Pulmonary Administration of GW0742, a High-Affinity Peroxisome Proliferator-Activated Receptor Agonist, Repairs Collapsed Alveoli in an Elastase-Induced Mouse Model of Emphysema

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Pulmonary emphysema is a disease in which lung alveoli are irreversibly damaged, thus compromising lung function. Our previous study revealed that all-trans-retinoic acid (ATRA) induces the differentiation of human lung alveolar epithelial type 2 progenitor cells and repairs the alveoli of emphysema model mice. ATRA also reportedly has the ability to activate peroxisome proliferator-activated receptor (PPAR) β/δ. A selective PPARβ/δ ligand has been reported to induce the differentiation of human keratinocytes during wound repair. Here, we demonstrate that treatment using a high-affinity PPARβ/δ agonist, GW0742, reverses the lung tissue damage induced by elastase in emphysema-model mice and improves respiratory function. Mice treated with elastase, which collapsed their alveoli, were then treated with either 10% dimethyl sulfoxide (DMSO) in saline (control group) or GW0742 (1.0 mg/kg twice a week) by pulmonary administration. Treatment with GW0742 for 2 weeks increased the in vivo expression of surfactant proteins A and D, which are known alveolar type II epithelial cell markers. GW0742 treatment also shortened the average distance between alveolar walls in the lungs of emphysema model mice, compared with a control group treated with 10% DMSO in saline. Treatment with GW0742 for 3 weeks also improved tissue elastance (cm H₂O/mL), as well as the ratio of the forced expiratory volume in the first 0.05s to the forced vital capacity (FEV 0.05/FVC). In each of these experiments, GW0742 treatment reversed the damage caused by elastase. In conclusion, PPARβ/δ agonists are potential therapeutic agents for pulmonary emphysema.

Key words chronic obstructive pulmonary disease; alveolar repair; GW0742; pulmonary administration; differentiation; peroxisome proliferator-activated receptor β

Chronic obstructive pulmonary disease (COPD), the fourth leading cause of death according to a report in 2013,¹ is an increasing global health problem. COPD includes three pathological manifestations: chronic obstructive bronchitis, mucus plugging, and pulmonary emphysema. Pulmonary emphysema is a disease in which the lung’s gas-exchange structures, the alveoli, are irreversibly damaged, causing patient death due to compromised lung function unless a transplant is provided.²⁻⁴ The causes of COPD have not been clearly defined. However, many lipid mediators, inflammatory peptides, reactive oxygen and nitrogen species, chemokines, cytokines, and growth factors have been implicated in alveolar destruction.⁵

All-trans-retinoic acid (ATRA), a metabolite of retinol (vitamin A; also known as tretinoin) was initially reported to have the ability to repair lung tissue in elastease-induced pulmonary emphysema model rats in 1997.⁵ We recently reported that ATRA induced the differentiation of human lung alveolar epithelial type 2 progenitor cells (AEPCCs) into alveolar type II epithelial (AT-II) cells, which secrete surfactant protein, surfactant protein A (SP-A), and repair the alveoli of emphysema model mouse.⁶

ATRA has been used as an alternative therapy for acute promyelocytic leukemia (APL), a specific acute myeloid leukemia subtype characterized by a chromosomal translocation t(15; 17) involving the retinoid acid receptor (RAR) α gene on chromosome 17 and the promyelocytic gene on chromosome 15. ATRA can induce the differentiation of APL blasts both in vitro and in vivo via stimulation of RARα.⁷ ATRA is usually well tolerated, but a few major side effects can be observed; retinoic acid syndrome being the most important of them. Respiratory distress, fever, and weight change were the main clinical signs of retinoic syndrome.⁸

The pathophysiology of retinoic acid syndrome is still poorly understood, but the proposed mechanisms involve changes in the cytokine secretion and adhesive qualities of APL cells during ATRA-induced differentiation to white blood cells, which occurs via stimulation to RARα or β.⁹

In the field of therapy for lung tissue repairing, oral treatment with ATRA failed in clinical trial of patients with pulmonary emphysema, and it needed more trials to evaluate with higher-doses of ATRA.⁹ However, it seems difficult because there is some possibility that higher-doses of ATRA cause many side effects.¹⁰ Therefore, we expected that utilizing nuclear receptor selective agonist could avoid some severe side effects of ATRA.

ATRA has also been shown to activate the nuclear receptor peroxisome proliferator-activated receptor (PPAR) β/δ as an alternative to RAR.¹¹ A selective PPARβ/δ ligand was reported to induce the expression of differentiation markers in a human keratinocyte cell line, and PPARβ/δ upregulation is observed when keratinocyte differentiation occurs during wound repair. Interestingly, this PPARβ/δ upregulation did not occur during normal epidermis renewal; rather, it occurred during inflammation caused by wound injury.¹² Therefore, the hypothesis of this study was that activation of PPARβ/δ could play a key role in repairing the damage caused by pulmonary emphysema.
Elastase treatment is generally used as an animal model of emphysema, based on the hypothesis that COPD is caused by an imbalance between protease activity and anti-protease activity. Elastase destroys alveolar walls, leading to airspace enlargement, loss of elastic recoil, and lung hyperexpansion.  

In this study, we assessed the ability of GW0742, a high-affinity PPARβ/δ agonist, to repair collapsed alveoli without the side effects of ATRA. Elastase-induced COPD model mice were treated with GW0742 via pulmonary administration to avoid first pass effects and induce their ability to repair lung tissue.

**MATERIALS AND METHODS**

**Reagents** [4-[[2-[(3-Fluoro-4-(trifluoromethyl)phenyl)-4-methyl-5-thiazolyl]methyl]thio]-2-methyl phenoxy]-acetic acid (GW0742) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). This chemical was dissolved in dimethyl sulfoxide (DMSO) for biochemistry purchased from Wako Chemical Industries, Ltd. (Osaka, Japan) and stored in the dark at –20°C before use. Antibodies against SP-A (cat. Sc-7699) were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, U.S.A.) and anti-surfactant protein D antibody (cat. ab101703) were obtained from Abcam Inc. (Cambridge, U.K.). Donkey anti-goat immunoglobulin G-fluorescein isothiocyanate (IgG-FITC) (cat. AP180F) was purchased from EMD Millipore Corporation (Billerica, MA, U.S.A.) and donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 594 conjugate (cat. A-21207) were obtained from Thermo Fisher Scientific (Waltham, MA, U.S.A.). 4,6-Diamino-2-phenylindole (DAPI) was purchased from Roche Diagnostics (Basel, Switzerland). Mayer’s hematoxylin, eosin and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries.

**Elastase-Induced Emphysema Model Mouse and Administration of GW0742** All animal procedures followed the guidelines established by the Animal Care and Use Committee of the Tokyo University of Science.

For evaluation of the expression level of AT-II cells and the toxicity of GW0742 on elastase-induced emphysema in mice, we measured changes in weight (%), using the following equation:

\[
\text{Weight change} \% = \frac{\text{The body weight of indicated treatment day (g)}}{\text{The body weight of day 0 (g)}} - 100
\]

**Pulmonary Physiology, Histology, and Morphometry** After 2 weeks of first treatment with GW0742 or 10% DMSO in saline, the mice were then killed by excessive inhalation of isoflurane. Preparation of thin frozen lung tissue sections were performed according to Kawamoto’s Film Method using Cryostat CM 3050 S (Leica Biosystems, Nussloch, Germany). Tissue sections were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and blocked with 1% BSA in PBS and incubated overnight at 4°C with anti-SP-A antibody (1:200) or anti-SP-D antibody (1:300). After washing with PBS, the well was incubated 1.5 h at room temperature with DAPI (20 µg/mL) and fluorescein isothiocyanate (FITC)-conjugated antibody (1:200) for anti-SP-A antibody or Alexa 594-conjugated antibody (1:200) for anti-SP-D antibody and washed with PBS-T.

Determination of surface markers was performed by a direct immunofluorescence assay using the following samples were observed by fluorescent microscopy (BZ-9000, KEYENCE, Osaka, Japan).

Fluorescent intensity was converted into numerals using an image processing program, ImageJ (http://imagej.nih.gov/ij/) and relative fluorescent units were calculated by the fluorescent intensity of FITC divided by one of DAPI.

**Evaluation of the Toxicity of GW0742 on Body Weight Change** To evaluate the toxicity of GW0742 on elastase-induced emphysema in mice, we measured changes in weight (%), using the following equation:

\[
\text{Weight change} \% = \frac{\text{The body weight of indicated treatment day (g)}}{\text{The body weight of day 0 (g)}} - 100
\]

**Measurement of Lung Mechanics, Airway Resistance, and Forced Expiratory Volume (FEV) 0.05%/Forced Vital Capacity (FVC)** Measurement of lung mechanics and airway resistance in mice was evaluated with the flexiVent™ system (flexiVent™ SCIREQ©, Montreal, Canada). The flexiVent™ system equips a precision piston pump and is capable of measuring the pneumodynamics. All data were analyzed using FlexiVent software. Using flexiVent™, it is possible to evaluate the respiratory capacity per unit time (the ratio of forced expiratory volume in 0.05 s; FEV 0.05) in mice, which is similar to the forced expiratory volume in 1 s/forced vital capacity ratio clinically used as an index of the elasticity and respiratory function of the lung, as well as measurement of airway resistance and compliance.

**Statistical Analysis** Normally distributed data are presented as the mean±standard error (S.E.). Dunnet method or the Student’s t-test for unpaired results was used to evaluate
RESULTS

Effects of GW0742 on the Expression of Surfactant Proteins A and D  
In this study, the level of differentiation of mouse lung tissue was evaluated using immunofluorescence to determine the levels of SP-A (Fig. 1) and SP-D (Fig. 2), which is also known as a marker of AT-II cells. Nuclei were labeled with DAPI.

After pulmonary administration of elastase (7.5 U/head once a week for 1 week and then 4.05 U twice a week for 1 week), treatment with GW0742 (1.0 mg/kg twice a week for 2 weeks) increased the level of SP-A expression (Fig. 1A lower panel) compared with that of a control group treated with 10% DMSO in saline (Fig. 1A upper panel). Positive cells, demonstrating the presence of SP-A-antibody complexes, could be observed in sections of lung tissue damaged by elastase (Fig. 1A). This fluorescence was quantified and normalized to the fluorescence of the nuclear stain DAPI. These data demonstrate that the level of SP-A expression was significantly greater (around 1.37-fold) in the lungs of GW0742-treated mice than in those of the control group (Fig. 1B).

SP-D is also known as AT-II cells marker. Treatment with GW0742 increased the level of SP-D expression (Fig. 2A lower panel) compared with that of the control group, which was treated with 10% DMSO in saline (Fig. 2A upper panel). Moreover, the red fluorescence that arose from the SP-D antibody complex was quantified and normalized to the fluorescence of the nuclear stain DAPI. These data demonstrate that the expression of SP-D was significantly increased (1.62-fold) compared with that of the control group (Fig. 2B).

Effect of GW0742 on Body Weight Change  
Treatment of the mice with GW0742 (1.0 mg/kg twice a week for 2 weeks) showed no significant toxicity compared with the control group treated with 10% DMSO in saline (Fig. 3).

Effect of GW0742 on the Average Distance between Alveolar Walls  
To evaluate the effect of GW0742 on lung sections, the average distance between alveolar walls (Lm) in non-treated mice (non-treated group) was compared with the Lm in emphysema model mice treated with GW0742 (1.0 mg/kg twice a week for 2 weeks) or with 10% DMSO in saline (control group). The linear intercept of the alveolar airspace, which is indicative of the severity of the alveolar damage,
was 30.1±2.81 µm in the control group and 25.0±2.41 µm in the GW0742-treated group, demonstrating that the lungs of GW0742-treated mice recovered to a level similar to that observed in the non-treated group (23.3±1.91 µm) (Fig. 4).

Effects of GW0742 on Lung Tissue Elastance To evaluate the effect of GW0742 treatment on lung tissue elastance, lung tissue in non-treated mice (non-treated group) was compared with that in emphysema model mice (prepared by pulmonary administration of 7.5 U/head elastase once a week for 1 week) treated with GW0742 (1 mg/kg twice a week for 3 weeks) or with 10% DMSO in saline (control group). The lung tissue damage in emphysema model mice was evaluated by using a high-performance respiratory function analysis system (flexiVent®). Tissue elastance, which indicates the elasticity and surface tension of the lung, significantly increased in GW0742-treated group (15.0±2.04 cm H₂O/mL) compared with the control group (8.2±1.16 cm H₂O/mL) indicating recovery of the contraction/expansion function of the lung and demonstrating recovery to a similar level of that observed in the non-treated group (20.0±1.46 cm H₂O/mL) (Fig. 5).

Effects of GW0742 on Respiratory Function To evaluate the effect of GW0742 treatment on respiratory function, the ratio of forced expiratory volume in 0.05 s (FEV₀.₀₅) to forced vital capacity (FVC), which is a respiratory function index, of non-treated mice (non-treated group) was compared with those of emphysema model mice (prepared by pulmonary administration of 7.5 U/head elastase once a week for 1 week) treated with GW0742 (1.0 mg/kg twice a week for 3 weeks)
or with 10% DMSO in saline (control group). Respiratory function was evaluated using a high-performance respiratory function analysis system (flexiVent™). The FEV₀.05/FVC ratio increased to 65.5±5.68% in the GW0742-treated group, which was significantly higher than that of the control group (45.6±4.55%), demonstrating recovery of respiratory function to a level similar to that observed in the non-treated group (82.8±3.58%) (Fig. 6).

DISCUSSION

The data from this study showed that treatment with elastase caused significantly enlarging the average distance between alveolar walls and decreasing lung tissue elastance, and respiratory function. Then, treatment with GW0742, a high affinity PPARβ/δ agonist, significantly repaired these deteriorations. This suggests that GW0742 plays an important role in the differentiation of alveolar epithelial cells and increases surfactant protein secretion by AT-II cells. Thus, PPARβ/δ agonists may have therapeutic utility in the treatment of pulmonary emphysema.

Keratinocyte growth factor (KGF) was originally reported to induce the differentiation of AEPCs to AT-II cells. In our previous study, we demonstrated that ATRA induces human AEPCs to differentiate into AT-II cells and significantly increases the expression of SP-A, a known AT-II cell marker. Pulmonary administration of ATRA repaired the collapsed alveoli of emphysema model mice by alveolar repairing.

PPARβ/δ is one of the nuclear receptors that can be activated by ATRA. Stimulation of PPARβ/δ affects many cellular processes, including glucose metabolism, fatty acid metabolism, and keratinocyte, adipocyte and sebocyte differentiation. We focused on the ability of PPARβ/δ activation to induce differentiation.

It has also been reported that mice have epithelial stem/progenitor cells, as do humans. Epithelial stem/progenitor cells have the ability to differentiate into alveolar type II epithelial (AT-II) cells. AT-II cells secrete surfactant proteins that reduce alveolar surface tension and maintain the shape of alveoli. Moreover, AT-II cells can differentiate to AT-I cells. AT-I cells cover a majority of the surface area of the alveoli. Therefore, it expected that the differentiation of human AEPCs to AT-II cells plays an important role in this aspect of alveolar repair.

In this study, we evaluated the levels of SP-A and SP-D expression. Surfactant protein, a lipoprotein complex, was originally described for its essential role in reducing surface tension at the air–liquid interface of the lung. However, it is
by smoking 

It has been reported that SP-D is released into the blood during certain types of lung injury. Therefore, it has been proposed that serum SP-D level will be a useful biomarker in patients with lung injury, such as idiopathic pulmonary fibrosis. SP-D has been analyzed in bronchoalveolar lavage fluid (BALF) and serum and it is clear that the level of SP-D expression in COPD patients is lower than the normal value in BALF and higher than the normal value in serum. In addition, a significant positive correlation was observed between BALF SP-D levels and respiratory function (FEV/FVC). In our study, stimulation of PPAR/β/δ by GW0742 increased the expression of SP-D in lung tissue and improved FEV 0.05/FVC in emphysema model mice.

The three PPAR isoforms, PPARα, PPARβ/δ and PPARγ, have also been observed in human keratinocytes. PPARβ seems to be the prevalent isotype, because its expression remains high during the differentiation of human keratinocytes, although PPARα and γ are expressed at lower levels.

A selective PPARβ/δ agonist was reported to induce the expression of differentiation markers in a human keratinocyte cell line. In mouse keratinocyte primary cultures, a PPARβ/δ agonist was shown to accelerate the differentiation of cultured cells.

Moreover, it has been reported that keratinocyte differentiation via PPARβ/δ up-regulation did not occur during normal epidermis renewal, but instead during the inflammation following wound repair. It has been suggested that one of the roles of PPARβ/δ expression after injury is to induce differentiation during the re-epithelialization phase of tissue repair, like the action of other factors, such as KGF, which modulates keratinocyte behavior. In this study, we demonstrated the possibility that ATRA and PPARβ/δ-selective agonists can repair lung tissue damage similar to that of keratinocytes.

COPD is characterized by progressive chronic inflammation. In the lung of COPD patients, it has been suggested that an increase in macrophage numbers and the upregulation of matrix metalloproteinases (MMPs) cause increasing proteolysis in the small airways and respiratory units. These changes activate inflammatory mediators and cause the pathology that occurs during COPD.

It is thought that lung tissues originally have the ability to self-repair. However, it has also been reported that the levels of fatty acid binding protein (FABP) 5, known to transport self-repair. However, it has also been reported that the levels of fatty acid binding protein (FABP) 5, known to transport

Surfactant proteins are synthesized and packaged into the AT-II cells secretory organelle called the lamellar body. Surfactant proteins, particularly SP-A and SP-D, are hydrophilic surfactant proteins and pattern recognition molecules of the collectin family of C-type lectins. They are a part of the innate immune system and regulate the functions of other innate immune cells.

now recognized to be a critical component of lung immune host defense. Surfactant proteins are synthesized and packaged into the AT-II cells secretory organelle called the lamellar body. Surfactant proteins, particularly SP-A and SP-D, are hydrophilic surfactant proteins and pattern recognition molecules of the collectin family of C-type lectins. They are a part of the innate immune system and regulate the functions of other innate immune cells.

The role of PPARβ/δ stimulation in regulating lung development has not yet been revealed probably because PPARβ/δ
selective agonists were administered to mice through oral\textsuperscript{5}\textsuperscript{5} or peritoneal\textsuperscript{36,37} routes in previous research. In this study, we administered GW0742, a PPAR\(\beta/\delta\)-selective agonist, via the pulmonary route. Direct delivery of GW0742 to alveoli led to effective repair.

The non-selective agonist ATRA has been utilized as an alternative therapy for APL via its ability to stimulate RAR and induce differentiation of leukemia cells.\textsuperscript{8,9} It was also reported that the low body weight has negative effect on survival in patients with severe COPD.\textsuperscript{40} However, ATRA has been reported to have severe side effects; some of them, such as teratogenicity, weight loss, and skin disorder, have been shown to arise via RAR stimulation.\textsuperscript{0,11} On the other hand, it was reported that stimulation to PPAR\(\beta/\delta\) suppressed inflammation and greatly enhanced ATRA differentiation potential.\textsuperscript{10} In this study, no significant weight change was observed by treatment with PPAR\(\beta/\delta\) selective agonist, GW0742. Therefore, PPAR\(\beta/\delta\)-selective agonists could avoid these side effects from RAR. Moreover, stimulation of RAR reportedly increases the expression of CYP26A1, an enzyme of ATRA metabolism.\textsuperscript{43} The failure of ATRA as an effective therapy for emphysema patients in clinical tests was attributed to this enzyme.\textsuperscript{44}

CONCLUSION

In conclusion, this study is the first to demonstrate improved respiratory function in emphysema model mice through stimulation of PPAR\(\beta/\delta\). Although further studies are required to establish the role of PPAR\(\beta/\delta\) in lung tissue, this study indicates the possibility that PPAR\(\beta/\delta\) agonists may be useful as new therapeutic agents for pulmonary emphysema.

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Conflict of Interest The authors declare no conflict of interest.

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