic protein (MCP)-1 and transforming growth factor (TGF)-β in BALF were measured. The left lung was fixed for histopathological examination and the right lungs were used to measure hydroxyproline content and collagen I, III, matrix metalloproteinase (MMP)-2, 9, tissue inhibitor of MMPs (TIMP)-1, 2 and fibroblast specific protein (FSP)-1 mRNA levels.

[Results]

At day 10, in the SALINE groups, body weight was lower while relative lung weight was higher in the 30 μg ZnO-exposed mice compared with the vehicle control (Table 1). Similar trend was observed in the BLM groups. Four out of seven mice died after day 5 following exposure to 30 μg of ZnO nanoparticles in the BLM groups.

Hematoxylin and eosin (H&E)-stained micrographs of the lungs showed moderate to severe inflammatory infiltration in mice exposed to ZnO nanoparticles in the SALINE groups and BLM groups (Figure 1). The dose-dependently increased pulmonary inflammation induced by ZnO nanoparticles was also demonstrated by the total inflammation score, as shown in Figure 2. However, no distinct fibrotic change was observed.

BALF total cell, neutrophil and lymphocyte counts increased in a dose-dependent manner after administration of ZnO nanoparticles compared with the vehicle control (Fig. 3), which is consistent with the pathological findings. The cytokines levels in BALF were analyzed by enzyme-linked immunosorbent assay (ELISA). The level of IL-1β in BALF increased dose-dependently following exposure to ZnO nanoparticles, and an increasing trend of MCP-1 was observed in the BLM groups (Fig. 4).

The interaction between treatment with BLM and exposure to ZnO nanoparticles was tested by analysis of covariance (ANCOVA). At day 10, interaction between BLM treatment and ZnO exposure was significant for body weight and levels of IL-1β and MCP-1 in BALF. Furthermore, the absolute values of coefficients of body weight (2 fold) and levels of IL-1β (7 fold) and MCP-1 (14 fold) in BALF were higher in the BLM group than the SALINE group, suggesting synergistic effects of BLM and ZnO nanoparticles on these parameters (Table 2).

IL-1β and MCP-1 are considered to promote pulmonary fibrosis by triggering the activation and proliferation of fibroblasts and stimulate collagen production. Thus, we conducted another set of animal experiments in which we examined the effects at day 14, in order to examine whether exposure to ZnO enhances or accelerates pulmonary fibrosis induced by BLM. Because high mortality was found in BLM-treated mice exposed to ZnO at 30 μg, the high dose of ZnO nanoparticles was adjusted to 20 μg for the 14-day experiment.

Recovery of pulmonary inflammation was observed at day 14 (Figure 5). To check pulmonary fibrosis, Masson's trichrome staining and alpha smooth muscle actin (α-SMA) immunohistochemistry were conducted. The results showed that exposure to ZnO nanoparticles up to 20 μg had no obvious effects on collagen deposition or fibroblast proliferation both in the SALINE and BLM groups. The level of TGF-β in BALF and MMP-2 mRNA level in lung tissue were found to increase in the SALINE group after exposure to 20 μg ZnO nanoparticles (Figure 6). However, there was no ZnO-induced change in collagen I, III, MMP-2, MMP-9, TIMP-1, TIMP-2 and FSP-1 mRNA levels in either the SALINE or BLM groups. Moreover, hydroxyproline content, which showed the amount of collagen in tissue, did not change after exposure to ZnO nanoparticles.

[Discussion]

The present study demonstrated that pharyngeal aspiration of ZnO nanoparticles induced severe but transient pulmonary inflammation. Marked inflammation was observed in mice at 10 days after exposure to ZnO nanoparticle, but recovery of inflammation was demonstrated at day 14. This transient inflammation induced by ZnO nanoparticles is compatible with the well-known metal fume fever: influenza-like symptoms typically appear within 4-12 hr after inhalation of fumes and resolve within 24-48 hr.

At the both time points, no distinct fibrotic change was found in histopathological observation or biochemical analysis in BALF and lung tissues. Such minimal fibrotic effect of ZnO nanoparticles found in the present study is consistent with previous studies in mice after treatment of ZnO nanoparticles by intratracheal instillation or inhalation. On the other hand, previous studies on rats reported increase in TGF-β levels in BALF and collagen deposition in lung sections. The discrepancy in the fibrotic changes between mice and rats might represent species differences in the pulmonary responses to ZnO nanoparticles. Similar outcome was described in rodents exposed to ultrafine titanium dioxide particles: rats developed severer inflammatory response compared to mice.

The synergistic effect of BLM treatment and ZnO exposure on pro-fibrotic cytokines in BALF detected at day 10 was attenuated at day 14. Moreover, the fibrotic changes induced by BLM did not seem to be promoted by a single bolus exposure to ZnO nanoparticles at this time point. As a limitation of this study, pharyngeal aspiration or intratracheal instillation is a less physiologic method of exposure compared with inhalation, and may alter the study results. A case report showed that welders exposed to condensation aerosol with high ZnO concentrations developed pneumoconiosis with exogenous fibrosing alveolitis, indicating the risk for individuals to develop pulmonary fibrosis after long-term exposure to ZnO nanoparticles. At present, there are very few animal studies which evaluated the fibrotic effect of pulmonary exposure to ZnO nanoparticles. It is worth trying continuous inhalation experiment, as it better mimics the exposure situation in human and might result in persistent irritation, leading to continuous interaction between BLM and ZnO.

[Conclusion]

Pharyngeal aspiration of ZnO nanoparticles in mice resulted in transient infiltration of inflammatory cells and upregulation of inflammatory cytokines in the lungs. The study demonstrated synergistic effect of pulmonary exposure to ZnO nanoparticles and subcutaneous infusion of BLM on secretion of pro-fibrotic cytokines in the lungs, but a single bolus exposure to ZnO nanoparticles did not induce distinct pulmonary fibrotic changes in mice.