Dasatinib Suppresses TGFβ-Mediated Epithelial–Mesenchymal Transition in Alveolar Epithelial Cells and Inhibits Pulmonary Fibrosis

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Abstract

Purpose Transforming growth factor β (TGFβ)-mediated epithelial–mesenchymal transition (EMT) of alveolar epithelial cells contributes to pulmonary fibrosis. Dasatinib (DAS), a potent and broad-spectrum tyrosine kinase inhibitor, has been widely studied as an anti-cancer agent. However, the therapeutic application of DAS for pulmonary fibrosis has not been clarified. Our purpose here is to investigate the effect of DAS on TGFβ1-induced EMT in human alveolar and bronchial epithelial cells in vitro and to evaluate the efficacy of DAS on lung fibrosis in vivo.

Methods TGFβ1-stimulated human alveolar epithelial (A549) and bronchial epithelial (BEAS-2B) cells were treated with or without DAS in vitro. Murine pulmonary fibrosis model was generated by injection of bleomycin (BLM).

Results A549 and BEAS-2B cells exposed to TGFβ1 underwent EMT, as indicated by downregulation of epithelial protein E-cadherin and induction of the mesenchymal proteins, fibronectin and type I and type IV collagen. These effects were dramatically suppressed by DAS treatment, which also prevented Smad2 and Smad3 phosphorylation. DAS inhibited TGFβ1-induced cell motility and migration. Furthermore, DAS administration significantly attenuated lung fibrosis in mice by histological analysis. Treatment with DAS also significantly reduced the levels of collagen and fibronectin and phosphorylation of Smad2 in the lung tissues of the murine model.

Conclusions These findings suggest that DAS inhibited TGFβ-mediated EMT of alveolar and bronchial epithelial cells and attenuated BLM-induced lung fibrosis in mice by suppressing the TGFβ/Smad pathway. DAS may be a promising and novel anti-fibrotic agent for preventing lung fibrosis.

Keywords Dasatinib · Pulmonary fibrosis · Epithelial mesenchymal transition · TGFβ

Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>DAS</td>
<td>Dasatinib</td>
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<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>TGF</td>
<td>Transforming growth factor</td>
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<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
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<tr>
<td>BLM</td>
<td>Bleomycin</td>
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<td>HE</td>
<td>Hematoxylin-Eosin</td>
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<td>MT</td>
<td>Masson-Trichrome</td>
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<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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Introduction

Idiopathic pulmonary fibrosis (IPF), a chronic pulmonary disorder of unknown etiology, is characterized by progressive deposition of extracellular matrix (ECM) proteins such as collagen and fibronectin [1]. Transforming growth factor (TGF) β plays a crucial role in inducing pulmonary fibrosis [2]. TGFβ can induce epithelial–mesenchymal transition (EMT) in human alveolar epithelial cells via Smad2 activation [3] and plays a role in ECM synthesis by activated mesenchymal cells, which results in the progression of fibrosis [4].

Pirfenidone and nintedanib are currently used as oral anti-fibrotic agents for patients with IPF [5, 6]. Although these agents can decrease the decline of focal vital capacity in a pulmonary function test, it has never been shown that any agent can inhibit or prevent fibrosis progression in IPF patients. Many recent studies have focused on attenuating TGFβ-mediated EMT of alveolar epithelial cells as a treatment strategy for IPF. It has been reported that the antibiotic, methacycline, a member of the tetracycline antibiotic family, can inhibit TGFβ-induced EMT in the human alveolar epithelial cell line A549 in vitro and attenuates bleomycin-induced pulmonary fibrosis in vivo [7]. Therefore, attenuating TGFβ-mediated EMT of alveolar epithelial cells may inhibit pulmonary fibrosis, and an agent that blocks EMT might be a new medicine for IPF.

Dasatinib (DAS) is an oral multitarget inhibitor of several tyrosine kinases, including Bcr-Abl family members, Src and Btk family members, c-Kit, PDGFR, and Eph receptors [8, 9]. DAS has been widely used and studied for treating cancers such as imatinib-resistant chronic myelogenous leukemia or triple-negative breast cancer [10, 11]. Recently, it was shown that DAS inhibits lung fibrosis in experimental models through suppressing myofibroblast activation via the inhibition of Src, PDGFR-α, and c-Abl [12]. It has also been reported that DAS inhibits TGFβ-induced myofibroblast differentiation through the Src-SRF Pathway [13]. However, the effect of DAS on TGFβ-mediated EMT of alveolar epithelial cells in pulmonary fibrosis has not been clarified.

In this study, we examined the effect of DAS on TGFβ-induced EMT in the human alveolar epithelial cell line A549 and bronchial epithelial cell line BEAS-2B in vitro, and bleomycin-induced pulmonary fibrosis in vivo. The molecular mechanism by which DAS attenuates pulmonary fibrosis was assessed.

Materials and Methods

Cells and Reagents

A549 cells (human type II alveolar epithelial cells) were obtained from the Riken Bioresource Center (Tokyo, Japan) as previously described [14]. BEAS-2B cells (human bronchial epithelial cells) were obtained from the American Type Culture Collection (Rockville, MD, USA). Dasatinib was purchased from Carbosynth (Compton, UK). Recombinant soluble human TGFβ1 was from eBioscience (San Diego, CA, USA). The details of cell culture and reagents are provided in the online supplement.

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) conditions and primer sequences used for detecting transcripts are described in Supplementary Materials and Methods.

Western Blotting

The details of the methods and antibodies used are provided in Supplementary Materials and Methods. All blots in this paper are representative and were performed at least twice.

Cell Wound Healing Assay and Migration Assay

The details of these assays are provided in Supplementary Materials and Methods.

Bleomycin-Induced Pulmonary Fibrosis Model

To develop the pulmonary fibrosis model, ICR mice were intravenously injected with bleomycin (BLM) dissolved in 200 µL of normal saline for five consecutive days [15]. Then, these mice were treated with DAS 30 mg/kg or vehicle from days 8 to 20, and were sacrificed at day 22. The details of this procedure are provided in the online supplement.

Fibrosis Score

Mouse lung specimens were obtained from all six lobes, and all available specimens were reviewed. Images of sections stained with Hematoxylin-Eosin (HE) and Masson-Trichrome (MT) were generated using a microscope (Axiophoto; Carl Zeiss Corporation; Oberkochem, Germany) with a CCD camera (SPOT; Diagnostic Instruments; Sterling Heights, MI) [16]. Fibrotic changes were scored by Ashcroft score [17]. Additionally, to evaluate the inter-observer
correlation of our method, the two physicians (M.K. and F.T.) independently reviewed the same specimen as previously described [18].

**Collagen Content**

The levels of collagen in lung tissues were examined by a Sircol collagen assay (Biocolor Ltd., Carrickfergus, Northern Ireland, UK) according to the manufacturer’s instructions.

**Immunohistochemistry**

Immunohistochemical staining of murine lungs for fibronectin and phospho-Smad2 was performed as described in Supplementary Materials and Methods.

**Statistics**

Statistical analysis was carried out using one-way ANOVA with Tukey’s multiple comparisons test using JMP 13 for Windows statistical software (SAS Institute Japan Inc., Tokyo, Japan). The differences were considered to be statistically significant at $p < 0.05$.

**Results**

**Dasatinib Inhibits TGFβ-Induced EMT in BEAS-2B Cells**

The hallmarks of EMT include reduced levels of epithelial markers such as E-cadherin and increased levels of mesenchymal markers such as fibronectin and collagen [4]. First of all, we examined the effect of Dasatinib (DAS) on cell viability at concentration of 1–10 µM for BEAS-2B cells. DAS did not influence the cell growth at these concentrations (Fig. S1A). BEAS-2B cells were incubated with or without 5 ng/mL TGFβ1 in the absence or presence of 5 µM DAS. After incubation, proteins were extracted, and their levels were measured by western blot analysis. As shown in Fig. 1a, b, TGFβ1 stimulation led to the decrease in expression of the epithelial marker E-cadherin, and the increase in expression of mesenchymal markers, N-cadherin, fibronectin, type I collagen, and type IV collagen, reminiscent of the EMT phenotype. Treatment with DAS suppressed the expression of these mesenchymal markers and restored E-cadherin expression (Fig. 1a, b).

We also examined mRNA expression of E-cadherin and mesenchymal markers including N-cadherin, fibronectin, collagen 1A1 (*COL1A1*), and collagen 4A1 (*COL4A1*) in BEAS-2B cells by qPCR analysis. BEAS-2B cells were incubated with or without 5 ng/mL TGFβ1 and various concentrations of DAS. As shown in Fig. 1c, TGFβ1 stimulation led to the loss of E-cadherin and gain of mesenchymal markers by qPCR analysis, and treatment with DAS significantly rescued expression of E-cadherin and inhibited expression of mesenchymal factors, respectively, in a dose-dependent manner. Transcription factors, including Snail and Slug, which are involved in TGFβ-mediated EMT, were upregulated by stimulation with TGFβ1, and this upregulation was significantly suppressed by DAS (Fig. 1c).

**Dasatinib Inhibits TGFβ-Induced EMT in A549 Cells**

Similar results to those described above were obtained in A549 cells (Fig. 2a, b). We examined the effect of DAS on cell viability at concentration of 0.1 to 1 µM for A549 cells. DAS did not influence the cell growth at these concentrations (Fig. S1B). A549 cells were incubated with or without 5 ng/mL TGFβ1 in the absence or presence of 1 µM DAS. Treatment with DAS suppressed protein expression of mesenchymal markers fibronectin, type I collagen, and type IV collagen, and rescued E-cadherin expression as determined by western blot analysis (Fig. 2a, b). We also examined mRNA expression of E-cadherin and mesenchymal markers including fibronectin and COL4A1 in A549 cells by qPCR analysis (Fig. 2c). Expression of Snail and Slug was significantly suppressed by DAS (Fig. 2c).

These findings suggest that DAS treatment inhibits TGFβ1-induced EMT in human bronchial epithelial cells and alveolar epithelial cells.

**Dasatinib Suppresses Smad2/3 Phosphorylation in BEAS-2B and A549 Cells**

Next, we examined the effect of DAS on TGFβ-mediated Smad2 and Smad3 activation. BEAS-2B cells were incubated with or without 5 ng/mL TGFβ1 in the absence or presence of 5 µM DAS. DAS attenuated TGFβ1-induced phosphorylation of Smad2 and Smad3 as determined by western blot analysis. (Fig. 3a, b). Similar results were obtained in A549 cells (Fig. 3c, d). A549 cells were incubated with or without 5 ng/mL TGFβ1 and 0.1 or 1 µM DAS. Treatment with DAS also suppressed TGFβ1-induced phosphorylation of Smad2 and Smad3 (Fig. 3c, d).

Collectively, these findings suggest that DAS reversed EMT by suppressing the TGFβ/Smad pathway in human bronchial epithelial cells and alveolar epithelial cells.

**Dasatinib Attenuates TGFβ-Induced Cell Motility in BEAS-2B and A549 Cells**

To evaluate cell motility, wound healing assays were performed. BEAS-2B cells were incubated with or without 5 ng/mL TGFβ1 in the absence or presence of 3 µM
Fig. 1 Dasatinib inhibited TGFβ-induced EMT in BEAS-2B cells. 

a BEAS-2B cells were incubated with or without TGFβ1 (5 ng/mL) in the absence or presence of 5 µM Dasatinib (DAS) for 48 h. Cell lysates were subjected to western blot analysis for E-cadherin, N-cadherin, fibronectin, collagen1, collagen4, and β-actin. Result is representative of two independent experiments. b The density of each band was quantified by densitometry using ImageQuant TL and was normalized to the density of β-actin. Result is representative of two independent experiments. c BEAS-2B cells were incubated with or without 5 ng/mL TGFβ1 and various concentrations (1, 5, and 10 µM) of DAS. mRNA expression of E-cadherin, N-cadherin, fibronectin, COL1A1, COL4A1, and transcription factors including Snail and Slug was analyzed by quantitative polymerase chain reaction (qPCR) in BEAS-2B cells. Data were normalized to actin expression. *p < 0.001 compared with the value of untreated cells, †p < 0.001, ‡p < 0.05 compared with the value of TGFβ1-treated cells.
Dasatinib inhibited TGFβ-induced EMT in A549 cells. A A549 cells were incubated with or without TGFβ1 (5 ng/mL) in the absence or presence of 1 µM DAS for 48 h. Cell lysates were subjected to Western blot analysis for E-cadherin, fibronectin, collagen1, collagen4, and β-actin. Result is representative of two independent experiments. B The density of each band was quantified by densitometry using ImageQuant TL and was normalized to the density of β-actin. Result is representative of two independent experiments. C A549 cells were incubated with or without 5 ng/mL TGFβ1 and various concentrations (0.1, 0.5, and 1 µM) of DAS. mRNA expression of E-cadherin, fibronectin, COL4A1, and transcription factors including Snail and Slug was analyzed by qPCR in A549 cells. Data were normalized to actin expression. *p < 0.01 compared with the value of untreated cells, †p < 0.01 compared with the value of TGFβ1-treated cells.

We also performed migration assays for BEAS-2B and A549 cells. DAS significantly attenuated TGFβ1-induced migration ability of BEAS-2B and A549 cells (Fig. 4b).
Dasatinib Attenuates BLM-Induced Pulmonary Fibrosis in Mice

To investigate the anti-fibrotic effects of DAS in vivo, intravenous administration of BLM was used for our mouse model of pulmonary fibrosis. We sacrificed these mice on day 23 based on the qPCR data together with histological findings. Masson-trichrome stain showed remarkable fibrosis on day 23 (Fig. S2). Additionally, the expression levels of fibrotic markers were also significantly upregulated and peaked on day 23 (Fig. S2). Quantitative histological analysis revealed that DAS administration significantly attenuated pulmonary fibrosis in mice treated with BLM (Fig. 5b). We evaluated the inter-observer variation for scoring the fibrotic changes by Ashcroft score by two physicians, which showed strong correlation (Fig. S3). Administration of DAS alone had no effect on the pathological findings of lung tissues in the control mice.

Dasatinib Inhibits Collagen and Fibronectin Expression and Smad2 Phosphorylation in the Lung Tissues of Mice Treated with BLM

Finally, we examined whether DAS inhibited the expression of extracellular matrix (ECM) proteins, including collagen and fibronectin, and phosphorylation of Smad2 in the lungs of a BLM-induced murine pulmonary fibrosis animal model. The collagen content of whole lung tissues was significantly lower in mice treated with BLM and DAS than that in mice treated with BLM and vehicle as determined by a Sircol collagen assay (Fig. 5c).

Expression of fibronectin and phospho-Smad2 was evaluated by immunohistochemical analysis. Fibronectin was upregulated by BLM treatment, and DAS administration significantly suppressed fibronectin expression (Fig. 5d). Quantification of phosphorylated Smad2-positive cell number in mice treated with both, BLM and

Fig. 3 Dasatinib suppressed Smad2 and Smad3 phosphorylation in TGFβ-treated BEAS-2B and A549 cells. a BEAS-2B cells were treated with or without 5 ng/mL TGFβ1 in the absence or presence of 5 μM DAS for 30 min. Then, equal amounts of lysates were analyzed by western blot analysis for p-Smad2, Smad2, p-Smad3, and Smad3. The levels of β-actin served as internal controls for equal protein loading in each lane. b The density of each band was quantified by densitometry using ImageQuant TL. The fold changes were calculated by setting the ratios of the phospho-protein/total protein band intensities in BEAS-2B cells. c A549 cells were treated with or without 5 ng/mL TGFβ1 in the absence or presence of 0.1 or 1 μM of DAS for 30 min. Then, equal amounts of lysates were analyzed by western blot analysis for p-Smad2, Smad2, p-Smad3, and Smad3, and β-actin. d The density of each band was quantified by densitometry using ImageQuant TL. The fold changes were calculated by setting the ratios of the phospho-protein/total protein band intensities in A549 cells.
DAS, were significantly lower than those in mice treated with both, BLM and vehicle (Fig. 5e). These findings suggest DAS inhibits ECM protein production and Smad2 phosphorylation in the lungs of a murine pulmonary fibrosis animal model.

Discussion

To our knowledge, this is the first study to investigate the efficacy of DAS on TGFβ-mediated EMT in human
Dasatinib attenuated BLM-induced pulmonary fibrosis in mice. ICR mice were intravenously injected with 10 mg/kg/day bleomycin or saline for 5 consecutive days (days 1–5). Mice were administered with either DAS (30 mg/kg/day) or 5% carboxymethyl cellulose or saline for 5 consecutive days. Mice were sacrificed at day 8 and lung specimens were obtained. A histological examination was performed by hematoxylin-eosin (HE) and Masson’s trichrome (MT) staining. Bar = 200 µm. Fibrotic changes in lungs were scored by Ashcroft score in each group of 4–6 mice. Data were expressed as mean ± SD. *p < 0.05. c The effect of DAS on whole lung collagen contents. Data were expressed as mean ± SD. *p < 0.05. d Effect of DAS on the expression of fibronectin by immunohistochemistry (IHC) in the murine lungs (magnification, ×40). 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 4–6 mice. *p < 0.0001. e IHC staining for phospho-Smad2 in the murine lungs (magnification, ×100). The phospho-Smad2-positive cells were counted in 6 fields at 100× magnification. Bar = 100 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 4–6 mice. *p < 0.001

alveolar epithelial cells in pulmonary fibrosis. This study has four key results: (A) DAS suppressed EMT and attenuated Smad2/3 phosphorylation in TGFβ-stimulated A549 and BEAS-2B cells; (B) DAS inhibited TGFβ1-induced cell motility of A549 and BEAS-2B cells; (C) DAS attenuated BLM-induced pulmonary fibrosis in mice; (D) DAS suppressed ECM production and Smad2 phosphorylation in the lungs of mice treated with BLM.

DAS is an inhibitor that targets Src family of kinases, Bcr-abl, and PDGF receptors, and is currently approved for treating a variety of neoplasia [19, 20]. Src kinases are involved in fibroblast activation, and the therapeutic inhibitory effect on Src kinases was shown in a BLM-induced pulmonary fibrosis model [21]. PDGF signaling has also been reported to attenuate pulmonary fibrosis [22, 23]. It has been demonstrated that DAS attenuates BLM-induced lung fibrosis in mice, and suppresses myofibroblast activation by inhibiting Src, PDGFR-α, and c-Abl [12]. DAS also inhibits TGFβ-induced myofibroblast differentiation through the Src-SRF Pathway [13]. Recently, it has also been reported that DAS reduces lung inflammation and fibrosis in acute experimental silicosis by inducing macrophage polarization toward the M2 phenotype [24]. This previous evidence suggests the potential efficacy of DAS for treating pulmonary fibrosis. However, the effect of DAS on TGFβ-mediated EMT of alveolar epithelial cells in pulmonary fibrosis has not been clarified.

In the pathogenesis of IPF, a possible role of TGFβ is to induce EMT in alveolar epithelial cells, and cells that have undergone EMT would contribute to the formation of fibroblastic foci and to abnormal ECM accumulation [25, 26]. EMT is characterized by loss of the epithelial marker E-cadherin, gain of mesenchymal markers including fibronectin, and enhanced cell motility. In this study, TGFβ1 stimulation led to EMT in A549 and BEAS-2B cells, and DAS suppressed TGFβ-induced EMT, especially the production of mesenchymal and ECM proteins, including collagen, and enhanced cell motility. These findings strongly suggest that DAS could block TGFβ-induced EMT and downregulate ECM production in human alveolar epithelial cells.

DAS has been widely studied for treating cancer. It has been reported that DAS effectively blocks TGFβ-induced Smad2/3 phosphorylation, cell migration, and TGFβ target gene expression, such as that of Snail and Slug, which are associated with EMT, in pancreatic adenocarcinoma cells [27]. In EGFR-mutated non-small cell lung cancers (NSCLC), EMt has been associated with acquired resistance to EGFR inhibitors [28]. Recent reports suggested that DAS was more effective against EGFR-mutated NSCLC cell lines with mesenchymal characteristics and overcome the EMT-associated resistance to EGFR inhibitors [29, 30]. These previous reports also support the results of this study that DAS suppresses TGFβ-induced EMT in human alveolar epithelial cells by inhibiting the Smad pathway.

It has been reported that DAS inhibits the progression of fibrosis in other organs. For instance, DAS prevents hepatic fibrosis induced by carbon tetrachloride via anti-inflammatory and antioxidant mechanisms [31]. DAS also attenuates pressure overload-induced cardiac fibrosis in a murine transverse aortic constriction model [32]. These findings suggest that DAS can be used as anti-fibrotic, anti-inflammatory, and anti-oxidative agent in multiple organs in clinical settings.

Pulmonary toxicities including interstitial lung disease is a major concern when treating patients with tyrosine kinase inhibitors. Although pleural effusions and pulmonary arterial hypertension are known to be associated with DAS, few studies have reported pulmonary infiltrates and interstitial changes associated with this drug. In one case series of 40 patients with imatinib resistance or intolerance who were treated with DAS (70 mg twice daily), 9 (22.5%) patients developed lung abnormalities, presumably related to DAS [33]. Although information is scarce regarding interstitial lung disease associated with DAS, it should be used with caution especially in patients with pre-existing pulmonary disease.

In conclusion, our findings suggest that DAS attenuates pulmonary fibrosis by suppressing TGFβ-mediated ECM protein production from mesenchymal cells via the inhibition of Smad2/3 phosphorylation. DAS might represent a promising and novel anti-fibrotic agent for preventing IPF.

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Conflict of interest The authors declare no conflicts of interest for this work.

Ethical Approval 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 approved by the Committee for Animal Experimentation of Juntendo University with the Approval No. 290031.

Research Involving Human Participants This article does not contain any studies with human participants performed by any of the authors.

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