All-*trans* retinoic acid attenuates pulmonary fibrosis through inhibition of EphA2-EphrinA1 signal in bleomycin-induced pulmonary fibrosis mouse model

Ah Young Leem

Department of Medicine

The Graduate School, Yonsei University

All-*trans* retinoic acid attenuates pulmonary fibrosis through inhibition of EphA2-EphrinA1 signal in bleomycin-induced pulmonary fibrosis mouse model

Directed by Professor Moo Suk Park

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Ah Young Leem

December 2016

This certifies that the Doctoral Dissertation of Ah Young Leem is approved.

Thesis supervisor: Moo Suk Park

Thesis Committee Member#1: Young Sam Kim

Thesis Committee Member#2: Jong Wook Shin

Thesis Committee Member#3: Jaeho Cho

Thesis Committee Member#4: Myung Hyun Sohn

The Graduate School
Yonsei University

December 2016

ACKNOWLEDGEMENTS

I would like to express deep and sincere gratitude to Professor Moo Suk Park, M.D., Ph. D. for encouraging me with patience and careful concern. Thanks to his supervision and suggestions, I could wrap up this study.

I am deeply indebted to Professor Young Sam Kim, M.D., Ph. D. who gives the opportunity to widen my visions and guides the academic directions.

I am grateful to Professor Jong Wook Shin, Professor Jaeho Cho and Professor Myung Hyun Sohn for precious academic discussion and advices.

I wish to express special thanks to Mrs. Mi Hwa Shin for her passion for experiment and her concrete help.

Lastly, I would like to express love to my family who always plays for my side.

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

All-*trans* retinoic acid attenuates pulmonary fibrosis through inhibition of EphA2-EphrinA1 signal in bleomycin-induced pulmonary fibrosis mouse model

Ah Young Leem

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Moo Suk Park)

Background: Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, irreversible, and usually lethal lung disease of unknown pathophysiology. All-*trans* retinoic acid (ATRA) is a biologically active metabolite of vitamin A, and is known to affect cell differentiation, proliferation, and development. Eph-Ephrin signaling mediates various cellular processes including vasculogenesis, angiogenesis, cell migration, axon guidance, fluid homeostasis and repair after injury. Only a few studies about the effect of ATRA of pulmonary fibrosis have been reported. And the

relationship between pulmonary fibrosis and ephrin type-A receptor 2 (EphA2) signaling remains unclear.

Purpose: The aim of this study is to evaluation the role and related signal pathways of ATRA in the bleomycin-induced pulmonary fibrosis mouse model and whether the mechanism involves the regulation of pre-existing pathways such as and Eph-Ephrin, PI3k-Akt or Wnt/ß-catenin signaling pathway.

Methods and Materials: A549 epithelial cells and CCD-11 Lu fibroblast were incubated and stimulated with or without ATRA, TGF-β_1, and PI3K inhibitor, respectively. The levels of Smad2/3 and p-Smad2/3 were analyzed by western blotting. For animal models, we studied four experimental mice groups. There were PBS control group (PBS exposure), bleomycin + PBS group (PBS instillation after bleomycin exposure), bleomycin + ATRA group (peritoneal ATRA injection after bleomycin exposure), bleomycin + EphA2 monoclonal antibody group (EphA2 mAb instillation posttreatment after bleomycin exposure). On day 21, eight mice of each group were sacrificed. The cell counts and protein concentration in the bronchoalveolar lavage fluid (BALF), changes in histopathology, hydroxyproline assay, and the expression of several signal pathway proteins including EphA2-EphrinA1, PI3K-Akt, Wnt/ß-catenin, and cytokine levels were compared among the groups.

Results: In this study, ATRA attenuates PI3K/Akt signaling in fibroblast. We report that bleomycin exposure significantly upregulated EphA2 and EphrinA1 expression at day 21 after bleomycin exposure. ATRA posttreatment attenuated lung injury score and reduced protein concentration of BALF. The expression of EphA2, Ephrin, and PI3K 110γ protein was significantly increased after bleomycin instillation, and decreased after ATRA posttreatment. ATRA led to a decrease of IL-6 and TNF-α

production in the bleomycin + ATRA group compared to the bleomycin + PBS group (all, $P < 0.05$). Inhibiting EphA2 receptor by intranasal EphA2 mAb instillation attenuated pulmonary fibrosis, reduced cell counts and protein concentration of BALF (all, $P < 0.05$). Furthermore, bleomycin exposure upregulated the expression of Wnt5a and PI3K, and inhibiting EphA2 receptor downregulated both of them.

Conclusion: The present data suggest that ATRA attenuated bleomycin-induced pulmonary fibrosis, and that it may regulate the EphA2-EphrinA1 signaling and PI3K dependent pathway. Furthermore, EphA2 antagonism attenuates the PI3K dependent pathway and Wnt/ß-catenin-independent pathway, and decreased pulmonary fibrosis. Therefore, ATRA and inhibitor for EphA2 may have a protective effect in bleomycin-induced pulmonary fibrosis.

Key words: bleomycin, pulmonary fibrosis, All-*trans* retinoic acid, EphA2 protein, PI3K, Wnt

All-*trans* retinoic acid attenuates pulmonary fibrosis through inhibition of EphA2-EphrinA1 signal in bleomycin-induced pulmonary fibrosis mouse model

Ah Young Leem

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Moo Suk Park)

Ⅰ. INTRODUCTION

Idiopathic pulmonary fibrosis (IPF), the most common form of the idiopathic interstitial pneumonias, is a chronic, progressive, irreversible, and usually lethal lung disease of unknown cause.[1] The cause of IPF is unknown, but it appears to be a disorder likely arising from the interplay between environmental and genetic factors. It is a progressive and fatal diffuse parenchymal lung disease with unclear pathophysiology and there is no clear consensus on treatment. In 2011, the recent

American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin America Thoracic Association jointly published guidelines based on evidence-based idiopathic pulmonary fibrosis (IPF), but in the end still it did not give a clear and effective plan of treatment.[2]

All-*trans* retinoic acid (ATRA) is a biologically active metabolite of vitamin A. It is known to affect cell differentiation, proliferation, and development, and has been widely used for differentiating therapy of acute promyelocytic leukemia, with the ability to overcome promyelocytic leukemia (PML)/retinoic acid receptor (RAR) fusion protein. And, ATRA is used to ameliorate various models of autoimmunity, including rheumatoid arthritis, type 1 diabetes, and experimental encephalomyelitis.[3-5] However, the therapeutic effects of retinoic acid in such diseases remain controversial. Some studies considered that ATRA does not prevent or ameliorate lung fibrosis.[6] But several previous thought that ATRA can reduce the pulmonary fibrosis of both in irradiated and bleomycin-treated mouse lung tissues.[7-9] In these studies, ATRA ameliorates pulmonary fibrosis by regulating protein kinase C (PKC)-δ/NF-κB, p38MAPK/NF-κB, and TGF-β1/Smad3 pathways in mice model.

On the other hand, recent studies about EphA related pathway have been reported. Eph receptors constitute a large family of receptor tyrosine kinases that mediate cell-cell communication when engaged by ephrin ligands found on the surfaces of naboring cells.[10] Menges *et al.* reported that EphA2 plays an important role in tumor suppressor effects by inhibiting downstream MAP kinases and possibly also the PI3 kinase-Akt pathway.[11]

Although Eph receptors and ephrins have been studied in the context of embryonic development and tumor progression, but only a few studies about lung fibrosis have

been reported. Carpenter *et al.* reported that EphA2 regulates permeability and inflammation in the injured lung, and that EphA2 thus contributes to the pathophysiology of acute lung injury in bleomycin-induced mice model.[12] However, the previous studies were mainly focused on early-stage lung injury, not on the pulmonary fibrosis. The relationship between pulmonary fibrosis and ephrin type-A receptor 2 (EphA2) signaling remains unclear.

The aim of this study is to evaluation the role and related signal pathways of ATRA in the bleomycin-induced pulmonary fibrosis mouse model and whether the mechanism involves the regulation of pre-existing pathways such as and Eph-Ephrin, PI3k-Akt or Wnt/ß-catenin signaling pathway.

Ⅱ. MATERIALS AND METHODS

1. Cell culture

A. Human epithelial cell line

A549 epithelial cell line (American Type Culture Collection, Rockville, MD) and CCD-11Lu fibroblast cell line (American Type Culture Collection, Rockville, MD) were cultured and used for the experiment.

B. Chemicals and antibodies

TGF-β_1 was purchased from Calbiochem (San Diego, CA, USA). Three types of retinoic acid including all-*trans* retinoic acid (ATRA), 9-*cis* RA, and 13-*cis* RA were from Sigma (St. Louis, MO, USA). Primary antibodies of Smad proteins for western blot were obtained from Cell Signaling Technology (Denver, MA, USA)

and Zymed (South San Francisco, CA, USA). β-actin was used for negative control and obtained from Delta Biolabs (Gilroy, CA, USA). LY294002 (PI3K inhibitor) was purchased from Cell Signaling Technology (Beverly, MA, USA).

C. Cell culture and chemical stimulation

A549 cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL). CCD-11Lu cells were cultured EMEM medium (Beckton Dickinson & Company, Lincoln Park, NJ, USA). Cells were subcultured 2-3 times weekly to maintain a log phase growth and incubated at 37℃ with 5% CO_2 for 24 hours durations.

As a negative control, cells were incubated with only cell culture medium. To test the effects of RA on the activation of Smad induced by TGF-β_1, A549 cells were stimulated as follows: 1) control, 2) TGF-β_1, 3) RA, and 4) TGF-β_1 in combination with RA: pre-stimulated TGF-β_1 prior to RA treatment, and 5) TGF-β_1 in combination with RA: pre-treated RA prior to TGF-β_1 stimulation, respectively. After stimulation, A549 cells were incubated for 24 hours, and then processed for western blot as described below. Beside, to investigate the different effect of TGF-β_1 between A549 cells and CCD-11Lu cells, each cell was pretreated with none or PI3K inhibitor. These pretreated cells were stimulated above-mention same methed; 1) control, 2) TGF-β_1, 3) RA, and 4) TGF-β_1 in combination with RA: pre-stimulated TGF-β_1 prior to RA treatment, and 5) TGF-β_1 in combination with RA: pre-treated RA prior to TGF-β_1 stimulation, respectively.

D. Western blotting analysis

$2x10^6$cells were dissolved in 100 mm dish plates and pre-incubated 2 hours with

RPMI 1640 medium. After 24 hours with or without chemical stimulation, cells were washed twice with cold PBS, scraped and solubilized in a RIPA-B buffer containing 0.5% nonidet P-40, 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM DTT, 50 μg/ml PMSF, 20 μg/ml aprotinine, and 20 μg/ml leupeptine. After 30 minutes in 4℃, cell lysates were cleared of debris by centrifugation with 4℃, 12,000 rpm, and 20 minutes. The 20 μg of protein lysates were applied to the 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked at room temperature with 5% skim milk in TBST (1X TBS, 0.1% Tween 20). After washing twice in TBST, the membranes were incubated with the primary Smad antibodies for 2 hours at room temperature. The membranes were then washed triple times for 10 minutes in TBST, and incubated with the relevant HRP-conjugated secondary antibodies (1:1000 dilated with 3% BSA in TBST) for 1 hour. After washing triple times in TBST, the reactive proteins were visualized by an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Pittsburg, PA, USA).

2. Bleomycin-induced pulmonary fibrosis mouse model

All animal protocols were approved by the institutional animal care committee of the Medical College of Yonsei University (2014-0368). All animal experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Wild type male C57BL/6J mice, 7 weeks of age and weighing 18~22 g were purchased from Orient Bio (Sungnam, Republic of Korea). All animals were

supplied with food and water and were subjected to a similar day and night light cycle.

Thirty two mice were divided to four groups: (A) control group with PBS inhalation (n = 8), (B) bleomycin-induced pulmonary fibrosis group with PBS posttreatment (n = 8), (C) bleomycin-induced pulmonary fibrosis group with ATRA (0.5 mg) posttreatment (n = 8), (D) bleomycin-induced pulmonary fibrosis group with EphA2 monoclonal antibody (mAb) posttreatment (n = 8). Additionally, total cell count, protein concentration, and lung injury scores of bleomycin-induced pulmonary fibrosis group with ATRA (1.0 mg) posttreatment (n = 4) group was compared with the results of ATRA (0.5 mg) posttreatment group.

The experiment was performed by intranasal administration. Mice were lightly anesthetized by inhalation of isoflurane (Abbott Laboratories). The mouse was held in a supine position with the help of microsyringe (Hamilton Company cat# 7637-01). We adjusted the rate of release so as to allow the mouse to inhale the solution without trying to form bubbles.

50 μl of sterile phosphate-buffered saline (PBS) was administered by intranasal instillation for the control group. Except for the control group, bleomycin (Sigma, St Louis, MO, USA) 5 U/kg in 50 ul PBS was administered by intranasal instillation. As posttreatment, 0.5 mg of ATRA (sigma, St Louis, MO, USA) powder dissolved in 0.1 mL DMSO was injected for the bleomycin + ATRA group. Injections were repeated 3 times weekly. For the bleomycin + EphA2 mAb group, 4 μg of EphA2 mAb (R&D Systems, Cat# MAB639) were intranasally administered 3 times weekly.

On day 21, mice of each group were sacrificed. The bronchoalveolar lavage fluid (BALF) and lung specimens were collected.

A. Analysis of bronchoalveolar lavage fluid (BALF)

21 days after bleomycin/PBS inoculation, all mice were humanely euthanized by lethal overdose of xylazine. BAL was performed through a tracheal cannula using with a two 1 ml aliquots of sterile saline. The BAL fluid was centrifuged (4˚C, 1500~5000 rpm, 10 min) and the supernatant was stored at 80°C for further analysis. The cell pellet was reconstituted in 100 μl PBS and used for quantitative and qualitative cell counts. Total cell numbers were counted from each sample using a hemocytometer (Marienfield, Germany) according to the manufacturer's protocol. The slide chambers were prepared by inserting slide into frame with Poly-L lysine coating up and clamping with clips on either side. 90 μl aliquot of each sample was transferred into the slide chambers that were inserted into cytospin with the slide facing outward. Spinning was done at 600 rpm for 6 minutes. The slides were removed from cytocentrifuge and dried prior to staining. Diff Quick (Sysmex corporation) staining was used. The slides were immersed in three Diff Quickfluid (Fixative, Solution I, Solution II) for 5 seconds and rinsed with purified water.

The protein content of the BAL supernatant was measured using Coomassie Brilliant Blue G-250 technique (Quick StartTM Bradford Protein Assay, US). 25 μl of each sample and 200 μl of working reagent were pipetted into a microplate well and mixed thoroughly on a plate shaker for 30 seconds. After incubation for 30 min at 37°C, the plate was cooled and read at 595 nm by spectrophotometer.

B. Lung tissue harvest and histologic examination

The right lung was isolated and stored at -80°C prior to protein extraction, after flushing the pulmonary vasculature with saline under low pressure. The left lung was inflated via the tracheotomy with low-melting point agarose (4%) in PBS at 25

cm H_2O pressure and until the pleural margins became sharp. The lungs were then excised and fixed overnight in 10% formaldehyde in PBS and embedded in paraffin for sectioning at 5-μm thickness. Left lung sections were stained with H&E and Masson subjectively evaluated under light microscopy. The histopathology was reviewed in a blinded manner by two qualified investigators. Five easily identifiable pathologic processes were scored by a weighted scale presented in the official ATS workshop report.[13]

C. Hydroxyproline assay

The total collagen content of the left lung was measured using a colorimetric assay to determine lung hydroxyproline content. Every 10 mg of lung tissue was homogenized in 100 μL of distilled water. After that, 100 μL of 12 N hydrochloric acid was added, and we hydrolyzed the sample for 3 hours at 120°C. After cooling briefly, 5 mg of activated charcoal was added. Then, the sample was centrifuged at 10,000 x g for 5 minutes at 4 ˚C. The supernatant was collected and transferred to a new tube. Hydroxyproline assay kit (Cell biopabs, US) was used for the analysis. The results were calculated as μg hydroxyproline per mg.

D. Western blotting and cytokine ELISA

Frozen right lung were mechanically disrupted using a homogenizer in homogenization buffer, PRO-PREPTM Extraction solution (iNtRON BIOTECHNOLOGY cat# 17081). The amount of solution is 600 μl per 10mg tissue. Cell lysis was induced by incubation for 20-30 min on ice or freezer at -20˚C. The samples were centrifuged at 13,000 x g for 30 min at 4˚C. Equal amounts of protein

were separated by SDS-PAGE and transferred to nitrocellulose membrane before immunoblotting with primary Abs as indicated. Membranes were incubated with anti-rabbit or anti-mouse IgG HRP conjugated Abs and developed using Super-Signal West Pico chemiluminescence detection kit (Pierce). The band images were quantified by Alpha Ease FC software (Innotech, version 4.1.0).

The antibodies used in this study included rabbit EphA2 (Thermo Fisher Scientific, Germany), goat EphrinA1 (Thermo Fisher Scientific, Germany), rabbit PI3 Kinase 110γ (Cell Signaling Technologies, Beverly, MA), rabbit α-tubulin (Cell Signaling Technologies, Beverly, MA), rabbit phosphate–p70 S6 kinase (Cell Signaling Technologies, Beverly, MA), rabbit p70 S6 kinase (Cell Signaling Technologies, Beverly, MA), rabbit Wnt5a (Cell Signaling Technologies, Beverly, MA). The image densities were measured with NIH Image J for semiquantitative comparison.

Measurement of keratinocyte chemoattractant (KC/CXCL1), macrophage inflammatory protein 2 (MIP-2), interleukin (IL)-1ß, IL-6, and tumor necrosis factor-α (TNF-α) were performed on whole-lung homogenates using quantified EILSA kits (MILLIPLEXMAG Mouse Cytokine/Chemokine kit from Millipore, Billerica, MA, USA).

3. Statistical analysis

Statistical analysis was performed using Prism 5.0 (Graphpad Software, Durham, NC). The group comparisons were performed with an unpaired student t test or ANOVA with Bonferroni multiple comparisons tests. Data are expressed as means ± SD for each group. Differences were considered significant at $P < 0.05$.

III. RESULTS

1. Fibroblast growth patterns differ from the epithelial cell when treated with ATRA at suppression of PI3K signaling

Fibroblast growth differs compared with epithelial cells when treated with ATRA at 10^{-6} M and / or suppression of PI3K signaling with its specific inhitbitor LY294002. Both in epithelial cell and fibroblast, relative expression of p-Smad2 and p-Smad3 were increased with TGF-β alone. Compared with TGF-β alone group, relative expression of p-Smad2 and p-Smad3 were decreased significantly with TGF-β and ATRA (Figure 1, 2). Relative expression of p-Smad2 and p-Smad3 in TGF-β alone group were decreased when treated with PI3K inhibitor (LY294002) in epithelial cell (Figure 1). However, it was not in fibroblast. (Figure 2)

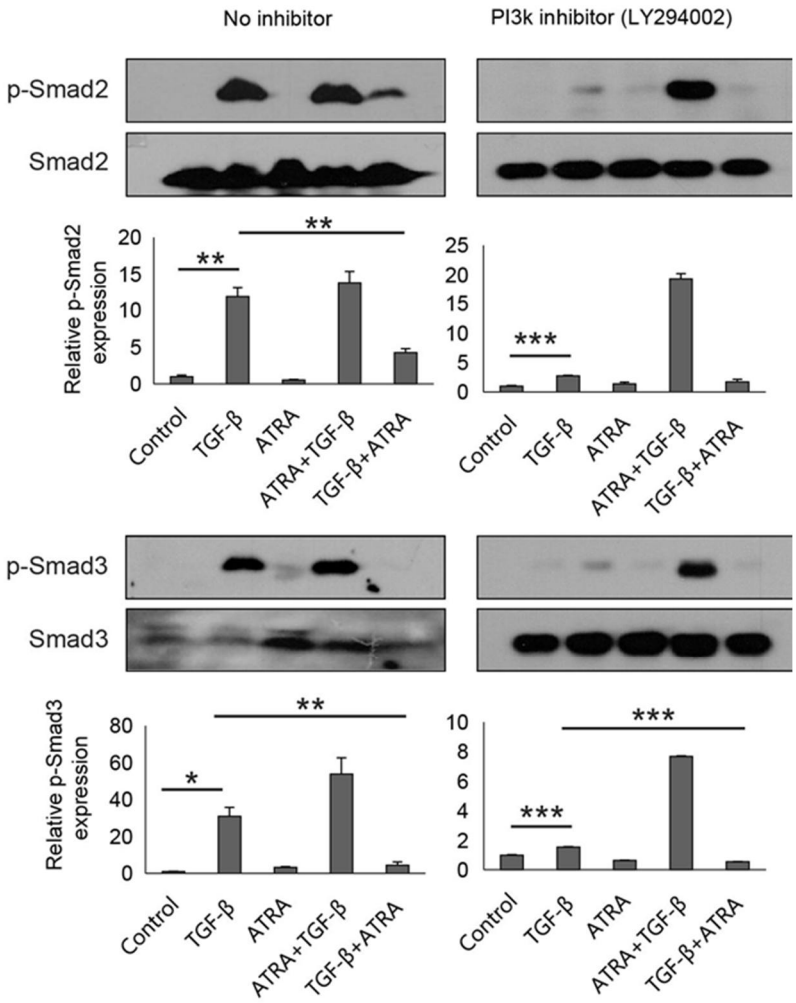


Figure 1. The expression of p-Smad2 and p-Smad3 with or without PI3K inhibitor (LY294002) in epithelial cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

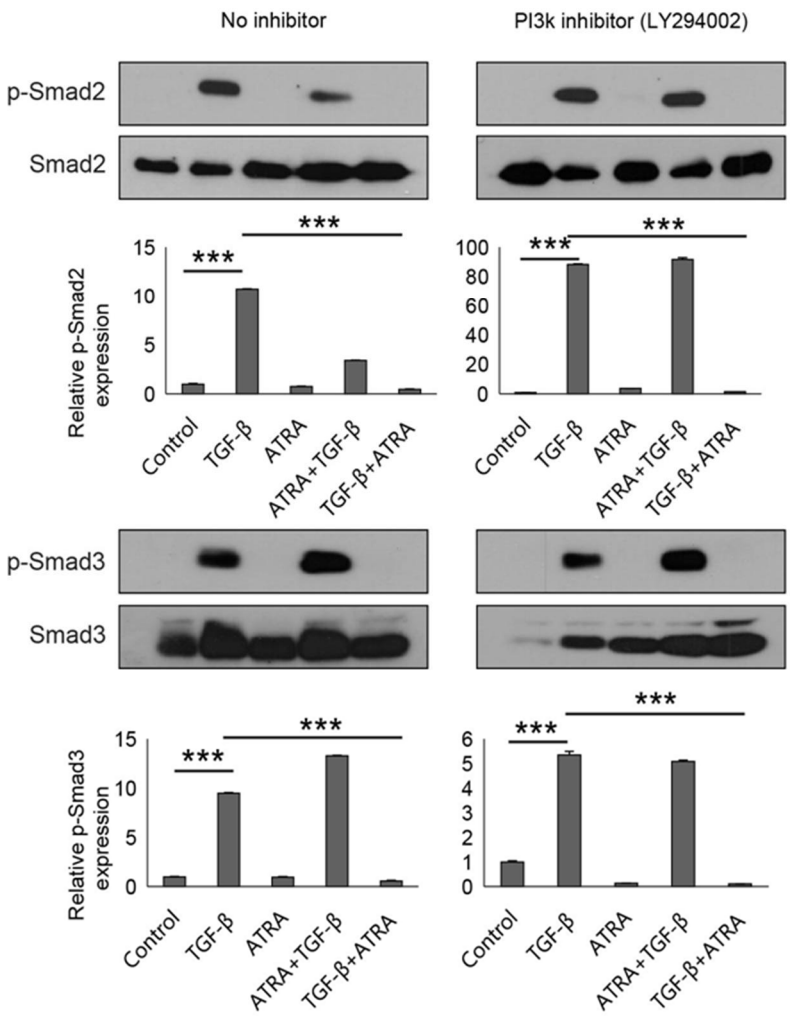


Figure 2. The expression of p-Smad2 and p-Smad3 with or without PI3K inhibitor (LY294002) in fibroblast. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

2. ATRA attenuates PI3K/Akt signaling in fibroblast

Fibroblast growth differs compared with epithelial cells when treated with ATRA at 10^{-6} M. In epithelial cell, relative expression of p-Akt was not significantly

decreased with TGF-β and ATRA group compared with TGF-β alone group. However, it was significantly decreased with TGF-β and ATRA group compared with TGF-β alone group (Figure 3).

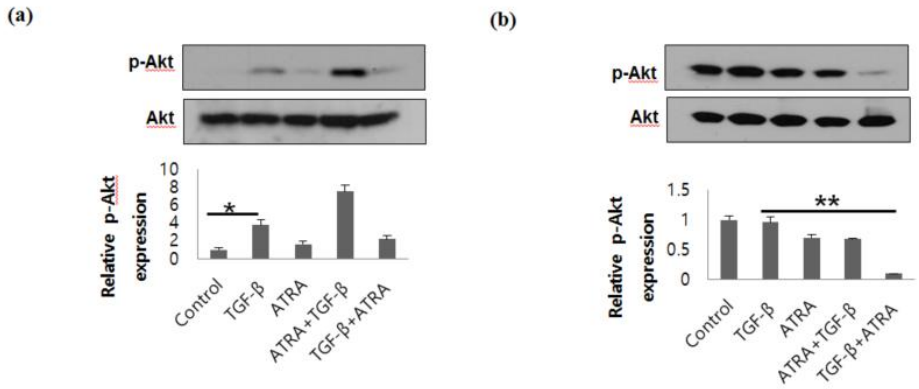


Figure 3. The expression of p-Akt with in (a) epithelial cell and (b) fibroblast. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. ATRA dose-dependently attenuates bleomycin-induced lung injury

We sought to determine whether ATRA dose dependently attenuates lung injury in bleomycin-induced pulmonary fibrosis mouse model.

As shown in Figure 4, bleomycin caused pulmonary fibrosis, as demonstrated by a significant increase in concentration of total protein (PBS control group: 0.09 ± 0.01 mg/ml, bleomycin + PBS group: 1.19 ± 0.27 mg/ml, all $P < 0.05$) and total cell count (PBS control group: 58.63 x 104 ± 13.42 x 104, bleomycin + PBS group: 384.10 x 104 ± 76.98 x 104, all P < 0.05) in BALF. Furthermore, histologic examination of the lungs of mice that received ATRA in addition to bleomycin instillation revealed a significant decrease in lung injury score compared with mice that received bleomycin and PBS posttreatment (bleomycin + PBS group: 70.50 ±

3.56, bleomycin + ATRA 0.5 mg group: 38.58 ± 3.91, bleomycin + ATRA 1.0 mg group: 29.45 ± 2.14 at day 21, all $P < 0.05$). ATRA posttreatment (0.5 mg and 1 mg) decreased in concentration of total protein in BALF compared with PBS control group. Compared with ATRA 0.5 mg group, total cell count was significantly decreased in ATRA 1 mg group. However, total protein and lung injury score were not significantly decreased in ATRA 1 mg group, compared with ATRA 0.5 mg group (Figure 4).

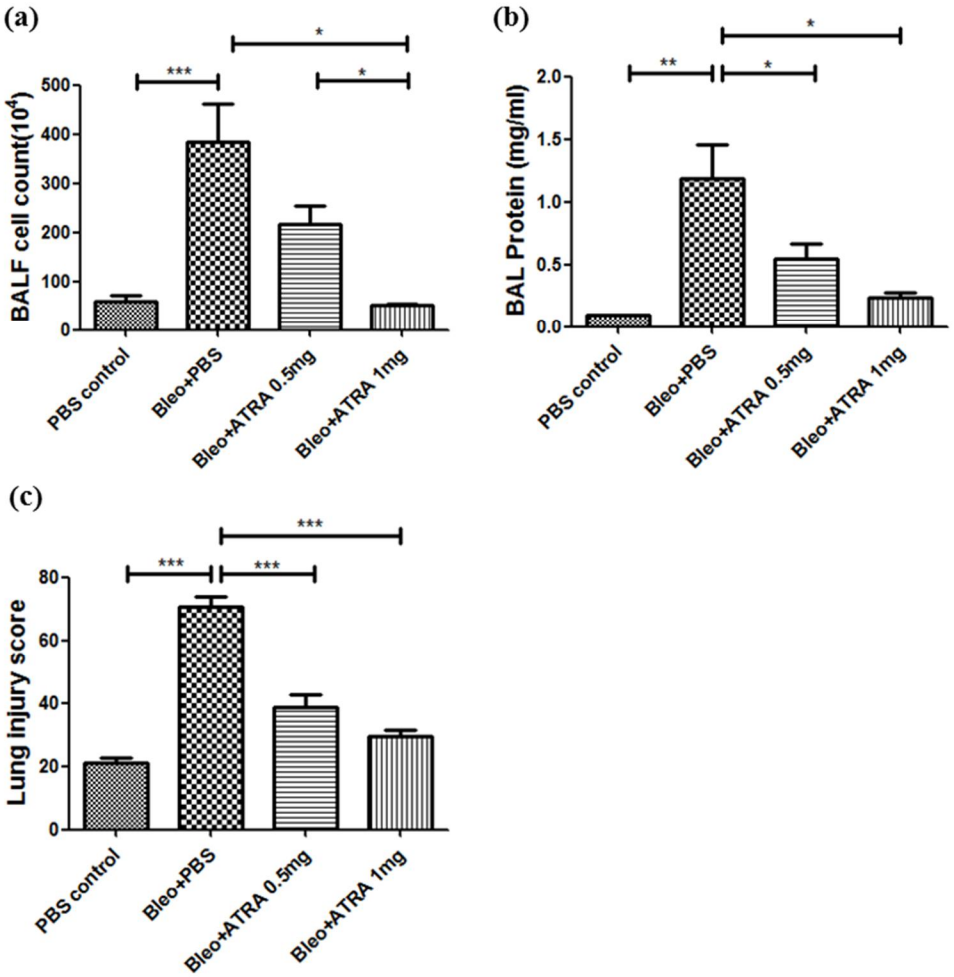


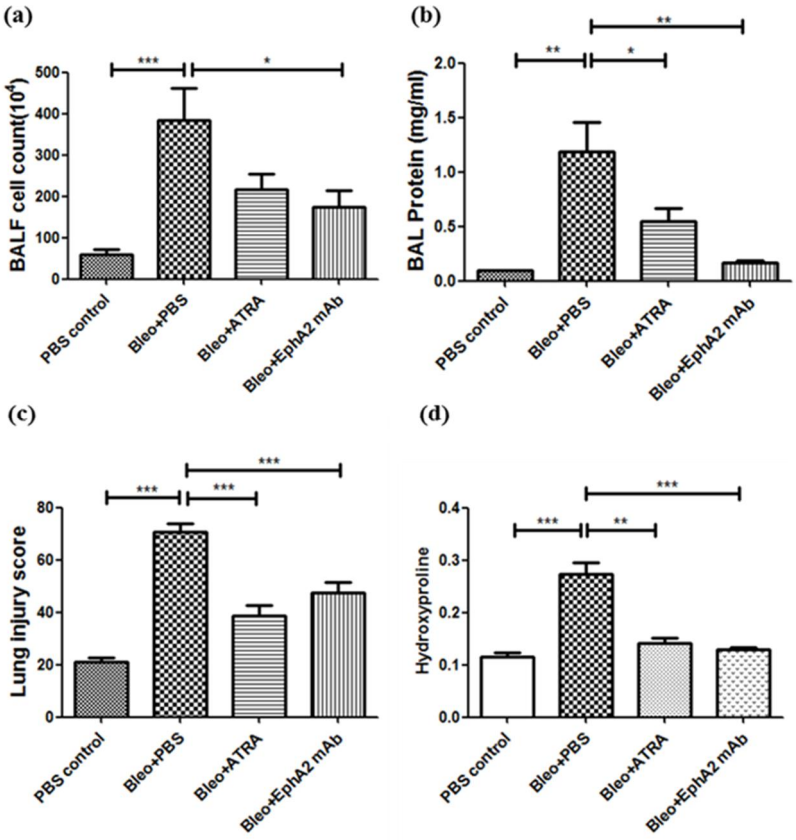
Figure 4. ATRA attenuates bleomycin-induced bleomycin-induced lung injury dose-independently at 21 days. (a) Total bronchoalveolar lavage fluid (BALF) cell counts. (b) Total BALF protein concentration. (c) The lung injury scores. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

4. ATRA and EphA2 antagonist attenuate bleomycin-induced lung injury and fibrosis

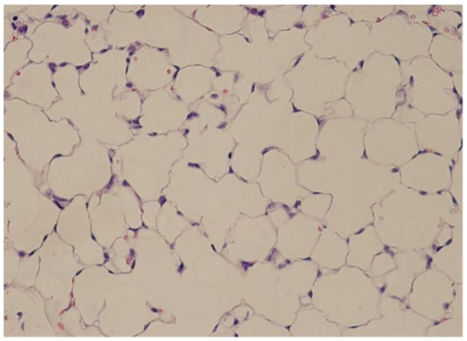
After finding that bleomycin-induced lung injury was associated with the increased expression of EphA2 and Ephrin A1 in the lung, we sougt to determine whether EphA2 contributes to pulmonary fibrosis by verifying the effects of EphA2 mAb posttreatment.

As shown in Figure 2, bleomycin caused pulmonary fibrosis, as demonstrated by a significant increase in concentration of total protein and total cell count in BALF. ATRA posttreatment decreased in concentration of total protein in BALF compared with PBS control group. EphA2 mAb posttreatment resulted in significant reduction in concentration of both total protein and total cell counts in BALF compared with PBS posttreatment after bleomycin instillation. Also, histologic examination of the lungs of mice that received ATRA and EphA2 mAb in addition to bleomycin instillation revealed a significant decrease in lung injury score compared with mice that received bleomycin and PBS posttreatment (bleomycin + PBS group: 70.50 ± 3.56, bleomycin + ATRA group: 38.58 ± 3.91, bleomycin + EphA2 mAb group: 47.64 ± 3.68 at day 21, all $P < 0.05$). The histologic changes are shown by hematoxylin and eosin and Masson staining. Pulmonary structure was clear, and inflammatory cells and collagen deposition did not exist in pulmonary alveoli in the

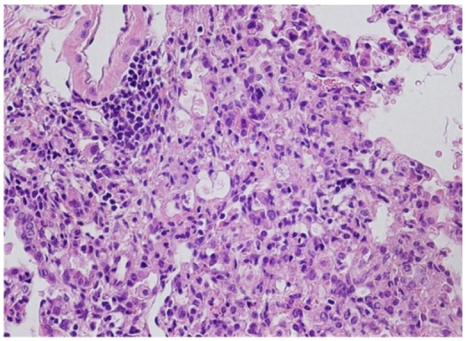
PBS control group. Hematoxylin and eosin staining showed the structure of lung tissue disordered, pulmonary interalveolar septa thickened and infiltrated by inflammatory cells, with collagen depositions in the interstitium, and a large number of alveolar collapsed and consolidated in the bleomycin group. Masson staining showed that fibroblasts and collagen matrix significantly increased, and the formation of fibrosis lesions with cord-like distribution in bleomycin group. Alveolitis and the pulmonary fibrosis were reduced in the ATRA group and EphA2 mAb group as compared with the bleomycin group. Furthermore, in hydroxyproline assay, both ATRA and EphA2 mAb decreased hydroxyproline content compared with PBS posttreatment after bleomycin instillation.



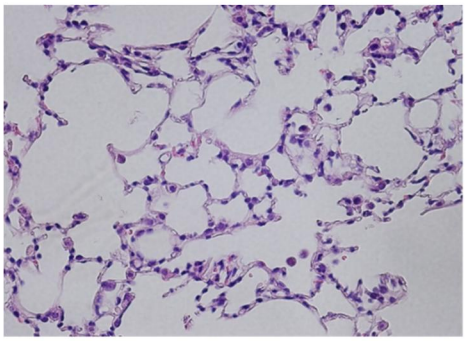
(e)



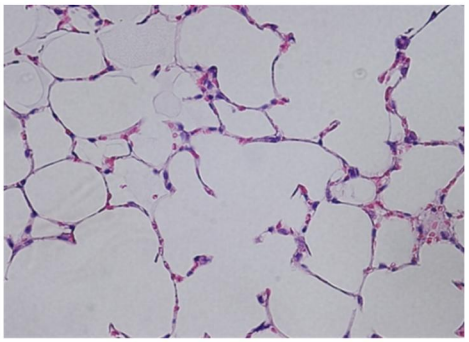
PBS Control (x400)



Bleo + PBS (x400)



Bleo + ATRA (x400)



Bleo + EphA2 mAb (x400)

(f)

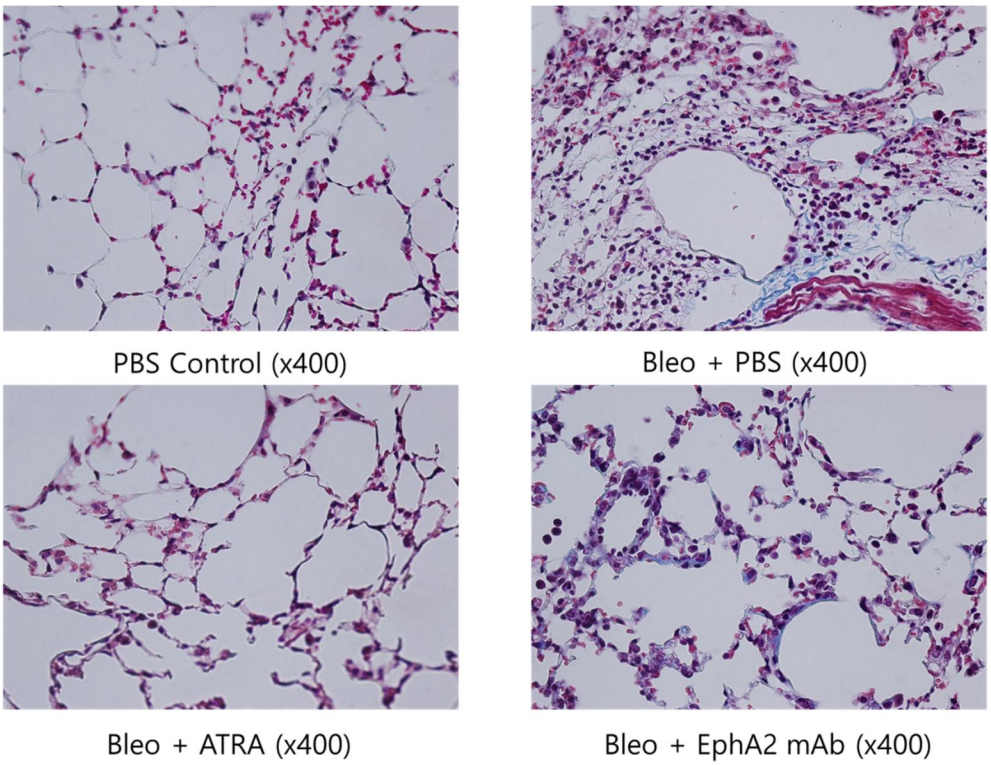


Figure 5. ATRA and EphA2 mAb attenuate bleomycin-induced pulmonary fibrosis at 21 days. (a) Total bronchoalveolar lavage fluid (BALF) cell counts. (b) Total BALF protein concentration. (c) The lung injury scores. (d) hydroxyproline assay. (e) The histologic changes are shown by hematoxylin and eosin staining. (f) The histologic changes are shown by Masson staining. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

5. Bleomycin upregulates EphA2 receptor and its ligand Ephrin A1 expression

As, measured by western blotting, the expression of EphA2 protein and EphrinA1 protein in lung tissue was increased after bleomycin instillation (EphA2: 1.38-fold,

EphrinA1: 1.21-fold), compared with PBS control at day 21 (Figure 1). Furthermore, increased expression of EphA2 and EphrinA1 in the lung were inhibited by ATRA posttreatment (Figure 6).

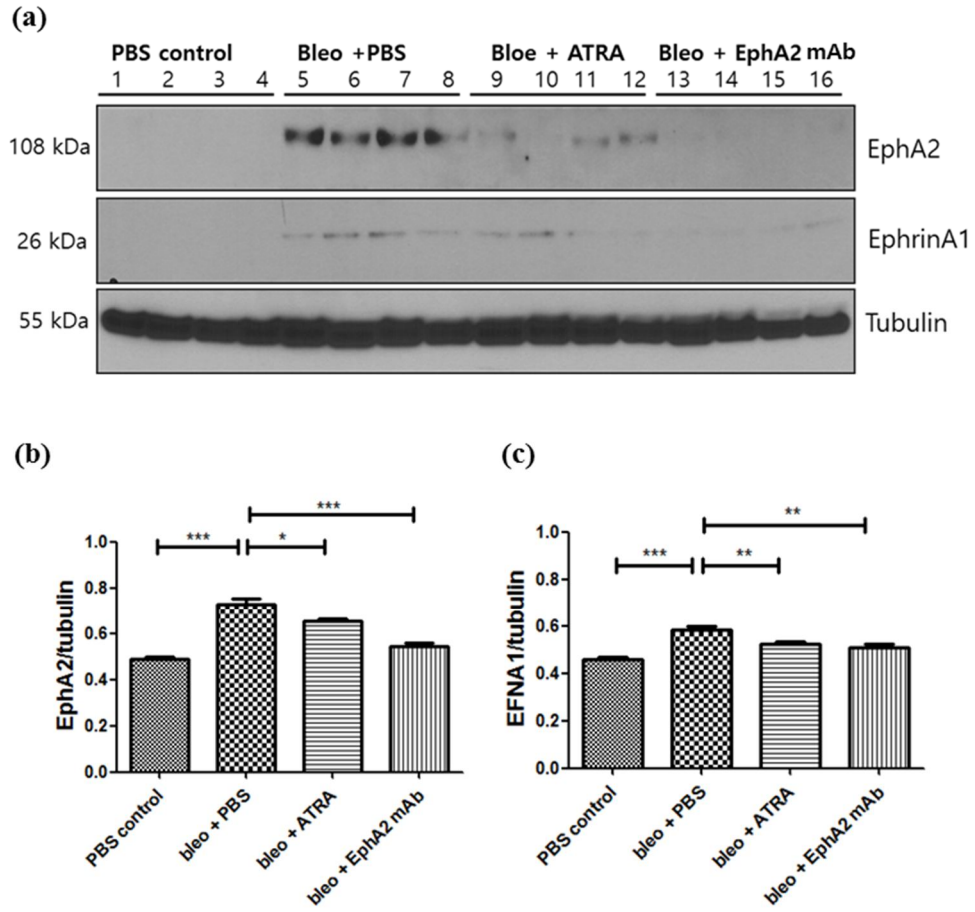


Figure 6-1. The expression of EphA2 and EphrinA1 protein in lung lysates, as shown by Western blots and densitometry. (a) western blots. (b) densitometry of EphA2. (c) densitometry of EphrinA1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

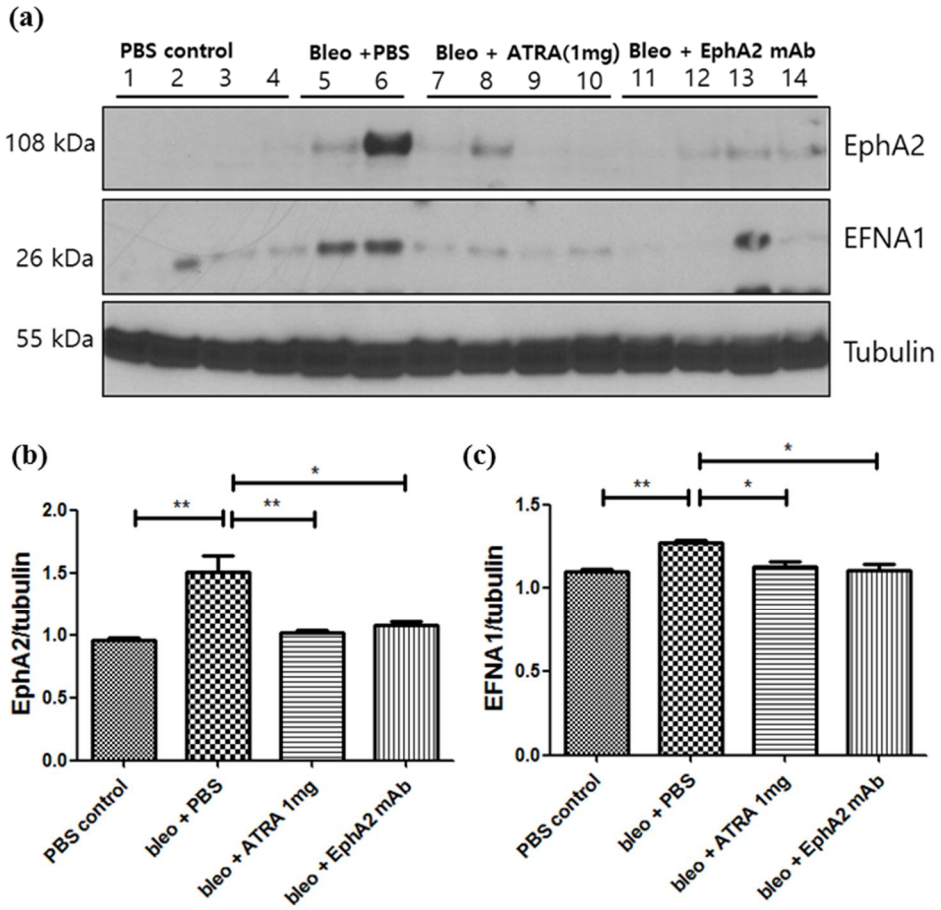


Figure 6-2. The expression of EphA2 and EphrinA1 protein in lung lysates, as shown by Western blots and densitometry (ATRA 1mg posttreatment). (a) western blots. (b) densitometry of EphA2. (c) densitometry of EphrinA1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

6. ATRA attenuates bleomycin-induced pulmonary fibrosis via downregulating EphA2-EphrinA1 and PI3K-Akt pathway

As, mentioned above, measured by western blotting, increased expression of EphA2 protein and EphrinA1 protein in lung tissue was increased after bleomycin

instillation was decreased by ATRA treatment (Figure 6).

Given the protective effect of ATRA in bleomycin induced lung injury, we wondered whether the mechanism involves the regulation of PI3K dependent signaling. To answer this question, the expression of PI3K 110γ was measured in all groups.

As shown in Figure 7-1, compared to the PBS control group, the bleomycin + PBS group showed significantly increased PI3K 110γ (PI3K 110: 1.85-fold). After ATRA posttreatment (0.5mg), the expression of PI3K 110γ was reduced. Futhermore, ther expression of Akt was significantly decreased after ATRA posttreatment (1.0mg) (Figure 7-2). It is showing that PI3K-Akt signaling is involved in bleomycin-induced pulmonary fibrosis. ATRA attenuated bleomycin-induced pulmonary fibrosis via regulation of PI3K-Akt pathway.

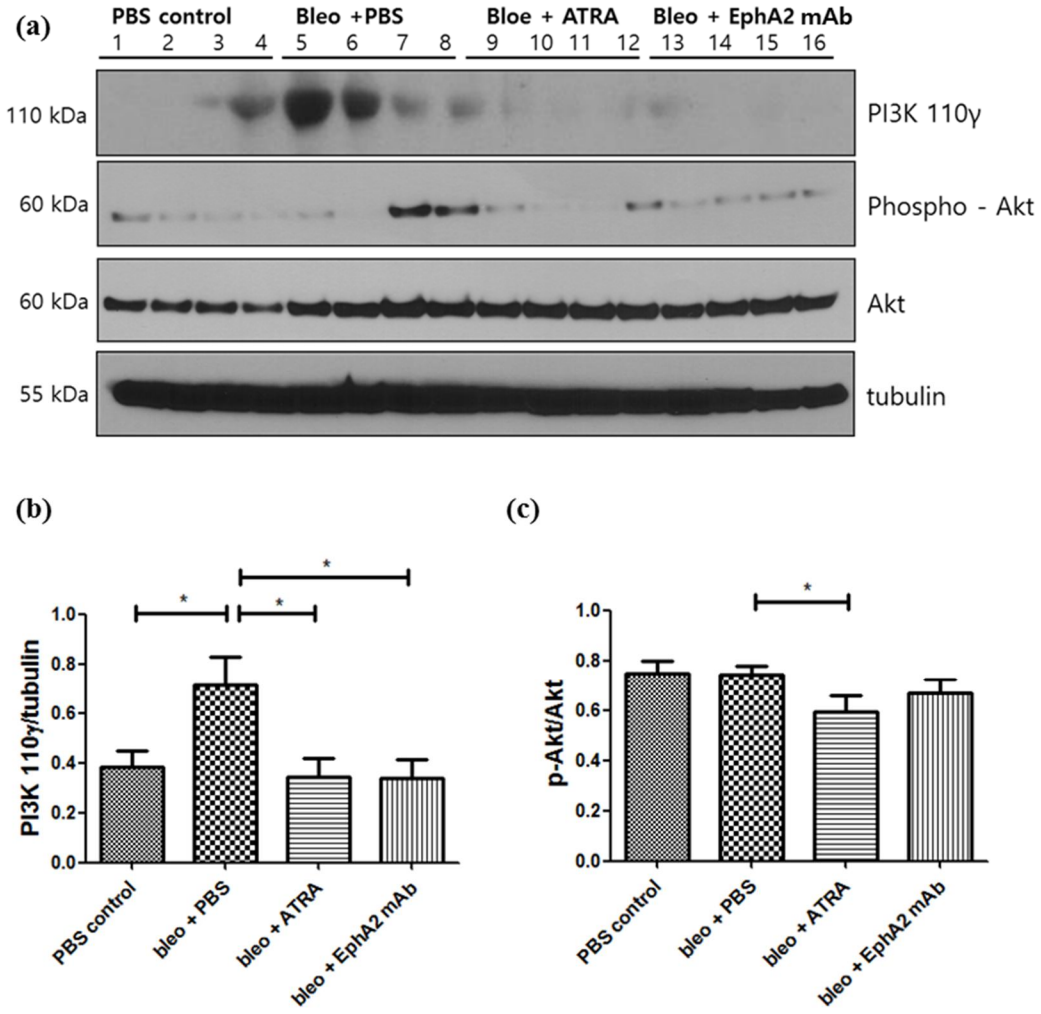


Figure 7-1. The expression of PI3K 110γ protein in lung lysates, as shown by (a) Western blots. (b) densitometry of PI3K 110 γ. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

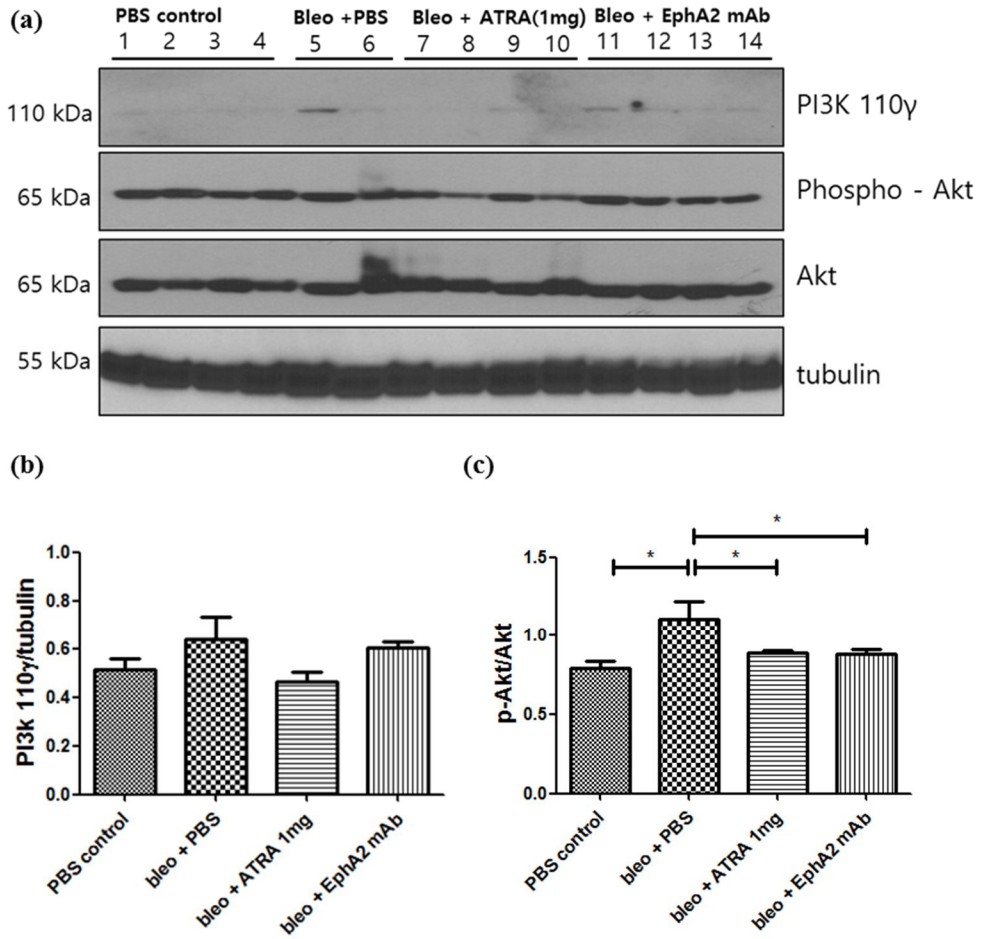


Figure 7-2. The expression of PI3K 110γ protein in lung lysates, as shown by (a) Western blots. (b) densitometry of PI3K 110 γ (ATRA 1mg posttreatment). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

7. Bleomycin upregulates Wnt and PI3K signaling pathway via EphA2 activation.

After finding that bleomycin-induced lung injury was decreased by EphA2 antagonism, we wondered whether inhibiting the EphA2-EphrinA1 signaling had an effect on the existing signal pathways. To answer this question, the expression of

various proteins in the lung tissue was measured in PBS group, bleomycin + PBS group, bleomycin + ATRA group, and bleomycin + EphA2 mAb group.

As shown in Figure 3, compared with the PBS control group, the bleomycin + PBS group showed significantly increased PI3K 110γ. After ATRA posttreatment, the expression of PI3K 110γ was reduced. EphA2 mAb seemed to attenuate bleomycin-induced pulmonary fibrosis via regulation of PI3K dependent step.

As shown in Figure 8, compared with the PBS control group, the bleomycin + PBS group showed significantly increased Wnt5a. However, β-catenin protein was not expressed in the bleomycin group. After EphA2 mAb posttreatment, the expression of Wnt5a was reduced significantly, showing that EphA2 signaling is involved in bleomycin-induced activation of Wnt/β-catenin-independent signaling pathway.

Inhibiting the EphA2-EphrinA1 signaling had no significant effect on expression of the proteins related to other signaling pathways, such as phosphate-S6K (data not shown).

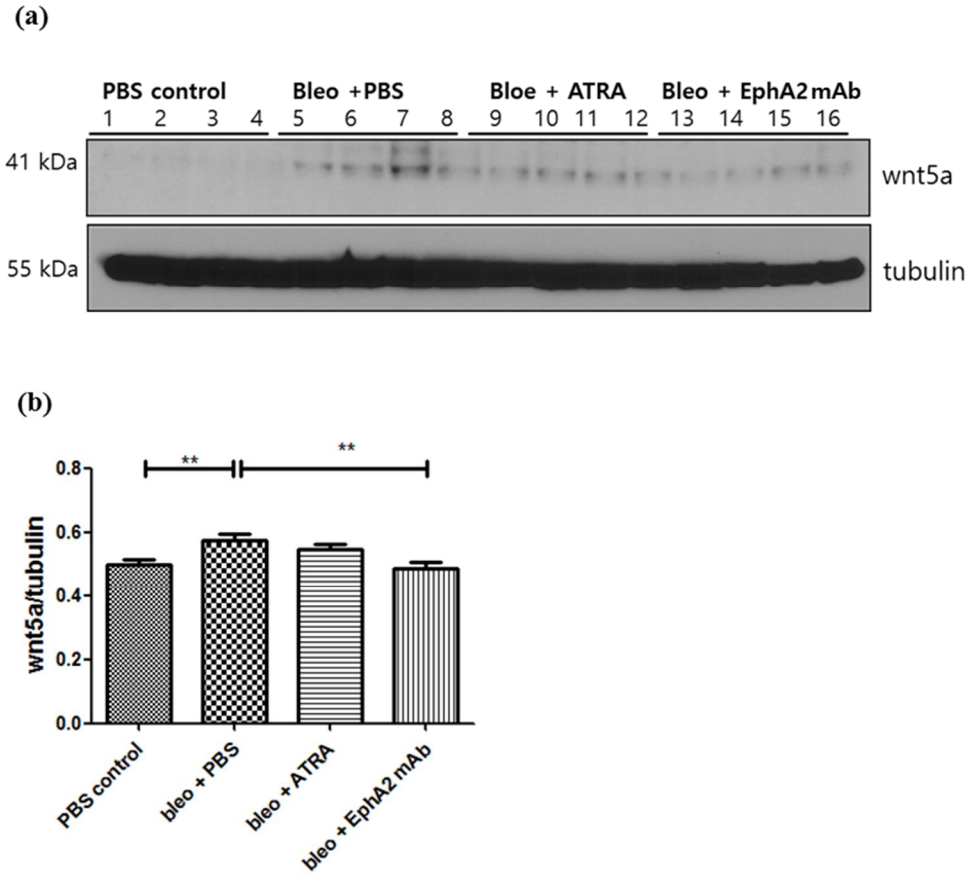
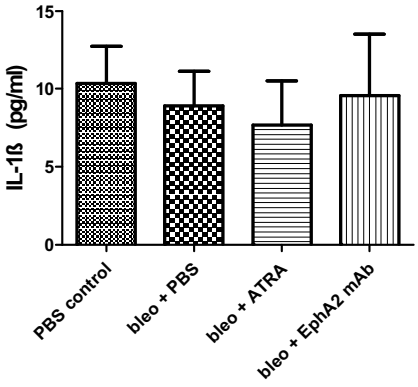


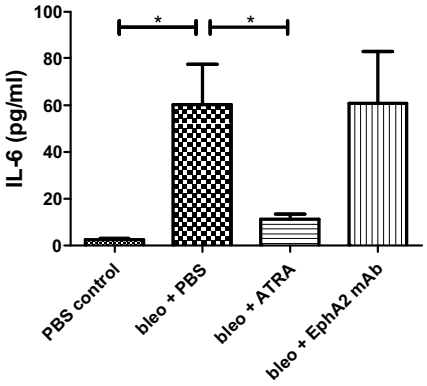
Figure 8. The expression of Wnt5a protein in lung lysates, as shown by (a) Western blots and (b) densitometry. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

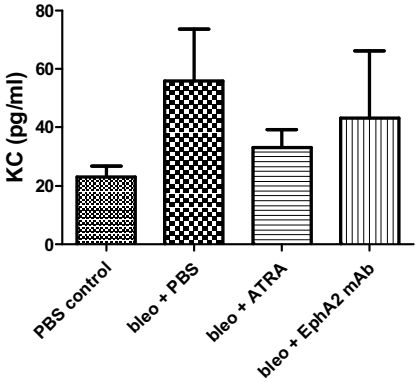
8. ATRA attenuates bleomycin-induced pulmonary fibrosis via decreasing IL-6 and TNF-α

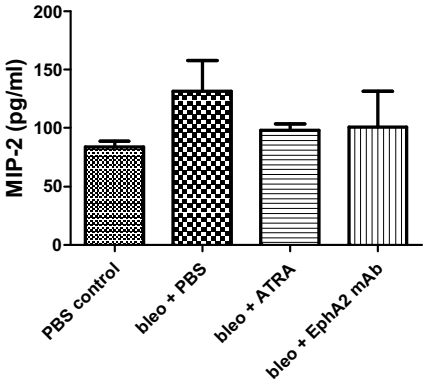
To determine whether ATRA and EphA2 mAb have a protective function in inflammatory response, we assessed the cytokine levels (IL-1ß, IL-6, KC, MIP-2, and TNF-α) at day 21 by ELISA. IL-6 and TNF-α expression were significantly

increased in bleomycin-induced pulmonary fibrosis. ATRA led to a decrease of IL-6 and TNF-α production in the bleomycin + ATRA group compared to the bleomycin + PBS group (Figure 9).









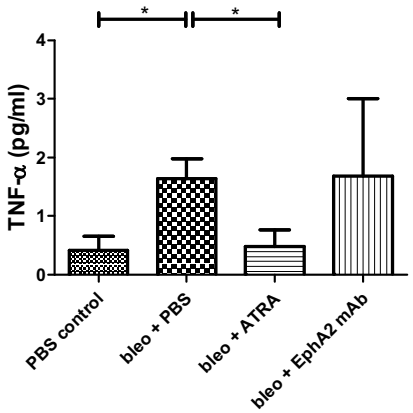


Figure 9. The expression of cytokines (IL-1ß, IL-6, KC, MIP-2, and TNF-α) in response to ATRA and EphA2 mAb pretreatment in a bleomycin-induced lung fibrosis model.

IV. DISCUSSION

Although pulmonary fibrosis accounts for significant morbidity and mortality in critically ill patients, there are no specific effective therapies. So, it is important to find a therapeutic target and verify the effectiveness of related treatment in pulmonary fibrosis.

In this study, both of ATRA and EphA2 contributed to the pathogenesis in bleomycin-induced pulmonary fibrosis of mice. ATRA attenuated bleomycin-induced pulmonary fibrosis. The expression of EphA2, EphrinA1, and PI3K protein was significantly increased after bleomycin instillation, and decreased after ATRA posttreatment. Both the expressions of EphA2 receptor and the ligand EphrinA1 were increased in the bleomycin-induced pulmonary fibrosis. Also, blocking the activation of EphA2 receptor by EphA2 mAb ameliorates fibrotic changes associated with lung injury. Furthermore, bleomycin exposure upregulated the expression of Wnt5a and PI3K, and inhibiting EphA2 receptor downregulated both of them. In the cytokine assay, ATRA led to a decrease of IL-6 and TNF-α production.

The results of ATRA posttreatment group in this study were similar to previous studies of mice model. In the study of Tabata *et al.* ATRA could reduce the pulmonary fibrosis of both in irradiated and bleomycin-treated mouse lung tissues.[7]

They found that ATRA reduced irradiation-induced IL-6 production through the protein kinase C (PKC)-δ/NF-κB pathway, and inhibited irradiation-induced TGF-β1 production through the p38MAPK/NF-κB pathway, resulting in the inhibition of cell differentiation and collagen synthesis. In another study of Tabata *et al.*, ATRA reduced irradiation-induced production of both IL-6 receptor (IL-6R) and its receptors in the lung fibroblasts and IL-6-dependent cell growth, and also directly inhibited the proliferation of lung fibroblasts after irradiation as well as anti-human IL-6R antibodies.[14] Dong *et al*. reported that ATRA may ease the bleomycin-induced pulmonary fibrosis by inhibiting the expression of IL-6 and TGF-β, shifting the regulatory T/T-helper 17 (Treg/Th17) ratio and reducing the secretion of IL-17A in mice model.[8] The study of Song *et al*. showed that ATRA ameliorates bleomycin-induced lung fibrosis by downregulating the TGF-β1/Smad3 signaling pathway.[9] Consistent with these studies, ATRA decreased bleomycin-induced pulmonary fibrosis in the current study. Similar to the results of Tabata *et al*.[14], ATRA reduced IL-6 expression of lung. Compared to previous researches which studied mice model of 4 weeks after the administration of bleomycin, the sacrifice time (3 weeks) of this study was earlier than in previous studies. Furthermore, this study suggest that ATRA attenuated bleomycin-induced pulmonary fibrosis via regulate the EphA2-EphrinA1 signaling and PI3K dependent pathway.

EphA2 signaling has been studied in angiogenesis, cell migration, fluid homeostasis and vascular assembly during early stages of development.[10,15] The interaction between Eph receptor and Ephrin ligand results in modification of cytoskeletal proteins and cell surface receptors.[10,15] The previous studies found that actin cytoskeleton rearrangement may be a key preceding event during the regulation of inflammatory responses in various cell populations.[16-19] The disruption of

endothelial cell junction by EphA2 signaling, allows the passage of fluid, protein and inflammatory cells into an injured tissue.[20,21] The various evidences suggest that EphA2 signaling may an important mediator in inflammation and injury.[12,22-24]

There are several studies demonstrate that EphA2 contributes to the pathogenesis in acute lung injury. In rats exposed to viral respiratory infection and hypoxia, EphA2 expression is markedly upregulated and EphA2 antagonism reduced vascular leakage in injured lung injury.[23] Similarly, in bleomycin-induced lung injury, EphA2 KO mice were protected from protein extravasation and inflammatory responses.[12] Both studies demonstrated that ligand EphrinA1 stimulation of lung endothelial EphA2 receptor leads to disruption of endothelial adherens junctions and increased permeability. Recently, Hong *et al*. reported that EphA2 and EphrinA1 were upregulated in lipopolysaccharide (LPS)-induced lung injury, and EphA2 antagonism inhibits the PI3K-Akt pathway and attenuates inflammation.[25] However, these previous studies were about acute lung injury, not about the pulmonary fibrosis model. In this study, the results of EphA2 mAb posttreatment groups were similar to previous studies in acute lung injury mice model. In our knowledge, this is the first study which investigates the role of EphA2-EphrinA1 signaling pathway in the pulmonary fibrosis mice model.

We found several potential mechanisms by ATRA and EphA2 signaling which can be related to the development of bleomycin-induced pulmonary fibrosis (Figure 10).

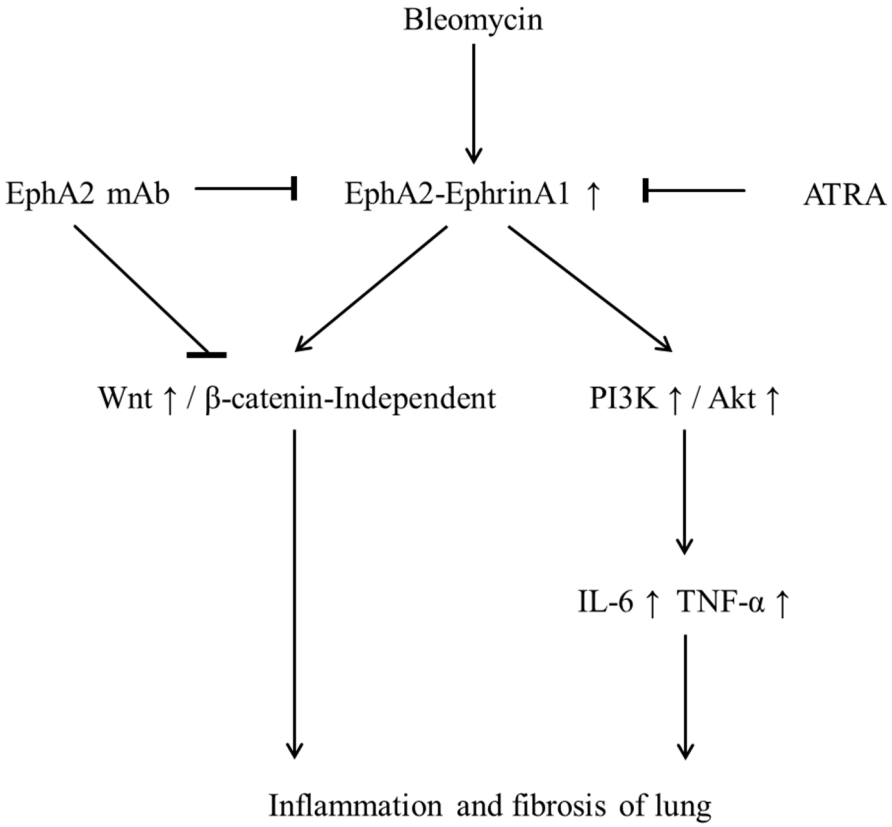


Figure 10. The potential mechanisms through which ATRA and EphA2 mAb may attenuate development of bleomycin-induced pulmonary fibrosis.

In this study, blocking EphA2 expression with EphA2 mAb posttreatment after bleomycin exposure downregulated the expression of PI3K and Wnt5a compared with PBS posttreatment after bleomycin exposure. ATRA treatment decreased the expression of EphA2, EphrinA1, and PI3K. The results showed bleomycin induced upregulation of PI3K pathway and Wnt/β-catenin-independent pathway were mediated through EphA2-EphrinA1 signaling pathway.

PI3K-Akt signaling pathway has been reported to have an important role in

regulating cell proliferation. Bakin *et al.* reported that PI3K-Akt activation was essential to the epithelial mesenchymal transition (EMT) process induced by TGF-β1 in tumor cell lines.[26] Hofmann *et al.* used TGF-β to stimulate primary hepatocytes in mice and demonstrated induction of the EMT and the activation of the PI3K-Akt signaling pathway.[27] The study of Conte *et el.* reported that the overexpression of PI3K p110γ was observed in IPF lung disuse and fibroblast cells.[28] Similar to previous studies, the expression of PI3K was increased after bleomycin exposure, and decreased with both of ATRA and EphA2 mAb treatment.

At the biochemical level, the EMT associated with gastrulation is dependent on and orchestrated by canonical Wnt signaling.[29,30] In previous studies, the Wnt/β-catenin signaling pathway has been implicated in the development of experimental and human pulmonary fibrosis.[31-33] The cell-specific mechanism by which Wnt/β-catenin signaling exerts its effect on cellular function remains unclear. However, crosstalk between TGF-β and Wnt/β-catenin signaling has recently been demonstrated as crucial for the EMT and the development of lung fibrosis.[34-36] Chen *et al.* reported that inhibiting Wnt/β-catenin signaling could suppress the production of the pro-fibrotic cytokines transforming growth factor-β1 (TGF-β1) and fibroblast growth factor 2 (FGF-2) in bleomycin-induced fibrotic lung tissue in mice model.[35] In this study, the expression of Wnt5a protein was increased with bleomycin treatment. However, β-catenin protein was not expressed in the bleomycin group. After EphA2 mAb posttreatment, the expression of Wnt5a was reduced significantly, showing that EphA2 signaling is involved in bleomycin-induced activation of Wnt/β-catenin-independent signaling pathway.

Previous studies which investigated the significance of EphA2 expression and epithelial-mesenchymal transition through the Wnt/β-catenin pathway in gastric

cancer cells have been reported.[37] However, there was no study about the relationship between EphA2 signaling and Wnt/β-catenin-independent pathway in pulmonary fibrosis model.[38] Our study provides evidence that bleomycin upregulates Wnt/ß-catenin-independent signaling pathway via EphA2 activation.

In this study, we demonstrated that ATRA attenuated bleomycin-induced pulmonary fibrosis in mice model, and it may regulate the EphA2-EphrinA1 signaling and PI3K dependent pathway. The possible role of ATRA and EphA2 inhibitor as a therapeutic agent in pulmonary fibrosis should be verified in human studies.

V. CONCLUSION

In conclusion, we demonstrate that ATRA attenuated bleomycin-induced pulmonary fibrosis, and that it may regulate the EphA2-EphrinA1 signaling and PI3K dependent pathway. Furthermore, EphA2 antagonism attenuates inhibits the PI3K dependent pathway and Wnt/ß-catenin-independent pathway and attenuates pulmonary fibrosis. Therefore, ATRA and inhibitor for EphA2 may have a protective effect in bleomycin-induced pulmonary fibrosis.

REFERENCES

1. King TE, Jr., Pardo A, Selman M. Idiopathic pulmonary fibrosis. Lancet 2011;378:1949-61.
2. Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, et al. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. Am J Respir Crit Care Med 2011;183:788-824.
3. Miyagawa N, Homma T, Kagechika H, Shudo K, Nagai H. Effect of synthetic retinoid, TAC-101, on experimental autoimmune disease. Pharmacology 2003;67:21-31.
4. Zunino SJ, Storms DH, Stephensen CB. Diets rich in polyphenols and vitamin A inhibit the development of type I autoimmune diabetes in nonobese diabetic mice. J Nutr 2007;137:1216-21.
5. Racke MK, Burnett D, Pak SH, Albert PS, Cannella B, Raine CS, et al. Retinoid treatment of experimental allergic encephalomyelitis. IL-4 production correlates with improved disease course. J Immunol 1995;154:450-8.
6. Segel MJ, Or R, Tzurel A, Lucey EC, Goldstein RH, Izbicki G, et al. All-trans-retinoic acid (ATRA) is of no benefit in bleomycin-induced lung injury. Pulm Pharmacol Ther 2001;14:403-7.
7. Tabata C, Kadokawa Y, Tabata R, Takahashi M, Okoshi K, Sakai Y, et al. All-trans-retinoic acid prevents radiation- or bleomycin-induced pulmonary fibrosis. Am J Respir Crit Care Med 2006;174:1352-60.

8. Dong Z, Tai W, Yang Y, Zhang T, Li Y, Chai Y, et al. The role of all-trans retinoic acid in bleomycin-induced pulmonary fibrosis in mice. Exp Lung Res 2012;38:82-9.

9. Song X, Liu W, Xie S, Wang M, Cao G, Mao C, et al. All-transretinoic acid ameliorates bleomycin-induced lung fibrosis by downregulating the TGF-beta1/Smad3 signaling pathway in rats. Lab Invest 2013;93:1219-31.

10. Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. Cell 2008;133:38-52.

11. Menges CW, McCance DJ. Constitutive activation of the Raf-MAPK pathway causes negative feedback inhibition of Ras-PI3K-AKT and cellular arrest through the EphA2 receptor. Oncogene 2008;27:2934-40.

12. Carpenter TC, Schroeder W, Stenmark KR, Schmidt EP. Eph-A2 promotes permeability and inflammatory responses to bleomycin-induced lung injury. Am J Respir Cell Mol Biol 2012;46:40-7.

13. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, et al. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. Am J Respir Cell Mol Biol 2011;44:725-38.

14. Tabata C, Kubo H, Tabata R, Wada M, Sakuma K, Ichikawa M, et al. All-trans retinoic acid modulates radiation-induced proliferation of lung fibroblasts via IL-6/IL-6R system. Am J Physiol Lung Cell Mol Physiol 2006;290:L597-606.

15. Kullander K, Klein R. Mechanisms and functions of Eph and ephrin signalling. Nat Rev Mol Cell Biol 2002;3:475-86.

16. Kustermans G, El Benna J, Piette J, Legrand-Poels S. Perturbation of actin

dynamics induces NF-kappaB activation in myelomonocytic cells through an NADPH oxidase-dependent pathway. Biochem J 2005;387:531-40.

17. Nemeth ZH, Deitch EA, Davidson MT, Szabo C, Vizi ES, Hasko G. Disruption of the actin cytoskeleton results in nuclear factor-kappaB activation and inflammatory mediator production in cultured human intestinal epithelial cells. J Cell Physiol 2004;200:71-81.

18. Kustermans G, El Mjiyad N, Horion J, Jacobs N, Piette J, Legrand-Poels S. Actin cytoskeleton differentially modulates NF-kappaB-mediated IL-8 expression in myelomonocytic cells. Biochem Pharmacol 2008;76:1214-28.

19. Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. Physiol Rev 2006;86:279-367.

20. Coulthard MG, Morgan M, Woodruff TM, Arumugam TV, Taylor SM, Carpenter TC, et al. Eph/Ephrin signaling in injury and inflammation. Am J Pathol 2012;181:1493-503.

21. Ojima T, Takagi H, Suzuma K, Oh H, Suzuma I, Ohashi H, et al. EphrinA1 inhibits vascular endothelial growth factor-induced intracellular signaling and suppresses retinal neovascularization and blood-retinal barrier breakdown. Am J Pathol 2006;168:331-9.

22. Ivanov AI, Romanovsky AA. Putative dual role of ephrin-Eph receptor interactions in inflammation. IUBMB Life 2006;58:389-94.

23. Cercone MA, Schroeder W, Schomberg S, Carpenter TC. EphA2 receptor mediates increased vascular permeability in lung injury due to viral infection and hypoxia. Am J Physiol Lung Cell Mol Physiol 2009;297:L856-63.

24. Cheng N, Chen J. Tumor necrosis factor-alpha induction of endothelial

ephrin A1 expression is mediated by a p38 MAPK- and SAPK/JNK-dependent but nuclear factor-kappa B-independent mechanism. J Biol Chem 2001;276:13771-7.

25. Hong JY, Shin MH, Chung KS, Kim EY, Jung JY, Kang YA, et al. EphA2 Receptor Signaling Mediates Inflammatory Responses in Lipopolysaccharide-Induced Lung Injury. Tuberc Respir Dis (Seoul) 2015;78:218-26.

26. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem 2000;275:36803-10.

27. Hofmann AF. In memoriam: Dr. Herbert Falk (1924-2008). Hepatology 2009;49:1-3.

28. Conte E, Gili E, Fruciano M, Korfei M, Fagone E, Iemmolo M, et al. PI3K p110gamma overexpression in idiopathic pulmonary fibrosis lung tissue and fibroblast cells: in vitro effects of its inhibition. Lab Invest 2013;93:566-76.

29. Skromne I, Stern CD. Interactions between Wnt and Vg1 signalling pathways initiate primitive streak formation in the chick embryo. Development 2001;128:2915-27.

30. Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, Bradley A. Requirement for Wnt3 in vertebrate axis formation. Nat Genet 1999;22:361-5.

31. Chilosi M, Poletti V, Zamo A, Lestani M, Montagna L, Piccoli P, et al. Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary

fibrosis. Am J Pathol 2003;162:1495-502.

32. Konigshoff M, Balsara N, Pfaff EM, Kramer M, Chrobak I, Seeger W, et al. Functional Wnt signaling is increased in idiopathic pulmonary fibrosis. PLoS One 2008;3:e2142.

33. Konigshoff M, Kramer M, Balsara N, Wilhelm J, Amarie OV, Jahn A, et al. WNT1-inducible signaling protein-1 mediates pulmonary fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. J Clin Invest 2009;119:772-87.

34. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, et al. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci U S A 2006;103:13180-5.

35. Li M, Krishnaveni MS, Li C, Zhou B, Xing Y, Banfalvi A, et al. Epithelium-specific deletion of TGF-beta receptor type II protects mice from bleomycin-induced pulmonary fibrosis. J Clin Invest 2011;121:277-87.

36. Zhou B, Liu Y, Kahn M, Ann DK, Han A, Wang H, et al. Interactions between beta-catenin and transforming growth factor-beta signaling pathways mediate epithelial-mesenchymal transition and are dependent on the transcriptional co-activator cAMP-response element-binding protein (CREB)-binding protein (CBP). J Biol Chem 2012;287:7026-38.

37. Huang J, Xiao D, Li G, Ma J, Chen P, Yuan W, et al. EphA2 promotes epithelial-mesenchymal transition through the Wnt/beta-catenin pathway in gastric cancer cells. Oncogene 2014;33:2737-47.

38. Ghahhari NM, Babashah S. Interplay between microRNAs and WNT/beta-catenin signalling pathway regulates epithelial-mesenchymal transition in

cancer. Eur J Cancer 2015;51:1638-49.

ABSTRACT (IN KOREAN)

bleomycin 으로 유도된 폐 섬유화 쥐 모델에서 EphrinA2-EphrinA1 신호 전달 체계의 조절을 통한 all-trans retinoic acid의 항섬유화 효과

<지도교수 박무석>

연세대학교 대학원 의학과

임 아 영

배경: 특발성 폐섬유화증은 만성적, 비가역적으로 진행하며, 종종 치명적인 결과를 초래하는 폐 질환으로, 그 병태생리는 명확하게 알려져 있지 않다. All-*trans* retinoic acid (ATRA)는 비타민 A의 활성화된 대사물로, 세포의 분화와 증식, 발달과 관계되는 것으로 알려져 있다. 한편, Eph-Ephrin 신호 전달 체계는 혈관의 형성, 세포의 이동, 세액의 항상성 및 손상 후의 복구를 포함한 다양한 세포 과정을 매개한다. 폐섬유화에 대한 ATRA의 효과에 대하여 소수의 연구가 보고된 바 있으며, 폐섬유화외 EphA2 신호 전달 체계의 상관 관계에 대하여는 아직 명확하게 밝혀져 있지 않다.

목적: 본 연구에서는 bleomycin으로 유도된 폐섬유화 마우스 모델에서 ATRA 의 역할과, 그 메커니즘이 Eph-Ephrin, PI3K-Akt 혹은 Wnt/ß-catenin과 같은 기존에 존재하는 신호 전달 체계와 연관되는지를 밝히고자 하였다.

방법: 세포 실험에서 epithelial cell과 fibroblast를 ATRA, TGF-β1, 그리고 PI3K 억제제의 유무에 따라 배양하였다. 동물 실험은 대조군, bleomycin 그룹, ATRA 그룹, EphA2 monoclonal antibody 그룹의 4 그룹으로 나누어 진행하였다. 21일째에 각 그룹의 mice로부터 기관지폐포세척액을 획득하여 세포 수와 단백질 농도를 그룹별로 비교, 분석하였다. 또한 폐 조직을 얻어 각 그룹의 조직학적 변화를 비교하고, hydroxyproline assay, EphA2-EphrinA1, PI3K-Akt, Wnt/ß-catenin 신호 전달 체계와 연관된 단백질의 발현 및 cytokine 농도를 각 그룹별로 비교, 분석하였다.

결과: 본 실험에서 ATRA는 fibroblast 에서 PI3K-Akt 신호 전달 체계를 억제하는 것으로 나타났다. 동물 실험에서 bleomycin 노출은 EphA2와 EphrinA1의 단백질 발현을 증가시켰다. ATRA의 투여는 lung injury score를 감소시키고 기관지폐포세척액의 단백질 농도를 감소시켰다. Bleomycin 비강 투여시 EphA2, EphrinA1 및 PI3K 110γ 단백의 발현이 증가하였으며, ATRA 투여 후 유의하게 감소하였다. IL-6와 TNF-α의 농도 또한 ATRA 그룹에서 bleomycin 그룹에 비하여 낮게 나타났다. EphA2 monoclonal antibody를 비강으로 투여하였을 때 폐섬유화가 감소하는 것으로 나타났으며, bleomycin 노출시 증가된 Wnt5a와 PI3K

단백은 EphA2 monoclonal antibody 그룹에서 유의하게 감소하는 것으로 나타났다.

결론: 이상의 결과는 ATRA가 EphA2와 PI3K 신호 전달 체계를 통하여 bleomycin으로 유도된 폐 섬유화 모델에서 폐 섬유화를 감소시키고, 또한, EphA2 수용체 억제제가 PI3K 신호 전달 체계와 ß-catenin 과는 독립적인 Wnt신호 전달 체계를 통하여 폐 섬유화를 감소시킴을 시사한다. ATRA와 EphA2 수용체 억제제의 폐 섬유화에 대한 약재로서의 잠재력을 확인하기 위한 추가적인 연구가 필요하다.

핵심되는 말: bleomycin, pulmonary fibrosis, All-trans retinoic acid, EphA2 protein, PI3K, Wnt