Genistein alleviates pulmonary fibrosis by inactivating lung fibroblasts

Seung-hyun Kwon1,#, Hyunjoo Chung2,#, Jung-Woo Seo2,# & Hak Su Kim1,*
1Veterans Medical Research Institute, Veterans Health Service Medical Center, Seoul 05368, 2Core Research Laboratory, Medical Science Research Institute, Kyung Hee University Hospital at Gangdong, Seoul 05278, Korea

INTRODUCTION
Pulmonary fibrosis is a serious lung disease that occurs predominantly in men. Genistein is an important natural soybean-derived phytoestrogen that affects various biological functions, such as cell migration and fibrosis. However, the antifibrotic effects of genistein on pulmonary fibrosis are largely unknown. The antifibrotic effects of genistein were evaluated using in vitro and in vivo models of lung fibrosis. Proteomic data were analyzed using nano-LC-ESI-MS/MS. Genistein significantly reduced transforming growth factor (TGF)-β1-induced expression of collagen type I and α-smooth muscle actin (SMA) in MRC-5 cells and primary fibroblasts from patients with idiopathic pulmonary fibrosis (IPF). Genistein also reduced TGF-β1-induced expression of p-Smad2/3 and p-p38 MAPK in fibroblast models. Comprehensive proteomic analysis confirmed that genistein exerted an anti-fibrotic effect by regulating various molecular mechanisms, such as unfolded protein response, epithelial-mesenchymal transition (EMT), mammalian target of rapamycin complex 1 (mTORC1) signaling, cell death, and several metabolic pathways. Genistein was also found to decrease hydroxyproline levels in the lungs of BLM-treated mice. Genistein exerted an anti-fibrotic effect by preventing fibroblast activation, suggesting that genistein could be developed as a pharmacological agent for the prevention and treatment of pulmonary fibrosis. [BMB Reports 2024; 57(3): 143-148]

RESULTS
Genistein suppresses the activation of MRC5 cells
The characteristics of progressive pulmonary fibrosis are ab-
Genistein alleviates pulmonary fibrosis
Seung-Hyun Kwon, et al.

normal accumulation of fibroblasts and excessive deposition of extracellular matrix components (20). To investigate the anti-fibrotic effects of genistein, we first evaluated its role in fibroblast activation. The activation of fibroblasts is regulated by various factors, including TGF-β1, leading to its differentiation into myofibroblasts, which is the activation form of fibroblasts (21). In MRC-5 cells (human lung fibroblasts), α-SMA and collagen type 1, which are markers of activated fibroblasts, were found to be induced by TGF-β1; however, genistein significantly decreased the expression of these proteins induced by TGF-β1 (Fig. 1A, B). TGF-β1 is involved in fibrotic pathology through the activation of Smad signaling pathway and non-Smad pathways, including the p38 MAPK pathway which is regulated by genistein and collagen (22-25). In this study, the phosphorylation levels of Smad2/3 and p38 MAPK were enhanced by TGF-β1 in MRC-5 cells (Fig. 1C, D). Genistein reduced the phosphorylation of Smad2/3 and p38 MAPK induced by TGF-β1 (Fig. 1C, D).

Genistein suppresses the activation of primary lung fibroblasts
To validate our in vitro results, we tested the anti-fibrotic effects of genistein in primary lung fibroblasts from patients with IPF. Genistein decreased the TGF-β1-induced expression of collagen type 1 and α-SMA in human primary lung fibroblasts, which was consistent with those obtained using MRC-5 cells (Fig. 2A, B). Genistein also reduced the TGF-β1-induced phosphorylation levels of Smad2/3 and p38 MAPK in primary lung fibroblasts (Fig. 2C, D). These results suggest that genistein reduces fibroblast activation. Collectively, these results indicate that genistein decreases the expression levels of α-SMA and collagen type 1 by reducing the TGF-β1-activating Smad and p38 MAPK pathways. Thus, genistein attenuates the activation of fibroblasts.

Proteomic profiling of the anti-fibrotic effects of genistein on MRC-5 cells
To investigate the therapeutic mechanisms of genistein in pulmonary fibrosis, we conducted proteomic analysis using nanoLC-ESI-MS/MS. In the MRC-5 cell models, our proteomic evaluation revealed a total of 6,476 proteins from four groups (control, genistein, TGF-β1, and TGF-β1 combined with genistein). Of the identified proteins, 6,013 were considered for further analysis as they were identified in all four groups (Supplementary Fig. 1A). Principal component analysis (PCA) revealed a separation between the four groups (Supplementary Fig. 1B). A total of 1,082 proteins, which had a Benjamini-Hochberg method-based FDR of < 0.05, were selected. Hierarchical clustering analysis (distance threshold = 3.32) was...
Genistein decreased the expression of the proteins induced by TGF-β1. Cluster 2 showed that genistein increased the expression of the proteins induced by TGF-β1, cluster 4 showed that genistein did not affect the proteins induced by TGF-β1, and cluster 6 showed that genistein decreased the expression of the proteins induced by TGF-β1. Thus, the proteins (410 proteins) belonging to Clusters 2 (301 proteins) and 6 (109 proteins) may serve as therapeutic targets for genistein. To further identify the positive effects of genistein on the activation of lung fibroblasts, proteomic analysis was conducted with protein clusters 2 and 6. Briefly, post-hoc Tukey’s HSD test for one-way ANOVA was performed, and 334 proteins were identified; these proteins appeared to be significantly affected by genistein compared to TGF-β1 (Fig. 3A). Of the 334 proteins, 240 were upregulated (Supplementary Table 1) and 94 were downregulated (Supplementary Table 2) by treatment with genistein compared to TGF-β1. Functional enrichment analysis of the 240 upregulated proteins revealed nine biological hallmarks that were significantly increased by genistein compared to TGF-β1; these hallmarks were mainly related to oxidative phosphorylation, fatty acid metabolism, adipogenesis, and apoptosis (Fig. 3B). Functional enrichment analysis of the 94 downregulated proteins revealed six biological hallmarks significantly reduced by genistein compared to TGF-β1; these hallmarks were mainly related to unfolded protein response, epithelial mesenchymal transition (EMT), protein secretion, and mTORC1 signaling (Fig. 3C). Next, functional enrichment analysis was performed for cluster 1 and cluster 5 to confirm the effect of genistein alone. Functional enrichment analysis of the 506 proteins in cluster 1 and cluster 5 revealed nine biological hallmarks that were significantly regulated by genistein compared to other groups; these hallmarks were mainly related to cell cycle-related proteins (mitotic spindle, Myc and E2F targets, and G2/M checkpoint), EMT, and mTORC1 signaling (Supplementary Fig. 3). The expression levels of the markers for fibroblast activation, such as fibronectin, collagen type 1, and α-SMA, were also significantly increased by TGF-β1 but decreased by genistein (Fig. 3D).

Genistein attenuates bleomycin-induced pulmonary fibrosis

Finally, we investigated the antifibrotic effects of genistein in a bleomycin-induced pulmonary fibrosis model. The bleomycin-treated group increased weight loss compared to the control group but treatment with genistein reduced the weight loss in the bleomycin-treated group (Fig. 4A). Histopathological analysis conducted for the 1,082 proteins, which generated six clusters (Supplementary Fig. 2). Clusters 1, 3, and 5 were proteins affected by genistein and were either upregulated or downregulated by genistein compared to the control. Clusters 2, 4, and 6 are proteins were found to be affected by TGF-β1. Cluster 2 showed that genistein increased the expression of the proteins induced by TGF-β1, cluster 4 showed that genistein did not affect the proteins induced by TGF-β1, and cluster 6 showed that genistein decreased the expression of the proteins induced by TGF-β1. Thus, the proteins (410 proteins) belonging to Clusters 2 (301 proteins) and 6 (109 proteins) may serve as therapeutic targets for genistein. To further identify the positive effects of genistein on the activation of lung fibroblasts, proteomic analysis was conducted with protein clusters 2 and 6. Briefly, post-hoc Tukey’s HSD test for one-way ANOVA was performed, and 334 proteins were identified; these proteins appeared to be significantly affected by genistein compared to TGF-β1 (Fig. 3A). Of the 334 proteins, 240 were upregulated (Supplementary Table 1) and 94 were downregulated (Supplementary Table 2) by treatment with genistein compared to TGF-β1. Functional enrichment analysis of the 240 upregulated proteins revealed nine biological hallmarks that were significantly increased by genistein compared to TGF-β1; these hallmarks were mainly related to oxidative phosphorylation, fatty acid metabolism, adipogenesis, and apoptosis (Fig. 3B). Functional enrichment analysis of the 94 downregulated proteins revealed six biological hallmarks significantly reduced by genistein compared to TGF-β1; these hallmarks were mainly related to unfolded protein response, epithelial mesenchymal transition (EMT), protein secretion, and mTORC1 signaling (Fig. 3C). Next, functional enrichment analysis was performed for cluster 1 and cluster 5 to confirm the effect of genistein alone. Functional enrichment analysis of the 506 proteins in cluster 1 and cluster 5 revealed nine biological hallmarks that were significantly regulated by genistein compared to other groups; these hallmarks were mainly related to cell cycle-related proteins (mitotic spindle, Myc and E2F targets, and G2/M checkpoint), EMT, and mTORC1 signaling (Supplementary Fig. 3). The expression levels of the markers for fibroblast activation, such as fibronectin, collagen type 1, and α-SMA, were also significantly increased by TGF-β1 but decreased by genistein (Fig. 3D).

Genistein alleviates pulmonary fibrosis

Seung-hyun Kwon, et al.

Fig. 3. Identification of differentially expressed proteins (DEPs) related to genistein in MRC-5 cells with TGF-β1. (A) Heatmap of DEPs that appeared to be significantly affected by genistein compared to TGF-β1 based on Post-hoc Tukey’s HSD test for one-way ANOVA. The expression levels of the protein are derived based on Z-score normalization and the DEPs were grouped into two clusters (upregulated or downregulated by genistein compared to TGF-β1). (B) Functional enrichment analysis of the 240 proteins upregulated by genistein compared to TGF-β1. (C) Functional enrichment analysis of the 94 proteins downregulated by genistein compared to TGF-β1. (D) Three protein markers for the activation of fibroblast (Fibronectin, Collagen 1, and α-SMA) were assessed as positive controls. * indicates P < 0.05 compared with the control, and # indicates P < 0.05 compared with TGF-β1 treatment alone (one-way ANOVA with Newman-Keuls multiple comparison test).

http://bmbreports.org
sis of the lung sections revealed that the intensity of fibrotic lung injury was lower in the bleomycin- and genistein-treated groups than in the bleomycin-treated group (Fig. 4B). To examine the collagen content in the lung tissues, we attempted to elucidate the hydroxyproline levels. The bleomycin-treated group had higher levels of hydroxyproline than the control group. However, treatment with genistein attenuated the bleomycin-induced increase in hydroxyproline levels (Fig. 4C). The phosphorylation levels of SMAD 2/3 (Fig. 4D) and p38 MAPK (Fig. 4E) were markedly augmented by bleomycin treatment and the augmentation was inhibited by genistein. Overall, these data suggest that genistein is a promising therapeutic target for lung fibrosis.

DISCUSSION

This study sought to reveal the anti-fibrotic effects of genistein using both in vitro and in vivo lung fibrosis models. Based on our findings, genistein could act as an effective anti-fibrotic agent by inhibiting fibroblast activation. The anti-fibrotic mechanism of genistein was comprehensively evaluated through proteomic analysis, which revealed that genistein exerted an anti-fibrotic effect by regulating various molecular mechanisms, such as unfolded protein response, EMT, mTORC1 signaling, cell death, and several metabolic pathways.

IPF is characterized by the excessive deposition of extracellular matrix components and abnormal accumulation of fibroblasts due to an imbalance between fibrolysis and fibrogenesis (26). Fibroblasts can differentiate into myofibroblasts expressing high levels of α-SMA and collagen, and secrete mediators that amplify epithelial cell injury (27). Therefore, the inhibition of fibroblast activation is a good strategy to alleviate the progression of pulmonary fibrosis. In our study, we showed that genistein decreased the TGF-β1-induced activation of lung fibroblasts by reducing the levels of α-SMA and collagen type 1. These data suggest that genistein prevents pulmonary fibrosis by inhibiting fibroblast activation.

TGF-β1 is widely recognized as a key player and regulates numerous intracellular signaling pathways in the pathogenesis of pulmonary fibrosis (28). The canonical TGF-β pathway stimulates Smad signaling via the phosphorylation of Smad2/3 (29). TGF-β also activates several other pathways, including phosphatidylinositol-3-kinase (PI3K), mitogen activated protein (MAP) kinases (p38, JNK and ERK), and EMT-related signaling (23). The transition from epithelial cells to myofibroblasts via EMT signaling is an important process in lung fibrosis (30, 31). In our study, genistein was demonstrated to reduce the TGF-β1-induced phosphorylation levels of Smad2/3 and p38 MAPK in MRC-5 cells and primary human lung fibroblasts based on western blot analysis. Proteomic analysis also revealed that genistein regulates mTORC1 signaling and EMT-related protein levels. Collectively, our data indicate that genistein inhibits the activation of fibroblasts by regulating multiple mechanisms, including the Smad2/3, p38 MAPK, mTORC1, and EMT-related signaling pathways.

Recent studies have suggested that pulmonary fibrosis is caused by an aberrant wound healing response after recurrent lung injury, and the dysregulation of several metabolic pathways, including metabolism of lipid and glucose, is related in the fibrotic pathogenesis of pulmonary (32-34). In pulmonary fibrosis, enhanced glucose metabolism affects autophagy, cell differentiation, proliferation, and the inflammatory response (35). Fatty acid oxidation in both the mitochondria and peroxisomes is a catabolic process in which fatty acids produce ATP. Pulmonary fibrosis is accompanied by decreased fatty acid oxidation and increased fatty acid synthesis (36). Using proteomic data, we showed that genistein could exert an anti-fibrotic effect by regulating various metabolic processes, such as oxidative phosphorylation, fatty acid metabolism, peroxisomes, and glycolysis.

In conclusion, our findings highlighted a major role of genistein in the inactivation of fibroblasts for the prevention and treatment of pulmonary fibrosis through the regulation of the TGF-β1-related pathways. Comprehensive data obtained through protein analysis provide targets for further rigorous studies to precisely understand the molecular mechanism of genistein in lung fibrosis. Our findings also suggest that genistein could be developed as a pharmacological agent for the prevention and treatment of pulmonary fibrosis.

MATERIALS AND METHODS

Cell culture

A normal human fetal lung fibroblast cell line, MRC-5 cells (ATCC, Rockville, MD, USA), were maintained in Eagle's minimal essential medium (ATCC) supplemented with 10% fetal bovine serum (FBS HyClone, Logan, UT, USA), and 100 g/ml of streptomycin and 100 unit/ml of penicillin (Invitrogen, Carlsbad, CA, USA), and at 37°C in a humidified incubator containing 5% CO₂. MRC-5 cells were treated with 5 ng/ml of TGF-β1 for 24 h in the presence or absence of genistein (Sigma-Aldrich, St. Louis, MO, USA). For the isolation of primary fibroblasts (33), idiopathic pulmonary fibrosis (IPF) lung tissues were cut into 1 × 1 mm² pieces and cultured at 37°C in a humidified atmosphere containing 5% CO₂ for 7-10 days, with medium changes every 3 days. Cells at passages 3-7 were used for all experiments.

Animal model

Mouse experiments were performed in accordance with the Guiding Principles for the Care and Use of Animals. Detailed methods refer to previous study (33). The protocols were approved by the Animal Care and Handling Committee of Kyung Hee University Medical Center (protocol #KH-2021-005). Seven-week-old female C57BL/6j mice were obtained from Orient Bio (Seongnam, South Korea) and acclimatized for one week before the experiments. Mice were housed under specific pathogen-free conditions in an air conditioned (22 ± 2°C).
and humidity-controlled (45-55%) room under a 12-h light and 12-h dark cycle with ad libitum access to food and water. Mice were randomly divided into four groups: 1) saline plus vehicle (n = 5), 2) saline plus genistein (n = 5), 3) bleomycin plus vehicle (n = 6), and 4) bleomycin plus genistein (n = 6). Mice were anesthetized with intraperitoneal injections of 50 mg/kg alfaxalone (Jurox, Australia) and 5 mg/kg xylazine (Bayer, Leverkusen, Germany). Saline or bleomycin (2 U/kg) was administered intratracheally, and 20 mg/kg of genistein was administered immediately after bleomycin via an intraperitoneal injection 3 days per week for 3 weeks. Mice were then sacrificed and their lungs were harvested on day 21, snap frozen in liquid nitrogen, and stored at −80°C.

**Hydroxyproline assay**
To estimate the amount of collagen in lung tissues, a hydroxyproline assay was performed using a commercial kit (Bio-Vision, Milpitas, CA, USA), according to the manufacturer’s protocol and previous study (33). The whole lungs were weighed, homogenized, and hydrolyzed in 10 N HCl for 3 h at 120°C. The hydrolyzed samples were then incubated with 4-dimethyl-amino) benzaldehyde for 90 min at 60°C, and the absorbance of the oxidized hydroxyproline was determined at 560 nm. The amount of hydroxyproline is expressed as g/mg of lung tissue.

**Data search, statistical analysis, and bioinformatic analysis**
The obtained MS/MS spectra were assigned to proteins using Sequest-HT on a Proteome Discoverer (Version 2.4, Thermo Fisher Scientific) and the UniProt human database (Apr 2022) (37). The identified proteins were analyzed and visualized using Perseus software (Version 2.0.7.0) (38). One-way analysis of variance (ANOVA) with Benjamini-Hochberg method-based false discovery rate (FDR) and a significance level of 0.05 was used to identify significant differences in the protein expression levels. Gene Ontology (GO) annotation of the proteins identified from the proteome analysis was performed using Proteome Discoverer (Version 2.4, Thermo Fisher Scientific). Functional enrichment analysis was performed using ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go/). Statistical analysis of the expression levels of selected individual proteins was performed using GraphPad Prism software (version 5.0). Significant differences were analyzed using one-way ANOVA followed by the Newman-Keuls multiple comparison test for more than three groups. All P values were two-tailed, with statistical significance set at P < 0.05.

**Supplementary methods**
A detailed description of the methods in provided in supplementary methods.

**ACKNOWLEDGEMENTS**
We would like to thank Editage (www.editage.co.kr) for English language editing. This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2020R1F1A1049629), a VHS Medical Center Research Grant from the Republic of Korea (VHSMC 21001) and a 2019 research grant from the Medical Science Research Institute of Kyung Hee University Hospital at Gangdong.

**CONFLICTS OF INTEREST**
The authors have no conflicting interests.

**REFERENCES**
Genistein alleviates pulmonary fibrosis
Seung-hyun Kwon, et al.

30. Camara J and Jarai G (2010) Epithelial-mesenchymal transition in primary human bronchial epithelial cells is Smad-dependent and enhanced by fibronectin and TNF-alpha. Fibrogenesis Tissue Repair 3, 2