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Pulmonary administration of phosphoinositide 3-kinase inhibitor is a curative treatment for chronic obstructive pulmonary disease by alveolar regeneration

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is an intractable pulmonary disease, causing widespread and irreversible alveoli collapse. The discovery of a low-molecular-weight compound that induces regeneration of pulmonary alveoli is of utmost urgency to cure intractable pulmonary diseases such as COPD. However, a practically useful compound for regenerating pulmonary alveoli is yet to be reported. Previously, we have elucidated that Akt phosphorylation is involved in a differentiation-inducing molecular mechanism of human alveolar epithelial stem cells, which play a role in regenerating pulmonary alveoli. In the present study, we directed our attention to phosphoinositide 3-kinase (PI3K)-Akt signaling and examined whether PI3K inhibitors display the pulmonary alveolus regeneration. Three PI3K inhibitors with different PI3K subtype specificities (Wortmannin, AS605240, PIK-75 hydrochloride) were tested for the differentiation-inducing effect on human alveolar epithelial stem cells, and Wortmannin demonstrated the most potent differentiation-inducing activity. We evaluated Akt phosphorylation in pulmonary tissues of an elastase-induced murine COPD model and found that Akt phosphorylation in the pulmonary tissue was enhanced in the murine COPD model compared with normal mice. Then, the alveolus-repairing effect of pulmonary administration of Wortmannin to murine COPD model was evaluated using X-ray CT analysis and hematoxylin–eosin staining. As a result, alveolar damages were repaired in the Wortmannin-administered group to a similar level of normal mice. Furthermore, pulmonary administration of Wortmannin induced a significant recovery of the respiratory function, compared to the control group. These results indicate that Wortmannin is capable of inducing differentiation of human alveolar epithelial stem cells and represents a promising drug candidate for curative treatment of pulmonary alveolar destruction in COPD.
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1. Introduction

Emphysema and chronic bronchitis that cause respiratory failure due to destruction of alveolar structures are collectively referred to as chronic obstructive pulmonary disease (COPD), which is currently the fourth leading cause of death worldwide [1]. The number of patients with COPD reportedly exceeds 200 million in the world including the potential ones on the basis of epidemiological surveys by WHO (Global Alliance Against Chronic Respiratory Diseases (GARD)). However, no therapeutic drug is available for curative treatment of COPD, and the development of a therapeutic agent to repair alveolar destruction is of particular urgency.

The targets of human lung alveolar remodeling are resident stem and progenitor cells in the lung that function in tissue repair and homeostasis. The adult lung consists of the following four major biologically distinct components: the trachea, bronchi, bronchioles, and alveoli. Each component is biologically distinct and has its own stem and progenitor population [2–5]. Alveoli are terminal structures of distal airways specialized for gas exchange. The gaseous alveolar surface is lined by alveolar type I cells (AT-I) and alveolar type II cells (AT-II) [6]. Recent studies have characterized resident alveolar stem cells in human [7]. However, potent regenerative compounds have not been identified for human alveolar stem cells.

We identified a differentiation-inducing agent for human alveolar epithelial stem cells responsible for the regeneration of pulmonary alveoli, and further studied its differentiation-inducing molecular mechanism. Based on this study, for first time, we reported that suppression of the phosphorylation of Ser/Thr kinase Akt leads to differentiation of human alveolar epithelial stem cells into AT-I and AT-II, which constitute pulmonary alveoli [8]. In this study, we focus on phosphoinositide 3-kinase (PI3K)-Akt signaling pathway. PI3K is categorized into class I, class II, and class III according to its primary structure and substrate specificity, with class I being involved in cellular survival and differentiation [9]. Class I PI3K is further divided into four subunits, α and β subunits involved in cellular survival, δ and γ subunits reported to be involved in inflammation [10,11]. While PI3K-δ and PI3K-γ have been reported

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to suppress inflammation in COPD [12,13], their differentiation-inducing effect on alveolar epithelial stem cells has yet to be elucidated.

Thus, in this study, we focused on phosphoinositide 3-kinase (PI3K), which phosphorylates Akt, and examined whether PI3K inhibitors induced the differentiation of human alveolar epithelial stem cells. We further examined the effectiveness of the PI3K inhibitors on the repair of pulmonary alveoli and improvement of respiratory function in a murine COPD model.

Pulmonary administration is a drug delivery system (DDS) that is superior to other methods of drug administration in delivering a drug to the lung. Previously, the efficacy of PI3K inhibitors has been studied primarily in oral administration, and no study has been reported on their pharmacological effects, including the representative antitumor effect, through pulmonary administration. This study is the first to report the effect of PI3K inhibitors on the lung when they are delivered by pulmonary administration. We demonstrated the alveolus-repairing effect of PI3K inhibitors in mice using a pulmonary administration method of breath-actuated inhalation, and found the possibility of an additional indication of PI3K inhibitors for COPD.

2. Materials and methods

2.1. Animals and cells

Male ICR mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). Animals were housed in a temperature-controlled (24 ± 1 °C) facility maintained on a light (12 h):dark (12 h) cycle with standard food available *ad libitum*. All animal procedures followed the guidelines established by the Animal Care and Use Committee of the Tokyo University of Science.

Six-week-old male mice were anesthetized with isoflurane, and a solution of porcine pancreatic elastase (Elastin Products Company, Owensville, MO) (4.05 or 7.5 U/50 μl of saline) was administered intratracheally. We administered pulmonary administration of 0.2 mg/kg Wortmannin to elastase-induced COPD model mouse twice a week and evaluated the results using X-ray CT scanning or hematoxylin and eosin staining. Mice were sacrificed after 4 weeks.

Human alveolar epithelial stem cells were provided by Dr. Hiroshi Kubo (Tohoku University, Sendai, Japan) [7] and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 20% ReproFF2 Medium (ReproCELL, Yokohama, Japan) in a humidified 5% CO_2 atmosphere maintained at 37 °C. Experiments using human cells were conducted in accordance with the guidelines of the Research Ethics Committee of the Tokyo University of Science and Tohoku University.

2.2. Induction of differentiation to AT-I or AT-II cells

To induce the differentiation of human alveolar epithelial cells to AT-I or AT-II cells, a culture system described previously was adopted with some modifications [14]. In brief, cells (5×10^5) at passages 3–6 were plated on cell culture inserts (BD Biosciences, Franklin Lakes, NJ) that had been coated with a mixture of 60% Matrigel (BD Biosciences, Franklin Lakes, NJ) and 40% rat tail collagen I (BD Biosciences, Franklin Lakes, NJ) in 5% FBS/E-MEM. After 4 h, media were changed to DMEM containing 5% FBS with or without Wortmannin, AS605240, PIK-75 Hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The media were changed every other day until analysis on day 7.

The cultured cells were fixed with 4% paraformaldehyde in phosphate buffer for 15 min at room temperature. Samples were blocked using Blocker BSA (Thermo Fisher, Waltham, MA) for 30min at room temperature. Cells were then incubated with the following primary antibodies overnight at 4 °C: goat anti-human CD90 (Thy-1) polyclonal antibody, goat anti-human aquaporin-5 (AQP-5) polyclonal antibody, and goat anti-human SP-A monoclonal antibody (Santa Cruz Biotechnology, Billerica, MA). Alexa Fluor 488-conjugated anti-goat IgG (each at 1:500, Molecular Probes, Carlsbad, CA) were used as secondary antibodies. After mounting the samples and staining nuclei using ProLong Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) (Molecular Probes, Carlsbad, CA), samples were observed using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) and an A1Rsi confocal laser scanning microscope system (Nikon, Tokyo, Japan). By using image analysis software, ImageJ (NIH), the number of positive cells in immunostaining images at 20× magnification was counted. Then the ratios of CD90, AQP-5, and SP-A positive cells were determined against the number of DAPI positive cells, *i.e.*, the nuclear marker. Ten views were taken of each sample, and CD90, AQP-5, and SP-A positive cell numbers were measured for each sample, for a total of 500 DAPI positive cells.

2.3. Evaluation of the safety of Wortmannin

Treatment dose-dependent cytotoxicity of Wortmannin on human alveolar epithelial stem cells was evaluated.

Wortmannin was dissolved in DMSO, and the resulting solution was diluted with DMEM to give Wortmannin solutions at final concentrations of 1, 10, and 100 μM containing DMSO at a final concentration of 0.1%. Human alveolar epithelial stem cells were seeded at 1×10^4 cells/cm^2, ATP derived from metabolically active cells was detected every 4 h for 24 h with the CellTiter-Glo™ Luminescent Cell Viability Assay kit (Promega, WI, USA), and the chemiluminescence signals were quantified with an ARVO (PerkinElmer, MA, USA). The proportion of viable cells was evaluated over time, using the cell viability at time 0 as 100%.

To evaluate body weight changes depending on the Wortmannin dose, Wortmannin was administered twice a week *via* the pulmonary route to elastase-induced COPD model mice at doses of 0.05, 0.1, 0.2, and 0.4 mg/kg, and the body weight was measured 1, 2, and 3 weeks after starting the administration.

2.4. Immunohistochemical analysis

Six-week-old male mice were anesthetized with isoflurane, and a solution of porcine pancreatic elastase (Elastin Products Company, Owensville, MO) (4.05 or 7.5 U/50 μl of saline) was administered intratracheally. After 4 weeks, the lung was fixed with 4% (w/v) paraformaldehyde in phosphate buffer, pH 6.9, and thereafter treated with Blocker BSA (Thermo Fisher, Waltham, MA) for 30 min at room temperature. Sliced lung tissues were incubated with the antibodies against p-Akt and Akt (1:200, Cell Signaling Technology, Inc., Boston, MA) at 4 °C for 12 h. After washing with cold PBS, cells were incubated with Alexa Fluor 546-conjugated anti-rabbit IgG (1:500, Santa Cruz Biotechnology, Billerica, MA) for 3 h. After mounting the samples and staining nuclei using ProLong Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) (Molecular Probes, Carlsbad, CA), samples were observed using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) and an A1Rsi confocal laser scanning microscope system (Nikon, Tokyo, Japan).

2.5. Oral, intraperitoneal, and pulmonary administration methods

Oral administration was conducted in the usual manner. Specifically, the tip of a gastric tube for oral administration (No. KN-348, for mouse, Natsume Seisakusho, Japan) was placed in the oral cavity of a retained mouse and inserted along the maxillary. Once the tip entered the stomach, the inner cylinder was pushed to inject the drug solution.

Intraperitoneal administration was conducted in the usual manner. Specifically, while the mouse was retained, an injection needle (NN-2613S, 26G, Terumo Corporation, Japan) was inserted subcutaneously to an approximate depth of 5 mm in the lower abdomen slightly left to the midline. The needle was then let stand upright and proceed to the peritoneal cavity, in which the drug solution was injected.

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