Tissue-Clearing Techniques Enable Three-Dimensional Visualization of Aerosolized Model Compound and Lung Structure at the Alveolar Scale

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In this study, we examined the usefulness of a tissue-clearing technique for the evaluation of the lung distribution of aerosolized drugs. An aerosol formulation of TexasRed dextran (70 kDa), a model compound of drug carrier for aerosolized drugs, was administered intrapulmonarily to mice using a MicroSprayer, and then DyLight 488-conjugated tomato lectin was administered intravenously to visualize general lung structure via the fluorescent labeling of alveolar and bronchial epithelial cells. Tissue clearing followed by laser scanning confocal microscopy enabled the three-dimensional visualization of intrapulmonary TexasRed dextran and the evaluation of its distribution at the alveolar scale without the preparation of thin tissue sections. These findings suggest that tissue-clearing techniques are useful for the evaluation of intrapulmonary distribution and development of pulmonary drug delivery systems.

Key words  pulmonary drug delivery system; intrapulmonary pharmacokinetics; ClearT2; laser scanning confocal microscopy; alveolus; alveolar epithelial cell

Respiratory diseases such as lung cancer, pulmonary fibrosis, pulmonary emphysema, and respiratory infections cause considerable morbidity and mortality worldwide.1–3) Effective therapy of these diseases requires the selective, efficient, and safe delivery of drugs to the focus site via drug delivery systems such as the intrapulmonary administration of aerosolized drugs. Various drug carriers for the pulmonary delivery to the focus site have been investigated.4–7) As part of the development processes of these systems, drug delivery efficiency in the lungs must be evaluated via visualization at the alveolar scale. Although X-ray computed tomography (CT), positron emission tomography (PET), and single-photon emission computed tomography (SPECT) enable the three-dimensional (3D) visualization of lung structure and the distribution of contrast agents administrated intrapulmonarily on a macroscopic tissue scale,8) visualization at the alveolar scale for the evaluation of drug distribution at focus sites is impossible with present technologies owing to their low resolution. On the other hand, evaluating drug distribution with micrographs of thin tissue sections of the lungs is neither as technically completely nor as accurate owing to the loss or distortion of sections that become torn or folded.

Optical tissue-clearing techniques have been developed for the depth-independent visualization and 3D imaging of fluorescent proteins and dyes in large tissues without the preparation of thin tissue sections.9,10) These techniques enable whole-organ imaging at a single-cell resolution using by laser scanning confocal microscopy. However, no studies have assessed the applicability of these techniques for the evaluation of drug distribution in tissues. In the present study, we examined the usefulness of a tissue-clearing technique (ClearT2)11) for evaluating the intrapulmonary distribution of aerosolized drugs.

MATERIALS AND METHODS

Materials and Animals  TexasRed dextran (70 kDa) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). DyLight 488-conjugated Lycopersicon esculentum lectin (tomato lectin) was purchased from Vector Laboratories Inc. (Burlingame, CA, U.S.A.). Formamide was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Polyethylene glycol (8 kDa) was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Male ICR mice (26–28 g) were purchased from Japan SLC (Shizuoka, Japan). The animal experimental protocol was approved by the Committee of the Laboratory Animal Center (No. H28-001) and conformed to the Guiding Principles for the Care and Use of Experimental Animals at Hokkaido Pharmaceutical University.

Mouse Lung Sample Preparation  The mice were anesthetized via intraperitoneal administration of sodium pentobarbital and butorphanol tartrate at doses of 50 and 5 mg/kg, respectively. TexasRed dextran, a model compound of drug carrier for aerosolized drugs, dissolved in phosphate-buffered saline solution (PBS, pH 7.4) was aerosolized into the lungs of the mice at a dose of 250 µg/250 µL/kg using a Liquid MicroSprayer® (Model IA-1C, PennCentury, Inc., Philadelphia, PA, U.S.A.). Five minutes after the administration of TexasRed dextran, fluorescent visualization of the general lung structure, DyLight 488-conjugated tomato lectin dissolved in PBS was administered intrapulmonarily to the mice at a dose of 2.5 mg/2.5 mL/kg. Ten minutes after the administration of the tomato lectin, the mice were transcardially perfused with 4% paraformaldehyde in PBS for tissue fixation. Lung samples were extracted and treated with 4% paraformaldehyde in PBS at 4°C overnight.

ClearT2 Tissue-Clearing Treatment  Tissue-clearing treatment of the lungs was performed with the ClearT2 technique reported by Kuwajima et al.11) Briefly, the samples were incubated in 25% formamide/10% polyethylene glycol solution with gentle rotation at room temperature for 1 h. Then, the samples were incubated in 50% formamide/20% polyethylene glycol solution for 1 h. Finally, the samples were transferred into fresh 50% formamide/20% polyethylene glycol solution.
Observation of Cleared Lung Tissue

For the evaluation of transparency uniformity, cleared lung tissues were cut using a tissue slicer. For the evaluation of general lung structure preservation, cleared lung tissues were cut using a cryostat into 5-mm-thick serial frozen sections and stained with hematoxylin–eosin (HE). For macroscopic visualization of intrapulmonary TexasRed dextran in whole lung scale, clearing lungs were observed using fluorescence zoom microscopy (Axio Zoom. V16; Zeiss, Oberkochen, Germany). For 3D visualization of intrapulmonary TexasRed dextran in alveolar scale, clearing lungs were placed on a glass-bottom dish and observed using laser scanning confocal microscopy (LSM 700; Zeiss).

RESULTS AND DISCUSSION

The present study evaluated the usefulness of a tissue-clearing technique for the evaluation of aerosolized drug distribution in the lungs after intrapulmonary administration. Transmitted images showed that the lungs become evenly transparent after ClearT2 tissue-clearing treatment (Fig. 1). Many tissue-clearing techniques for large-scale biological tissues, such as ClearT2,11) ScaleS,12) and CUBIC,13) have been reported. Among these, the ClearT2 technique can be completed in the shortest time (7 h), has a moderate clearing capability that causes no delipidation or alterations in tissue morphology, and preserves lipophilic dyes.10) Therefore, we chose the ClearT2 technique for lung clearing. The images of HE-stained lung sections showed that the ClearT2 clearing treatment did not change the tissue morphology (Fig. 2).

For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed. For the evaluation of drug distribution in clearing lungs, not only intrapulmonary drugs but also the lung structure must be observed.
be visualized with fluorescent labeling. The intravenous administration of fluorescence-tagged tomato lectin is a sensitive and reliable method for visualizing vascular structures in various organs. In the lung, tomato lectin binds specifically to many types of alveolar cells, including alveolar epithelial type I cells, the cilia of bronchial epithelium, and capillaries. Therefore, similar to HE staining, labeling via the intravenous administration of fluorescence-tagged (DyLight 488-conjugated) tomato lectin allowed the visualization of general lung structure (see Fig. 2). Compared with immunostaining techniques, this labeling technique requires a shorter treatment time after the sampling of fixed lung tissue.

The images in whole lung scale of TexasRed dextran in clearing lungs after intrapulmonary drug administration is shown in Fig. 3. Intrapulmonary TexasRed dextran in cleared lung tissue was clearly visibility than that in immediately after perfusion. Since TexasRed dextran using in this study has large molecular weight (70 kDa) and high hydrophilicity, the agent remained in the alveolus without diffusing from site of initially administration by passive diffusion during the clearing treatment. These findings indicate that the clearing method is useful for visualization of intrapulmonary large molecules of about 70kDa with low permeability, such as albumin (68kDa) and immunoglobulin G (150kDa) and large...
nanoparticle dendrimers (78 kDa). Therefore, this method is thought to be useful for evaluating the distribution in lungs after intrapulmonary administration of antibody drugs or drug carriers such as dextran-conjugate, albumin–drug complex, and dendrimers. In addition, drug carriers larger than TexasRed dextran, such as liposomes and polymeric micelles used for the pulmonary delivery might be observable because they are retained in the alveolar cells or alveoli. On the other hand, since small molecule drugs diffuse in the lung tissue through the alveolar walls during clearing treatment, it may be difficult to visualize and evaluate its intrapulmonary distribution.

The 3D reconstruction of confocal images of TexasRed dextran in clearing lungs after intrapulmonary drug administration is shown in Fig. 4. TexasRed dextran was visible as deep as 100 µm in cleared lung tissue, which is more than twice as deep as the visibility immediately after perfusion and before clearing treatment. The visible depth was sufficient to express the drug distribution in the alveoli, which have a radius of several tens of micrometers. In addition, because observations can be made without cutting the lung tissue, the complete alveolar structure can be maintained, as shown in the fluorescent staining with tomato lectin (see Fig. 4). Therefore, lung clearing is thought to enable the visualization of not only intracellular drugs but also extracellular drugs, such as TexasRed dextran, without alveolar leakage. These findings suggest that tissue-clearing treatment is useful for the study of intrapulmonary distribution and the development of pulmonary drug delivery systems targeting both the lung surface (e.g., epithelial lining fluid) and lung cells such as alveolar epithelial cells, alveolar macrophages, and lung fibroblasts. For the accurate and detailed evaluation of drug distribution in the lungs, future studies must develop a means of visually identifying these lung cell types.

CONCLUSION

The Clear tissue-clearing technique enabled the 3D visualization of both intrapulmonary TexasRed dextran as a model compound and lung structure at the alveolar scale. The results of this study indicate that tissue-clearing techniques are useful for the evaluation of drug distribution after the intrapulmonary administration of aerosolized drugs.

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Conflict of Interest The authors declare no conflict of interest.

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