INTRODUCTION

Evaluation of the pulmonary toxicity of air-borne materials involves exposure of animals via inhalation. Intratracheal instillation under anesthesia is a simple alternative technique for the inhalation exposure. Much attention is gathering to manufactured nanomaterials in terms of pulmonary (lung and plural cavity) toxicity in recent years. Multi wall carbon nanotubes moved from lung to thoracic cavity when intrapulmonary spraying in rats (Xu et al., 2012). The first contact surface encountered by inhaled materials is the epithelium of the respiratory tract and bronchial alveolar lavage fluid (BALF) can be used as a proper material for assessment of the volatile/mist chemical toxicity. The BALF has been used to assess inflammatory response in the lungs of experimental animals, whereas little information is available in pleural cavity lavage fluid (PLF) in the lungs. Evaluation of pulmonary toxicity including BALF and PLF analysis is expected to be essential to the assessment for manufactured nanomaterials. We treated rat with chemicals/zinc chloride intratracheally to induce the pulmonary toxicity, and evaluated the usefulness of BALF/PLF for the lung toxicity studies. We also compared diethyl ether and isoflurane for anesthesia of their compatibility.

MATERIALS AND METHODS

The studies were conducted in compliance with the Law Concerning the Protection and Control of Animals (1973) and Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (1980). The study was approved by the Institutional Animal Care and Use Committee and performed in accordance with the ethics criteria contained in the bylaw of the Committee of the BioSafety Research Center (BSRC).

Chemicals

As solvent/vehicle control, we used phosphate buffered saline (PBS, Life technologies Japan, Tokyo, Japan) and distilled water for injection (Otsuka Pharmaceutical Factory, Inc., Naruto, Tokushima, Japan). Distilled water...
is often used in toxicological study as negative/vehicle control, however, there is little information of use of distilled water in intratracheal instillation study. Therefore, we selected distilled water as negative/vehicle control and compared the effects on pulmonary function of PBS. We also used zinc chloride (Otsuka Pharmaceutical Factory, Inc.) as a model chemical. We used diethyl ether (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for anesthesia to compare and ensure the usability of isoflurane in this kind of study.

Animals
We used specific pathogen-free male Crl: CD (SD) rats (7 weeks old, Charles River Laboratories Japan Inc., Yokohama, Kanagawa, Japan) and Slc: Wistar rats (8 weeks old, Japan SLC, Inc., Hamamatsu, Shizuoka, Japan). The rats were housed in positive-pressure air-conditioned (19-25°C, 35-70% relative humidity) and 12:12-hr light/dark cycle animal facility.

Methods
Rats were euthanized with diethyl ether (five SD rats) or isoflurane (four Wistar rats) to compare the effects of anesthesia on respiratory tract. Lungs were removed and washed with 3 ml of PBS which had been warmed to 37°C to collect BALF. The lungs were gently manipulated after insertion of the PBS to withdraw the lavage fluid for cell counts (ADVIA120, Siemens AG, Munich, Germany). BALF cells were washed by centrifugation and re-suspended for differential analysis after staining with May-Grunwald Giemsa. The supernatant was analyzed for protein concentrations with an automatic analyzer (Model 7170, Hitachi, Ltd., Tokyo, Japan). The cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, GM-CSF, INF-γ, TNF-α, MCP-1, GRO/KC, IL-12, IL-18) were analyzed with a Luminex 200 (xPONENT System, Merck Millipore, Tokyo, Japan).

Statistical analyses
After F-test, Student t-test or Welch’s t-test was used to evaluate statistical significance. Differences were considered significant at p < 0.05.

RESULTS AND DISCUSSION

There was essentially no difference in the BALF volume, total protein contents, total number of cells or cell differentiation, between ether and isoflurane-anesthesia groups (Fig. 1A, 1B). From the viewpoint of occupational health and safety, diethyl ether has flammable characteristics (ISCs) and nasal mucosa irritation (Monticelli et al., 2011). Based on the data presented here, there is no problem in use of isoflurane instead of diethyl ether for this kind of studies.

As the solvent/vehicle control, both PBS and distilled water can be used because there was no difference in the BALF volume, total protein contents, total number of cells or cell differentiation (Fig. 1C, 1D).

There was no difference in the PLF volume, total number of cells or cell differentiation, between one and seven day(s) after treatment of PBS (Fig. 2A-2D). The number of cells per cubic millimeter in PLF was similar to that in BALF which was obtained in the previous experiment to evaluate the PBS and distilled water as negative control. The cell differentiation was slightly different between PLF and BALF. Percentage of macrophage in PLF was less than that of BALF which was obtained in the other experiments. Several papers reported usefulness of the PLF cytology on diagnosis of human lung cancer (Aokage et al., 2010; Enatsu et al., 2006; Li et al., 2008), however few paper reported usefulness for pulmonary toxicity assessment although the PLF cytology is considered to be a good method for pulmonary tox-
Fig. 1. BALF analysis of rats anesthetized with diethyl ether or isoflurane (A, B), and that of PBS or distilled water (C, D). (A, C) BALF volume, protein contents, and number of total cell, (B, D) Percent cell differential. Values are presented as the mean ± S.D.

Fig. 2. PLF analysis of rats instilled with PBS. (A) PLF volume, protein contents, and number of total cell, (B) percent cell differential, (C) photograph of PLF in day 1, bar = 50 μm. (D) photograph of PLF in day 7, bar = 50 μm. Values are presented as the mean ± S.D.
We are planning the intratracheal study of zinc chloride with PLF analysis in rats. As a model chemical, we treated animals with zinc chloride intratracheally. There were increases in protein content, LDH, total number of cells, neutrophils, lymphocytes, eosinophils, IL-5, IL-6, MCP-1, GRO/KC in the zinc chloride group compared to the distilled water group (Fig. 3A-3F). Other parameters, IL-1α, IL-1β, IL-2, IL-4, IL-10, GM-CSF, INF-γ, TNF-α, IL-12, IL-18, did not show differences between distilled water and zinc chloride groups (data are not shown). The BALF cytokine parameters such as IL-5, IL-6, MCP-1 and GRO/KC had good correlation to lung inflammation, but not IL-1α, IL-1β, IL-2, IL-4, IL-10, GM-CSF, INF-γ, TNF-α, IL-12, IL-18. Several papers reported usefulness of the BALF analysis on pulmonary damage (Henderson et al., 1985; Henderson, 2005; Kobayashi et al., 2010). Our present study confirmed the usefulness of the BALF for pulmonary toxicity assessment. We may conclude that the intratracheal treatment and combination usage of BALF...
and PLF as a target material is a good method for assessment of chemical pulmonary (lung and plural cavity) toxicity in rats.

ACKNOWLEDGMENTS

We would like to thank Prof. Hiroyuki Tsuda and his colleague of the Department of Molecular Toxicology, Graduate School of Medical Science, Nagoya City University, for instruction the PLF technique.

REFERENCES


