



Tranilast Inhibits Pulmonary Fibrosis by Suppressing TGF β /SMAD2 Pathway

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Motoyasu Kato, ^{1,2} Fumiyuki Takahashi,^{1,2} Tadashi Sato, ^{1,2} Yoichiro Mitsuishi,^{1,2} Ken Tajima,^{1,2} Hiroaki Ihara, ^{1,2} Fariz Nurwidya, ^{1,2} Hario Baskoro,^{1,2} Akiko Murakami,^{1,2} Isao Kobayashi,^{1,2} Moulid Hidayat, ^{1,2} Naoko Shimada,¹⁻³ Shinichi Sasaki,^{1,2} Reiko Mineki,⁴ Tsutomu Fujimura,⁵ Toshio Kumasaka,⁶ Shin-ichiro Niwa,⁷ Kazuhisa Takahashi ¹⁻³

¹Department of Respiratory Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan; ²Research Institute for Diseases of Old Ages, Juntendo University Graduate School of Medicine, Tokyo, Japan; ³Leading Center for the Development and Research of Cancer Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan; ⁴Laboratory of Proteomics and Biomolecular Science, Research Support Center, Juntendo University Graduate School of Medicine, Tokyo, Japan; ⁵Laboratory of Bioanalytical Chemistry, Tohoku Medical and Pharmaceutical University, Sendai, Miyagi, Japan; ⁶Department of Pathology, Japanese Red Cross Medical Center, Tokyo, Japan; ⁷Link Genomics, Incorporated, Tokyo, Japan

Correspondence: Motoyasu Kato
Department of Respiratory Medicine,
Juntendo University, Graduate School of
Medicine, Bunkyo-Ku, Tokyo 113-8431,
Japan
Tel +81-3-5802-1063
Fax +81-3-5802-1617
Email mtkatou@juntendo.ac.jp

Purpose: Idiopathic pulmonary fibrosis (IPF) is characterized by the accumulation of extracellular matrix (ECM) protein in the lungs. Transforming growth factor (TGF) β -induced ECM protein synthesis contributes to the development of IPF. Tranilast, an anti-allergy drug, suppresses TGF β expression and inhibits interstitial renal fibrosis in animal models. However, the beneficial effects of tranilast or its mechanism as a therapy for pulmonary fibrosis have not been clarified.

Methods: We investigated the in vitro effect of tranilast on ECM production and TGF β /SMAD2 pathway in TGF β 2-stimulated A549 human alveolar epithelial cells, using quantitative polymerase chain reaction, Western blotting, and immunofluorescence. In vitro observations were validated in the lungs of a murine pulmonary fibrosis model, which we developed by intravenous injection of bleomycin.

Results: Treatment with tranilast suppressed the expression of ECM proteins, such as fibronectin and type IV collagen, and attenuated SMAD2 phosphorylation in TGF β 2-stimulated A549 cells. In addition, based on a wound healing assay in these cells, tranilast significantly inhibited cell motility, with foci formation that comprised of ECM proteins. Histological analyses revealed that the administration of tranilast significantly attenuated lung fibrosis in mice. Furthermore, tranilast treatment significantly reduced levels of TGF β , collagen, fibronectin, and phosphorylated SMAD2 in pulmonary fibrotic tissues in mice.

Conclusion: These findings suggest that tranilast inhibits pulmonary fibrosis by suppressing TGF β /SMAD2-mediated ECM protein production, presenting tranilast as a promising and novel anti-fibrotic agent for the treatment of IPF.

Keywords: tranilast, idiopathic pulmonary fibrosis, TGF β , SMAD2

Background

Idiopathic pulmonary fibrosis (IPF) is a chronic pulmonary disorder of unknown etiology and is characterized by progressive deposition of extracellular matrix (ECM) proteins such as collagen and fibronectin.¹ Pathological findings of IPF include the formation of fibroblastic foci rich in ECM proteins in lung parenchyma of patients, and the number of fibroblastic foci is closely associated with progression and poor prognosis of the disease.² Transforming growth factor (TGF) β plays a crucial role in the induction of pulmonary fibrosis.³ TGF β can induce epithelial-mesenchymal transition (EMT) in human alveolar epithelial cells through the activation of SMAD2,⁴ and has been implicated in ECM synthesis by activated mesenchymal cells, resulting in the progression of fibrosis.⁵

Recently, much interests have been focused on the attenuation of the TGF β pathway as a treatment strategy for IPF. The antibiotic and tetracycline family

member, methacycline, reportedly inhibited the expression of TGF β -induced ECM proteins, such as collagen and fibronectin, in human alveolar epithelial cell line A549 cells, and attenuated bleomycin-induced pulmonary fibrosis in vivo.⁶ Therefore, attenuation of TGF β pathway may result in the inhibition of pulmonary fibrosis, and the agent that directly blocks the TGF β pathway may have potential therapeutic application in IPF.

Tranilast (*N*-3,4-dimethoxycinnamoyl-anthranilic acid) is an anti-allergic drug used for asthma as well as atopic and fibrotic pathologies, including keloid and scleroderma.^{7,8} Tranilast has been shown to inhibit renal and cardiovascular fibrosis in experimental models.^{9,10} Tranilast attenuated renal fibrosis induced by unilateral ureteral obstruction in rats through the reduction of TGF β and phospho-SMAD2 expression and modulation of EMT.¹¹ Although tranilast reportedly inhibited the progression of pulmonary fibrosis in mice, the mechanism of tranilast inhibition of pulmonary fibrosis has not been elucidated in detail.

In this study, we examined the effect of tranilast on TGF β -induced ECM synthesis and foci formation in A549 cells in vitro and BLM-induced pulmonary fibrosis in vivo.

Methods

Cells and Reagents

A549 cells are human lung cancer cell lines that maintain the characteristics of type II alveolar epithelial cells and were obtained from the RIKEN Bioresource Center (Tokyo, Japan). Tranilast was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA), while TGF β 2 and tumor necrosis factor alpha (TNF α) were obtained from eBioscience (San Diego, CA, USA). TGF β receptor inhibitor (SB431542) was purchased from TOCRIS Bioscience (Woburn, MA, USA). Details of cell culture and reagents are provided under [Supplementary Materials](#) and [Methods](#).

In vitro Proliferation Assay

Details of the method are provided under [Supplementary Materials](#) and [Methods](#).

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction conditions and primer sequences used for the detection of transcripts are described under [Supplementary Materials](#) and [Methods](#) and [Supplementary Table 1](#).

Western Blot

Details of the method and antibodies are provided under [Supplementary Materials](#) and [Methods](#).

Immunofluorescence

A549 cells were treated with TGF β 2 and TNF α and immunofluorescence for fibronectin was performed as described under [Supplementary Materials](#) and [Methods](#).

Cell Wound Healing Assay

Details of this method are provided under [Supplementary Materials](#) and [Methods](#).

Fibrotic Foci Assay

A549 cells were cultured for 48 h in a 24-well chamber, followed by incubation with 5 and 100 ng/mL of TGF β 2 and TNF α , respectively, for 48 h. Foci formation following treatment with TGF β 2 and TNF α were observed as described previously.¹² Fibrotic foci ratio (FFR) was calculated by dividing areas of foci with the area of the target field.

Bleomycin-Induced Pulmonary Fibrosis Model

Male 12-week-ICR mice, weighing about 35 g, were obtained from Japan Oriental Kobo (Tokyo, Japan). Mice were housed in a room under controlled temperature (25°C), humidity, and lighting (12/12-h light-dark cycle). Mice were categorized into four groups (A-D) at random, and mice in two groups (A and B) were injected with 10 mg/kg/day of bleomycin (BLM, Nippon Kayaku, Tokyo, Japan) dissolved in 200 μ L normal saline for 5 consecutive days,¹³ followed by treatment with tranilast 200 mg/kg. Mice in the other two groups (C and D) were injected with normal saline alone. Mice were administered either tranilast (200 mg/kg in 200 μ L 5% carboxymethyl cellulose [CMC]; groups A and C) or 5% CMC alone (groups B and D) by oral gavage twice daily from day 8 to 20 ([Supplementary Table 2](#)). All animals were sacrificed by intraperitoneal injection of pentobarbital at day 22 from the initiation of BLM injection. Both lungs were removed and frozen immediately in liquid nitrogen. Whole lungs were fixed in buffered 10% formalin solution (Wako Pure Chemical Industries, Tokyo, Japan) for histological examination. Fixed paraffin sections (3 μ m in size) were cut and stained with hematoxylin-eosin (HE) or Masson trichrome (MT) for the microscopic visualization of fibrotic lesions.

Fibrosis Score

Lung specimens were obtained from all five lobes, and all available specimens were reviewed. Images of sections stained with HE and MT were obtained using a microscope (Axiophoto; Carl Zeiss Corporation; Oberkochen, Germany) with a CCD camera (SPOT; Diagnostic Instruments; Sterling Heights, MI).¹⁴ Fibrotic changes were scored by Ashcroft score.^{15,16} In addition, two physicians (MK and FT) independently reviewed the same samples to evaluate the interobserver correlation of our method.

Collagen Content

The level of collagen in lung tissues was assessed with the Sircol collagen assay (Biocolor Ltd., Carrickfergus, Northern Ireland, UK) following the manufacturer's instructions.

Enzyme-Linked ImmunoSorbent Assay (ELISA) for Anti-TGFβ1 Antibody Detection

TGFβ1 concentration in BAL fluid was analyzed using ELISA sandwich, specifically developed with antibodies against canine TGFβ1 (Mouse/Rat/Porcine/Canine TGFβ1, MB100B, R&D Systems, Minneapolis, USA). ELISA was performed for each sample following the manufacturer's instructions. Reading was performed at 450 nm by using a microplate reader (Bio-Rad, Richmond, USA).

Immunohistochemistry

Immunohistochemical staining of mice lungs for fibronectin and phospho-SMAD2 was performed as described in [Supplementary Materials](#) and [Methods](#).

Ethics

All animal experiments were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology (Notice No. 71, 2006) and were approved by the Committee for Animal Experimentation of Juntendo University with the Approval No. 290,031.

Statistics

Statistical analysis was carried out using analysis of variance (ANOVA). The difference between the means was considered to be statistically significant at $p < 0.05$.

Results

Tranilast Inhibits TGFβ2-Induced ECM and Mesenchymal Protein Expression in A549 Cells

We initially examined the effect of various concentrations of tranilast on proliferation of A549 cells. At a concentration below 200 μM, tranilast did not influence the growth of A549 cells ([Figure S1](#)). Subsequently, A549 cells were treated with various concentrations of tranilast in the presence or absence of 5 ng/mL TGFβ2. As shown in [Figure 1A](#), qPCR analysis revealed that TGFβ2 stimulation alone resulted in the upregulation of mesenchymal markers, including N-cadherin, fibronectin, and collagen type IV alpha 1 chain (COL4A1); however, tranilast significantly inhibited TGFβ2-induced mRNA expression of these mesenchymal factors ([Figure 1A](#)). Further, Western blotting analysis showed that TGFβ2 stimulation led to the loss of the epithelial marker E-cadherin and increased the expression of mesenchymal markers such as fibronectin, type IV collagen, and N-cadherin ([Figure 1B](#)). Treatment with tranilast suppressed TGFβ2-induced expression of the mesenchymal markers and restored the expression of E-cadherin in a dose-dependent manner ([Figure 1C](#)). These findings suggest that tranilast inhibits TGFβ2-induced ECM and mesenchymal protein expression in A549 cells.

Tranilast Suppresses TGFβ/SMAD2 Pathway in A549 Cells

We examined the effect of tranilast on TGFβ/SMAD2 pathway. A549 cells were treated with different concentrations of tranilast in the presence of TGFβ2 for 72 h. Western blotting analysis revealed that tranilast attenuated TGFβ2-induced phosphorylation of SMAD2 ([Figure 1D and E](#)). As expected, TGFβ2 stimulation upregulated the expression of TGFβ1, TGFβ2, zinc finger E-box binding homeobox 1 (ZEB1), and SLUG, which are involved in TGFβ pathway; however, tranilast treatment suppressed the expression of these genes ([Figure 1F](#)).

Tranilast Attenuates TGFβ-Induced Cell Motility in A549 Cells

A wound healing assay was performed to evaluate the effect of tranilast on cell motility. A549 cells were treated with various concentrations of tranilast with or without 5 ng/mL TGFβ2, and the cell monolayer was scratched following a 48 h incubation. Migration of the cells into the

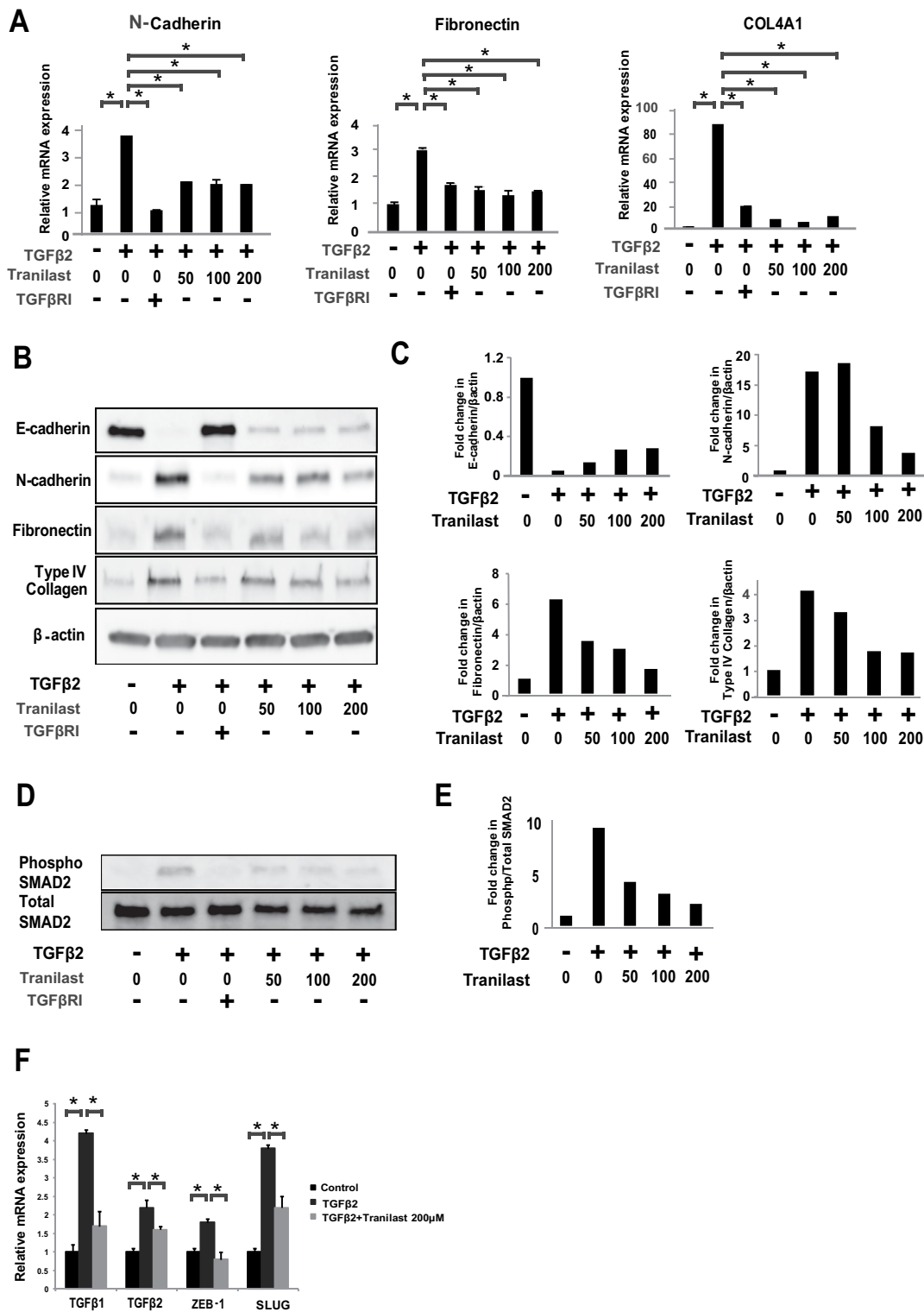


Figure 1 Tranilast inhibited TGFβ2-induced expression of mesenchymal markers and ECM synthesis via TGFβ/SMAD2 pathway in A549 cells. A549 cells were treated with various concentrations (50, 100, and 200 μM) of tranilast in presence or absence of 5 ng/mL TGFβ2 for 72 h. **(A)** Expression levels of N-cadherin, fibronectin, and COL4A1 mRNA was analyzed by quantitative polymerase chain reaction (qPCR). TGFβRI: TGFβ receptor inhibitor. **(B)** Expression of proteins E-cadherin, N-cadherin, fibronectin, and COL4A1 was analyzed by Western blotting. **(C)** Fold changes were calculated by setting the ratios of the any protein/β-actin band intensities. **(D)** A549 cells were treated with tranilast in presence or absence of 5 ng/mL TGFβ2 for evaluation of SMAD2. The phosphorylation level of Smad2 was analyzed by Western blotting. **(E)** Fold changes were calculated by setting the ratios of the phospho protein/total protein band intensities. **(F)** A549 cells with or without TGFβ2 stimulation were treated with tranilast for evaluation of TGFβ and transcriptional factors. Expressions of mRNA levels for TGFβ1, TGFβ2, ZEB-1, and Slug were evaluated by qPCR. Each bar represents mean ± SD of three independent experiments. *p < 0.01.

wound area was observed 48 h after scratching, and wound healing ratio (WHR) was evaluated in each well. Tranilast significantly attenuated TGF β 2-induced cell motility in A549 cells (Figure 2A).

Tranilast Suppresses TGF β 2 and TNF α -Induced Foci Formation in A549 Cells

Fibroblastic foci comprised of ECM proteins are characteristic features in the lungs of IPF patients, and the number of fibroblastic foci correlates with progression of IPF.¹⁷ To evaluate the effect of tranilast on the foci formation of human alveolar epithelial cells, A549 cells were cultured in the medium with 5 ng/mL of TGF β 2 and 100 ng/mL of TNF α for 48 h. As shown in Figure 2B, formation of cell foci was only induced by the stimulation with TGF β 2 and TNF α , but not by TGF β 2, TGF β 1, TNF α , or a combination of TGF β 1 and TNF α to (data not shown). These foci were strongly immunostained with fibronectin antibody, indicating that they were comprised of ECM proteins including fibronectin (Figure 2C). We calculated the focus formation ratio (FFR) by dividing the areas of foci with the area of the target field at six randomly selected fields (Figure 2D). Tranilast significantly suppressed foci formation in a dose-dependent manner (Figure 2E).

Tranilast Attenuates BLM-Induced Pulmonary Fibrosis in Mice

To investigate the anti-fibrotic effects of tranilast *in vivo*, we evaluated murine pulmonary fibrosis model induced by BLM injection. Lung tissues were stained with HE and MT stains (Figure 3A). Oral administration of tranilast significantly attenuated pulmonary fibrosis in mice treated with BLM, as evident from quantitative histological analysis (Figure 3B). Tranilast alone had no effect on lung tissues of control mice.

We evaluated the inhibitory effect of tranilast on TGF β 1 and ECM protein production, as well as on SMAD2 phosphorylation in lungs of murine pulmonary fibrosis model. BLM-treated mice exhibited an increased TGF β 1 level in bronchoalveolar lavage (BAL) fluid. However, enzyme-linked immunosorbent assay (ELISA) revealed that tranilast treatment significantly decreased TGF β 1 levels (Figure 4A). The Sircol collagen assay showed that tranilast-treated mice displayed significantly lower collagen content in the whole lung tissues than those treated with vehicle (Figure 4B).

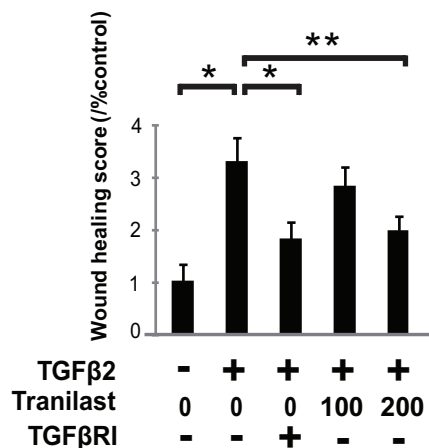
Expression of fibronectin (Figure 4C) and phospho-SMAD2 (Figure 4E) was evaluated by immunohistochemical analysis. BLM treatment upregulated the expression of fibronectin in murine lungs; however, tranilast administration significantly suppressed fibronectin expression (Figure 4D). In comparison to mice treated with BLM and vehicle, those treated with BLM and tranilast exhibited lower number of cells positive for phospho-SMAD2 in murine lungs (Figure 4F). Tranilast significantly attenuated BLM-induced phosphorylation of SMAD2, as revealed by Western blotting analysis (Figure 4G and H). These findings suggest that tranilast inhibited TGF β 1, ECM production, and SMAD2 phosphorylation in the lungs of a murine pulmonary fibrosis model.

Discussion

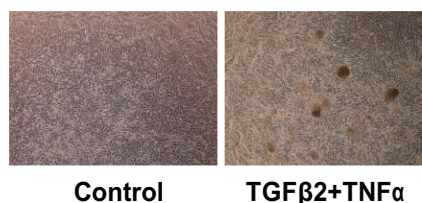
Our study highlights four main findings: (A) tranilast suppressed ECM protein expression and attenuated SMAD2 phosphorylation in TGF β 2-stimulated human alveolar epithelial cells (A549 cell line); (B) tranilast inhibited cell motility and foci formation in A549 cells; (C) tranilast attenuated BLM-induced pulmonary fibrosis in mice; (D) tranilast suppressed TGF β 1, ECM production, and SMAD2 phosphorylation in the lungs of mice treated with BLM.

TGF β contributes to the pathogenesis of IPF by inducing EMT in alveolar epithelial cells, leading to the formation of fibroblastic foci and abnormal accumulation of ECM proteins.¹⁸ EMT is characterized by the loss of the epithelial marker E-cadherin, gain of mesenchymal markers such as fibronectin, and enhanced cell motility. EMT is often associated with cancer invasion and metastasis;¹⁹ of note, ECM was also reported to be involved in the pathogenesis of IPF.^{20,21} In fact, several research studies on pulmonary fibrosis focused on EMT. However, at least one concluded that EMT is not involved in the pathogenesis of BLM-induced lung fibrosis and human IPF.²² Therefore, whether EMT associates with IPF pathogenesis is still controversial.²³ Although lung fibroblasts are undoubtedly related to the pathogenesis of IPF and are usually used for the research of lung fibrosis, the pathogenesis of IPF multifactorial.²⁴ In fact, the first step of lung fibrosis is alveolar epithelial damage caused by several factors; thus, type II alveolar epithelial cells will also have a role in the pathogenesis of IPF. Of note, several studies supporting an association between EMT and lung fibrosis have been published.^{25–30}

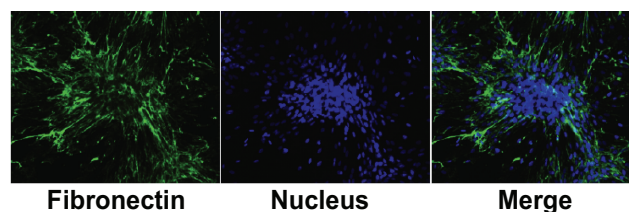
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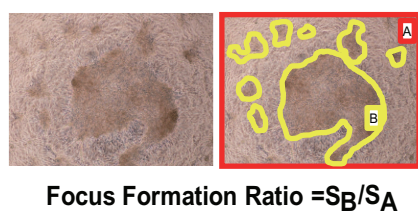
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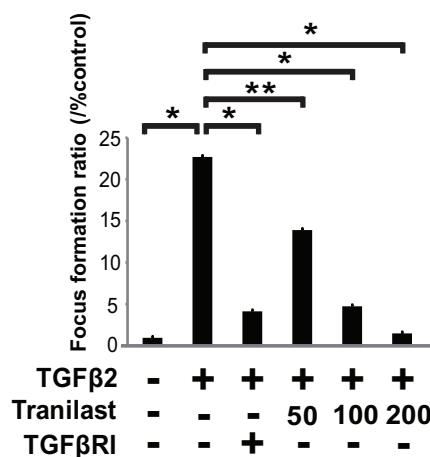


Figure 2 Tranilast attenuated cell migration and foci formation in A549 cells. **(A)** A549 cells with or without TGFβ2 (5 ng/mL) stimulation were incubated with 100 or 200 μM tranilast for 48 h and a wound was created by scratching the cell monolayer to assess cell migration; the wound area was evaluated. Photos of sections were taken under 200 × magnification using a microscope (Olympus, Japan) with a CCD camera. Wound areas were measured by ImageJ. Wound healing ratio in each well was calculated by dividing the wound areas. **(B)** To evaluate foci formation, A549 cells were incubated in presence of TGFβ2 (5 ng/mL) and TNFα (100 ng/mL) in 24-well chambers. Following 48 h stimulation, photos of sections were taken using a microscope fitted with a CCD camera. **(C)** Foci were strongly immunostained with fibronectin antibody. **(D)** Fibrotic foci ratio (FFR) was calculated by dividing the areas of foci with the area of the target field at six randomly selected wells using ImageJ. **(E)** The effect of several concentrations of tranilast (50, 100, and 200 μM) on foci formation in A549 cells. These cells in each group were observed after TGFβ2 stimulation with or without tranilast. The average of FFR was calculated for each group. Each bar represents mean ± SD of three independent experiments. **p* < 0.01, ***p* < 0.05.

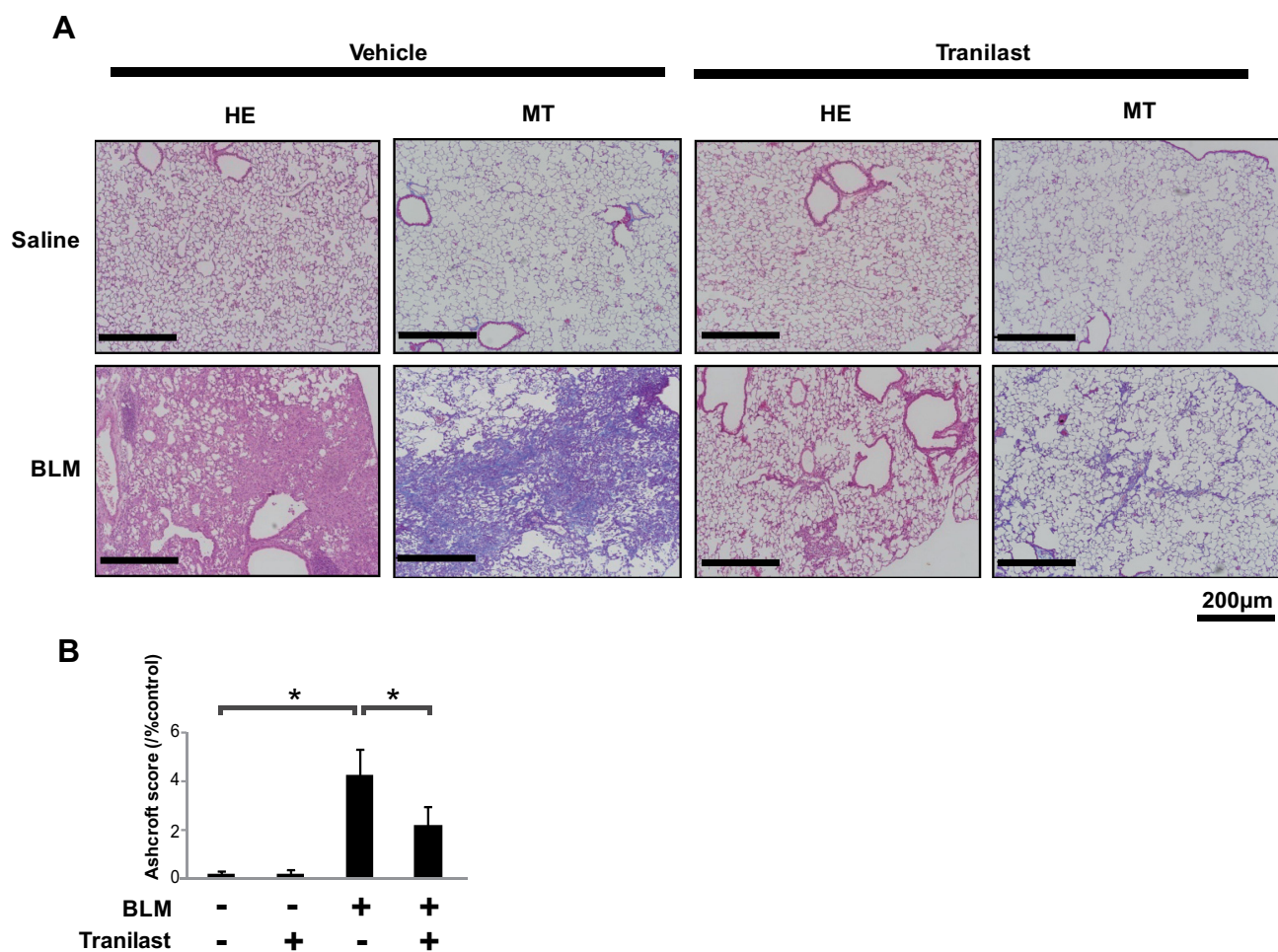


Figure 3 Tranilast attenuated BLM-induced pulmonary fibrosis in mice. **(A)** Histological examination was performed by hematoxylin-eosin (HE) as well as Masson trichrome (MT) staining. Bar = 200 µm. **(B)** The grade of pulmonary fibrosis was evaluated by the method described by Ashcroft and coworkers.¹⁹ Treatment groups were composed of saline-treated with vehicle (n = 10), saline-treated with tranilast (n = 10), BLM-treated with vehicle (n = 9), and BLM-treated with tranilast (n = 9). More than 30 randomized fields of each of the five lung lobes in mice were examined. Fibrotic score is presented as mean ± SD of all the fields examined in each group. *p < 0.01.

TGFβ is known as one of the pro-fibrotic key mediators in the pathogenesis of IPF and associates with EMT, fibroblast to myfibroblast transition, and senescence. Among the TGFβ family, TGFβ1 is an important key mediator of lung fibrosis; in fact, it is usually used as a biomarker for the analysis of lung fibrosis. TGFβ2 is also often used for the evaluation of lung fibrosis.^{27,31} In our research, we focused on the association between fibroblastic foci and EMT, as per a previous report.²¹ Therefore, we established an alveolar epithelial cells-induced foci assay system using an alveolar epithelial cell line. We tried to develop EMT-induced foci using several mediators; however, we could only establish foci via the stimulation with both TGFβ2 and TNFα. Of note, we used TGFβ2 in all of the assays in this study, precisely because we used TGFβ2 with TNFα for foci development.

Fibroblastic foci are one of the important pathological features associated with the progression of fibrosis in IPF.^{17,32,33} Therefore, we focused on fibroblastic foci as targets for the treatment of IPF. We designed an assay of fibroblastic foci formation using A549 cells stimulated with TGFβ2 and TNFα. Foci formation in our A549 in vitro model was associated with EMT; of note, foci comprised fibronectin. In theory, if a candidate drug suppresses the development of foci formation, it will attenuate the progression of lung fibrosis in IPF. In our study, tranilast treatment suppressed TGFβ-induced EMT as well as the production of mesenchymal and ECM proteins such as collagen and enhanced cell motility. Tranilast treatment also significantly inhibited foci formation in A549 cells.

Tranilast effectively prevents the progression of fibrosis in various organs, including inhibiting TGFβ1-induced

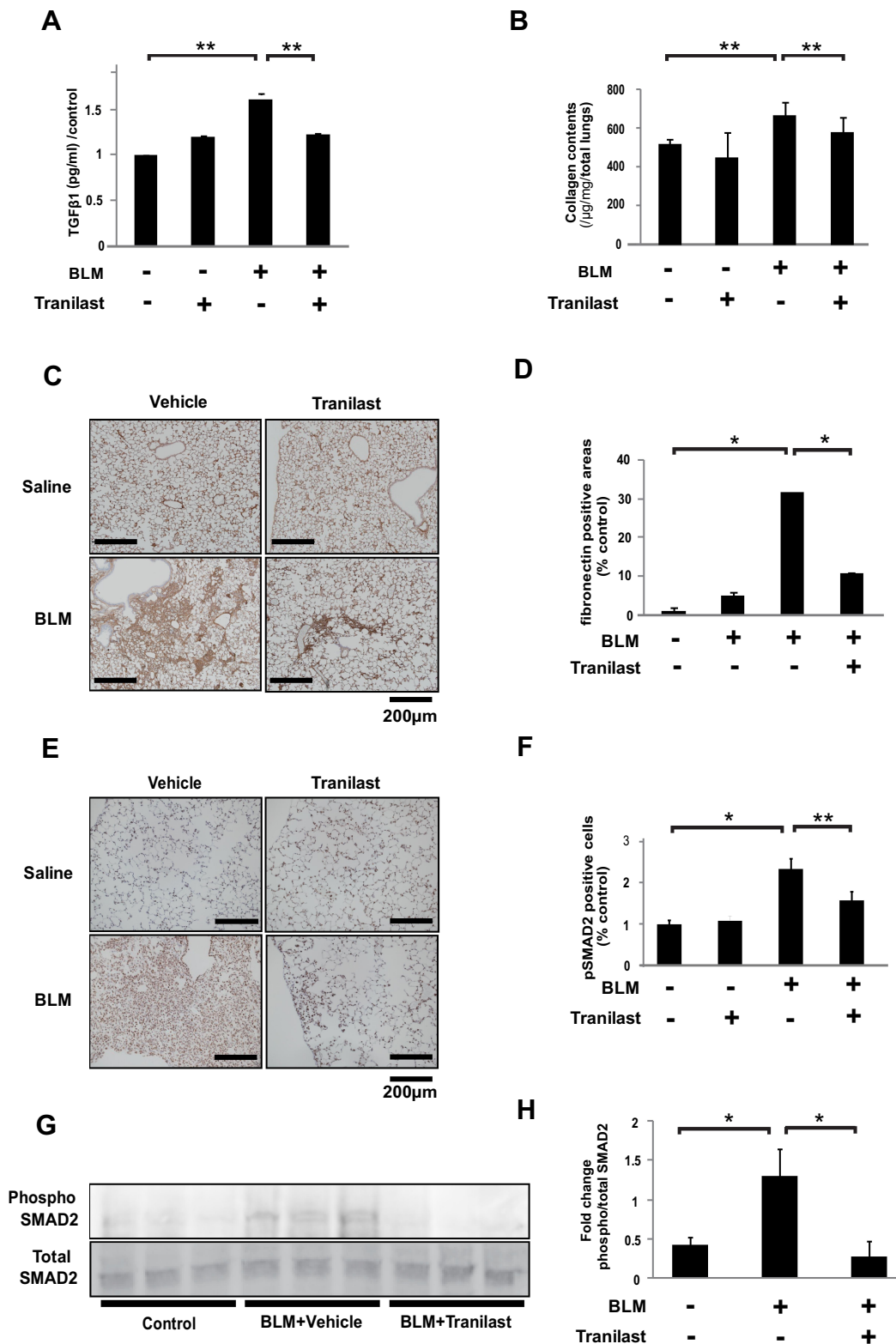


Figure 4 Tranilast attenuated BLM-induced ECM production and phosphorylation of SMAD2 in mice. **(A)** TGFβ1 concentration in BAL fluid was evaluated by ELISA. **(B)** Effects of tranilast on collagen contents. Data are presented as mean ± SD in each group of 10 mice. ***p* < 0.05. **(C)** Effect of tranilast on expression of fibronectin in the murine lungs by immunohistochemistry (IHC). Bar = 200 μm. **(D)** The average of the percentage of fibronectin positive ratio in each of the four groups was calculated by dividing the average of each group with that of the control group. Data are presented as mean ± SD in each group of 10 mice. **p* < 0.01. **(E)** IHC staining for phospho-SMAD2 in the murine lungs. **(F)** The phospho-Smad2-positive cells were counted in 10 fields at × 200 magnification. Bar = 200 μm. The average of the percentage of phospho-SMAD2-positive cells in each of the four groups was calculated by dividing the average of each group with that of the control group. Data are presented as mean ± SD in each group of 10 mice. **p* < 0.01, ***p* < 0.05. **(G)** Levels of phospho- and total-SMAD2 in the whole lung lysates of mice by Western blotting analysis. **(H)** Fold changes were analyzed by setting the ratios of the phospho/total protein band intensities.

ECM production in the proximal tube cells of the kidney and modulating renal fibrosis induced by unilateral ureteral obstruction in rats.¹¹ In addition, tranilast inhibited TGF β 1 expression, prevented atrial remodeling, and suppressed atrial fibrillation development in a canine model of cardiovascular fibrosis.³⁴ Moreover, tranilast was reported to inhibit pulmonary fibrosis by suppressing alveolar macrophage activation in alveolar macrophage (MAC-1 positive) cells and suppressing the production of reactive oxygen species in a BLM-induced lung fibrosis model.^{35,36} In addition, tranilast reportedly inhibits lung fibrosis by suppressing BLM-induced activation of neutrophils and alveolar macrophages in mast cell-deficient WBB6F1-W/Wv mice.³⁷ Our current study focused on the TGF β /SMAD pathway in epithelial cells and also shows that tranilast inhibits ECM protein production, which is consistent with previous reports.

Our study has several limitations. First, we did not assess the effect of lung fibrosis attenuation/prevention by anti-fibrotic agents, in comparison with that of tranilast. Of note, almost all of the previous studies also did not compare the effects of the candidate medicine studied with those of approved drugs. For instance, one of the existing anti-fibrotic agents, nintedanib, was not compared pre-clinically with another existing anti-fibrotic agent, pirfenidone, in the context of prevention of lung fibrosis.³¹ Of note, the effect of tranilast on lung fibrosis is still low; this said, we still need to compare the efficacy of tranilast on lung fibrosis (in comparison with positive control drugs, including nintedanib or pirfenidone) in the future. Second, we used the A549 cell line as representative of type II alveolar epithelial cells. The A549 cell line is a human alveolar epithelial lung cancer cell line with a *KRAS* mutation.³⁸ Therefore, these cells have characteristics of cancer cells such as fast cell proliferation and immortalization. Ideally, we should have used primary type II alveolar epithelial cells for the analysis of lung fibrosis. However, it is difficult to culture primary human alveolar epithelial cells. To perform some experiments, particularly for the evaluation of EMT using primary alveolar epithelial cells is also quite challenging. Thus, many researchers, ourselves included, evaluate EMT and epithelium in IPF using A549 cells instead of human primary type II alveolar epithelial cells.^{4,6,39–43} This said, we should have in mind the different cell characteristics of A549 and primary human alveolar epithelial cells in the analysis of the experimental results.

Conclusions

Our results showed that tranilast attenuates pulmonary fibrosis through the inhibition of SMAD2 phosphorylation and suppression of TGF β -mediated ECM protein production in mesenchymal cells.

Abbreviations

IPF, idiopathic pulmonary fibrosis; ECM, extracellular matrix; TGF, transforming growth factor; EMT, epithelial–mesenchymal transition; BLM, bleomycin; CMC, carboxymethylcellulose; HE, hematoxylin-eosin; MT, Masson trichrome; FFR, focus formation ratio; qPCR, quantitative polymerase chain reaction; TGF β RI, TGF β receptor inhibitor.

Data Sharing Statement

Not applicable.

Consent for Publication

Not applicable.

Ethics Approval and Consent to Participate

The Institutional Review Board at the Juntendo University School of Medicine approved the procedures. (No. 290,031).

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Disclosure

The authors declare no conflicts of interest for this work.

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