Anti-fibrotic Effects of Cyclosporine A on Pulmonary Fibrosis: Role of Hypoxia-inducible Factor-1α

(肺線維症におけるシクロスポリン A の抗線維化作用の解明: 転写因子 Hypoxia-inducible Factor-1α の関与)

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1. INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and lethal disease associated with an extremely poor prognosis in most patients (Velme *et al*., 2007). The median survival of patients with IPF from the time of diagnosis is only 2 to 4 years, which is shorter than that of patients with various types of cancers (Meltzer and Noble, 2008). A prominent pathological characteristic of IPF is the formation of fibrotic foci consisting of myofibroblasts and the aberrant expression of extracellular matrix (ECM) proteins in the lungs, and the extent of fibrotic foci has been correlated with the mortality of patients (King *et al*., 2001; Kendall and Feghali-Bostwick, 2014). As Fig. 1 shows, the progression of fibrotic formation is associated with the accumulation of fibroblasts and promotion of fibroblast to myofibroblast differentiation (FMD) and also results in dyspnea due to the excessive scarring of lung tissue (King *et al.*, 2011). Therefore, myofibroblasts are responsible cells in fibrogenesis under pathological environments in the lung.

Myofibroblasts are characterized by the up-regulation of α-smooth muscle actin (αSMA), fibronectin, and collagens (Hashimoto et al., 2001; Hu et al., 2003). Among the many factors and cytokines regulating lung fibrosis, transforming growth factor-β1 (TGF-β1) is a critical factor promoting FMD. TGF-β1 was previously shown to be up-regulated in the lungs of patients with IPF, indicating that TGF-β1 is a strong profibrotic factor (Evans *et al.*, 2003). However, blocking TGF-β1 itself is not realistic: TGF-β1 also plays important roles in cell growth, differentiation, apoptosis, migration, immune cell functions, and TGF-β1-knock out mice die due to severe systemic inflammation (Shull et al., 1992). Therefore, revealing the responsible signaling in fibrosis would be required. As one of the *in vitro* assay models for examining the anti-profibrotic effects of various agents, the measurement of TGF-β1-induced FMD is a useful strategy. In addition, the inhibition of FMD may contribute to the development of fundamental treatments for IPF.

Although there is no effective pharmacological therapy to improve the survival of patients with IPF, the optimal strategy for the management of patients by steroid and/or immunosuppressants as well as clinical trials for new drugs have been actively investigated (du Bois, 2010; Staitieh, 2015). The beneficial effects of immunosuppressant-based combination therapy such as prednisone/azathioprine (AZA) and prednisone/AZA/N-acetylcysteine (PANTHER) on IPF have been proven in some cases (Raghu *et al.*, 1991; American Thoracic Society, 2000). Similarly, the acute exacerbation of IPF defined as the acute and clinically significant deterioration of an unidentifiable cause in patients with IPF is ameliorated by immunosuppressants (Sakamoto *et al.*, 2010). However, it currently remains unclear whether immunosuppressant-based therapies inhibit fibrosis, i.e., myofibroblast formation. On the other hand, increased risks of death and hospitalization were recently reported in PANTHER-IPF studies, and the survival of patients with the acute exacerbation of IPF previously treated with immunosuppression was less than that of never treated patients (Idiopathic Pulmonary Fibrosis Clinical Research Network, 2012; Papiris *et al.*, 2015). Thus, the effectiveness of immunosuppressant-based combination therapy for IPF patients is controversial. An evaluation of the effects of immunosuppressants on fibrosis may lead to the discovery of new indications such as anti-profibrotic actions. Furthermore, studies are needed in order to elucidate whether the intratracheal administration of estimated anti-profibrotic immunosuppressants is effective in an experimental IPF model for the following reasons: 1) a lower dose of an immunosuppressant than the usual dose systemically administered may be directly delivered to the fibrotic wounds associated with alveolar wall damage, which may increase therapeutic efficacy. 2) To the best of our knowledge, the effects of the intratracheal administration of immunosuppressants on pulmonary fibrosis *in vivo* have not yet been assessed in detail.

We herein screened the anti-FMD effects of various types of immunosuppressants *in vitro*,

and showed that cyclosporine A (CsA) inhibited TGF-β1-induced myofibroblast formation by enhancing the protein degradation of hypoxia-inducible factor (HIF)-1α. Similarly, CsA and the inhibition of HIF-1α dedifferentiated myofibroblast-like cells (MyoLCs) derived from patients with pulmonary fibrosis. Furthermore, the intratracheal administration of CsA or HIFi, even at the early fibrotic stage of bleomycin (BLM)-induced lung injury, markedly ameliorated the development of lung fibrosis.

2. ABBREVIATIONS

αSMA, α-smooth muscle actin; AZA, azathiopurine; BALF, bronchoalveolar lavage fluid; BLM, bleomycin; CN, calcineurin; CsA, cyclosporine A; DAPI, 4’6’-diamino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; dpi, days post instillation; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; FMD, fibroblast to myofibroblast differentiation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hematoxylin-eosin; HIF, hypoxia-inducible factor; HIFi, hypoxia-inducible factor-1α inhibitor; IPF, idiopathic pulmonary fibrosis; MT, Masson’ s trichrome; MyoLCs, myofibroblast-like cells; PFD, pirfenidone; PHD, prolyl hydroxylase; pSmad3, phosphorylated Smad3; qPCR, quantitative polymerase chain reaction; S100A4, S100 calcium-binding protein A4; SIS3, Smad3 inhibitor; TGF-β1, transforming growth factor-β1; VHL, von Hippel-Lindau

3. MATERIALS AND METHODS

Cell culture. Human fetal lung fibroblasts, HFL-1 cells and WI-38 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and RIKEN Cell Bank (Tsukuba, Ibaraki, Japan), respectively. Cells were maintained in culture medium, Dulbecco's modified Eagle's medium (DMEM) (Wako, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA, USA), and 0.1 mg/ml streptomycin and 100 U/ml penicillin (Meiji Seika, Tokyo, Japan) at 37˚C in a humidified atmosphere (5% CO_2). In some experiments, the human lung adenocarcinoma epithelial cell line, A549 (European Collection of Cell Cultures, Salisbury, UK) was used to measure TGF-β1-induced decreases in E-cadherin levels, a marker of epithelial cells, in the absence or presence of a designated agent.

In vitro FMD assay. After 24 h of serum deprivation in DMEM, cells were treated with 10 ng/ml human recombinant TGF-β1 (PeproTech, Rocky Hill, NJ, USA) for a further 48 h in the absence or presence of the designated agent. Control cells were subjected to serum deprivation for 72 h. Cell lysates were subjected to immunoblotting to estimate the expression of FMD markers. In some experiments, serum-deprived cells were treated with TGF-β1 for short time periods in the absence or presence of the designated agent, and cell lysates were subjected to measurements of phosphorylation signaling and protein levels.

Regarding the effects of immunosuppressants and inhibitors for signaling molecules on FMD, the following designated concentrations of the reagent were applied to HFL-1 cells or WI-38 cells under TGF-β1-induced FMD, and the levels of αSMA in cells were estimated by immunoblotting: CsA (a calcineurin (CN) inhibitor, Enzo Life Science, Farmingdale, NY, USA), FK506 (a CN inhibitor, LC Laboratories, Woburn, MA, USA), cyclophosphamide (a DNA alkylating agent, Sigma-Aldrich, St. Louis, MO, USA), AZA (an inosine

monophosphate dehydrogenase inhibitor, Santa Cruz Biotech., Santa Cruz, CA, USA), rapamycin (a mammalian target of rapamycin (mTOR) inhibitor, LC Laboratories), hydrocortisone (a glucocorticoid class of steroid hormones, Wako), pirfenidone (PFD, an anti-fibrotic drug for the treatment of IPF, Shionogi & Co., LTD., Osaka, Japan), 6,7-dimethoxy-2-((2E)-3-(1-methyl-2-phenyl-1H-pyrrolo[2,3-b]pyridine-3-yl-prop-2-enoyl)-1,2,3,4-tetrahydroisoquinoline (SIS3, a Smad3 inhibitor, Calbiochem, La Jolla, CA, USA), methyl 3-[[2-[4-(2-adamantyl)phenoxy]acetyl]amino]-4-hydroxybenzoate (a HIF-1α inhibitor (HIFi), Santa Cruz).

Immunoblotting. Cultured cell-derived lysates and lung-derived protein samples were electrophoresed on 10% SDS-PAGE and transferred to PVDF membranes. Washed and blocked membranes were reacted with the following primary antibodies (for antibodies' information, see Table1) at room temperature for 1 h. After washing, the membranes were incubated with a HRP-conjugated secondary antibody (1:2000-1:4000, anti-mouse IgG or anti-rabbit IgG) at room temperature for 1 h. Immunoreactive bands were visualized using a chemiluminescent reagent, the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA). Results were analyzed using a LAS-1000 plus system equipped with Science Lab software (Fuji-film, Tokyo, Japan). The intensity of chemiluminescence was measured with NIH ImageJ software.

RNA interference. WI-38 cells were transfected with siHIF-1α using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) in Opti-MEM (Thermo Fisher Scientific) for 6 h and incubated in culture medium a further 24 h. After 24 h of serum deprivation, cells were treated with TGF-β1 for 48 h, and these cell lysates were subjected to immunoblotting for αSMA.

Nuclear translocation of pSmad3. WI-38 cells were treated with TGF-β1 in the absence or presence of HIFi (5 µM), fixed, and subjected to an immunofluorescent study. Cells were incubated with the anti-pSmad3 antibody, following by the reaction with Alexa Flour 488-conjugated anti-rabbit IgG, and observed under a confocal fluorescence microscope (LSM 780, Zeiss, Oberkochen, Germany). Nuclei were stained with 4'6'-diamino-2-phenylindole (DAPI).

Assay for cellular CN activity. Cellular CN activity was examined using an assay kit (Enzo Life Sci, Farmingdale, NY, USA). In brief, WI-38 cells were cultured with DMEM for 24 h, and treated with 10 µM CsA or 10 µM FK506 for a further 30 min. Washed cells were suspended in the lysis buffer, lysates after centrifugation were applied to a column to remove free phosphate, and CN activity was measured according to the manufacturer's protocol.

Total RNA isolation and quantitative polymerase chain reaction (qPCR). After 24 h of serum deprivation in DMEM, cells were treated with TGF-β1 for a further 24 h in the absence or presence of the designated agent. Total RNA was isolated from WI-38 cells using Isogen II (Nippon Gene, Toyama, Japan) according to manufacturer's instructions. The total RNA samples (0.5 µg) were reverse-transcribed into cDNAs with ReverTraAce® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Relative gene expressions were quantified in Pikoreal 96 (Thermo Scientific) by using PowerUp™ SYBR™ Green Master Mix (Thermo Scientific). Expression levels of target gene were corrected for Gapdh expression levels by using ΔΔCt method (Pikoreal software 2.2). The primer sequences were as follows: *Acta2* (5'-CTGGCATCGTGCTGGACTCT-3' and 5'-GATCTCGGCCAGCCAGATC-3'), *FN1* (5'-GAGCTATTCCCTGCACCTGATG-3' and 5'-CGTGCAAGGCAACCACACT-3'), and *Gapdh* (5'-GCACCGTCAAGGCTGAGAAC-3'

and 5’- TGGTGAAGACGCCAGTGGA-3’). Amplification parameters were as follows: one cycle of 50˚C for 2 min and 95˚C for 2 min, followed by 40 cycles of 95˚C for 15 s, 55˚C 15 s, and 72˚C 60 s.

Degradation assay of transiently overexpressed HIF-1α. HIF-1α cDNA (GenBank Accession No: BC012527, Thermo Fisher Scientific) was subcloned into an expression vector with the CMV promoter. HEK293T cells were transiently transfected with the vector for HIF-1α using Lipofectamine 2000 (Invitrogen) in Opti-MEM for 6 h and cultured in culture medium for a further 24 h. Cells were then treated with 10 µM CsA, 10 µM MG132 (a proteasome inhibitor, Merck Millipore, Billerica, MA, USA), or 10 µM CsA plus 10 µM MG132 for 30 min.

Primary culture of human lung MyoLCs. Lung tissue from a 19-year-old male patient who underwent lung transplantation due to severe pleuroparenchymal fibroelastosis was obtained after surgery. The patient provided informed consent, and the study was approved by the Ethics Committee of Chiba University, Graduate School of Medicine and Hospital. Lung tissue was minced into small pieces. After rinsed with phosphate-buffered saline (PBS), pieces were incubated in DMEM supplemented with 1 mg/ml collagenase type I (Worthington, Lakewood, NJ, USA), 0.5 mg/ml dispase (Life Technologies), 2 U/ml DNase (QIAGEN, Valencia, CA, USA), 0.1 mg/ml streptomycin, and 100 U/ml penicillin at 37˚C for 15 min with gentle shaking. After washing twice with DMEM, the resulting pieces transferred to an 80-cm^2 culture flask (Thermo Fisher Scientific) were dipped with culture medium and cultured at 37˚C and 5% CO_2. The outgrowth of cells was monitored weekly with changes in culture medium every 4 days. When the flask reached confluence, outgrown cells were harvested as cells at passage 0. Expanded cells at passage 3 were used in

experiments.

In the immunofluorescent study, cells were incubated with the mouse anti-αSMA antibody, the rabbit anti-S100 calcium-binding protein A4 (S100A4) antibody, or mouse anti-fibronectin antibody, following by a reaction with Alexa Flour 594-conjugated anti-mouse IgG and Alexa Fluor 488 phalloidin, and were then observed under a fluorescence microscope (Axio Imager A2, Zeiss, Oberkochen, Germany). Phalloidin was used to detect actin filaments. Nuclei were stained with DAPI.

Animals. Male C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan) and were used at 8-12 weeks of age. Animals were housed in the Animal Resource Facility of Chiba University under pathogen-free conditions and cared for according to the animal care guidelines of Chiba University. Experiments were performed according to an animal protocol approved by the Animal Welfare Committee of Chiba University.

BLM-induced lung injury model. Mice under anesthesia were given a single intratracheal injection of BLM hydrochloride (3 mg/kg, Nippon Kayaku, Tokyo, Japan) dissolved in saline, using a Microsprayer® atomizer (PennCentury, Philadelphia, PA, USA). Control mice received a sham treatment with saline. At the designated days post-instillation (dpi) of BLM, mice under anesthesia were intracardially perfused with ice-cold PBS to thoroughly wash out blood cells in the lungs and were then sacrificed. The left lung lobes were separated from the trachea and main bronchi. The upper parts were homogenized and centrifuged at 4˚C to prepare supernatants as protein samples for immunoblotting. The lower parts of the lobes were fixed, dehydrated, and frozen for histopathological analyses and immunofluorescent studies. Freshly cut lung sections (thickness of 5 μm) placed on poly-L-lysine-coated slides were stained with hematoxylin-eosin (HE) or Masson' s trichrome (MT) in order to visualize

inflammatory or fibrotic lesions. The semi-quantitative elucidation of lung fibrotic changes was performed according to Ashcroft and co-workers' method with a modification (Kobayashi *et al.,* 2015). Briefly, under x100 magnification, the grade of lung fibrosis was scored on a scale of 0 to 8 (Hubner *et al.,* 2008) by examining 10 randomly selected fields in each section (more than 4 sections/each group), and the average score was calculated. All sections were analyzed in a blinded manner by three independent researchers. In some experiments, sections were subjected to double staining with the anti-HIF-1α antibody in combination with the anti-αSMA antibody, rabbit anti-S100A4 antibody, rabbit anti-ionized calcium-binding adaptor molecule (Iba)-1 antibody (Wako), hamster anti-podoplanin/gp36 antibody (Abcam), or rabbit anti-pro-surfactant protein (pro-SP)-C antibody (Tanaka *et al.,* 2014), and this was followed by a reaction with the appropriate fluorescein-conjugated secondary antibodies.

In order to elucidate the effects of CsA or HIFi on BLM-induced lung injury, mice were intratracheally administered CsA dissolved in castor oil (0.5 mg/kg/day) or HIFi (0.1 mg/kg/day) at 7, 8, and 9 dpi. Mice were divided into the following 6 groups: a control group (saline at 0 dpi, castor oil/saline at 7-9 dpi); CsA or HIFi-control group (saline at 0 dpi, CsA or HIFi at 7-9 dpi); BLM group (BLM at 0, castor oil/saline at 7-9 dpi); and BLM-CsA or HIFi group (BLM at 0 dpi, CsA or HIFi at 7-9 dpi), and differences in pathological outputs between these groups were estimated at the designated times. In some experiments, bronchoalveolar lavage fluid (BALF) was collected with 1 ml PBS using a 20-gauge catheter at 14 dpi. After centrifuging BALF at 400 x *g* for 10 min, the cell pellet was resuspended in PBS and subjected to a cell count using a hemocytometer.

Measurement of soluble collagen. Collagen contents in lung samples were measured using Sircol™ Soluble Collagen Assay kit (biocolor life science assays, Carrickfergus, UK)

according to the manufacturer's protocol. In brief, the minced left lung samples were digested in 0.1 mg/mL pepsin/0.5 M acetic acid at 4˚C overnight. The digested samples were centrifuged to remove tissue debris, and the resulting supernatants were subjected to the kit.

Statistical analysis. Data are expressed as the mean ± S.E. Statistical analyses were conducted using Graphpad Prism Version 6 (GraphPad Software Inc., San Diego, CA). The significance of differences was assessed using the Student's *t*-test or an analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ were considered significant.

4. RESULTS

CsA prevented TGF-β1-induced FMD. In HFL-1 cells, αSMA levels were detected under serum-starved conditions, and the treatment with 10 ng/ml TGF-β1 for 48 h markedly increased αSMA levels, as described previously (Kawashima *et al.,* 2012). We examined the effects of several drugs (CsA, FK506, cyclophosphamide, AZA, rapamycin, hydrocortisone, and PFD) used clinically for TGF-β1-induced FMD by monitoring changes in the expression of αSMA, a marker for myofibroblasts (Hinz *et al.,* 2007). Among them, only CsA dose-dependently exerted a significant counter effect on the TGF-β1-induced up-regulated expression of αSMA (in my main publication, Fig. 1A and Fig. S1). CsA significantly inhibited the expression levels of αSMA, even under the TGF-β1-free state. In the presence of 10 μM CsA, αSMA levels in cells stimulated with TGF-β1 were similar to control levels under the resting state, indicating that CsA exerts anti-FMD effects. The treatment with 5 mM PFD, an orally active drug approved for the treatment of IPF, slightly inhibited the TGF-β1-induced up-regulated expression of αSMA. However, its anti-FMD efficacy was weak and not significant, at least in our assay system (in my publication, Fig. S1). Although CsA and FK506 both act as inhibitors of CN, FK506 did not inhibit the TGF-β1-induced up-regulated expression of αSMA (in my publication, Fig. S1). The anti-FMD efficacy of CsA in comparison with FK506 was also investigated in WI-38 cells, another cell line of human lung fibroblasts. CsA significantly inhibited the TGF-β1-induced expression of not only αSMA, but also fibronectin, another myofibroblast marker (in my publication, Fig. 1, B and C). In contrast, the two markers up-regulated by TGF-β1 were not inhibited by the treatment of cells with FK506 (in my publication, Fig. 1D). Although a marked difference was observed in anti-FMD efficacy between CsA and FK506, both agents significantly inhibited the activity of CN in the lysate from WI-38 cells (in my publication, Fig. 1E). These results indicate that the anti-FMD effects of CsA are caused by the targeting of some molecules other than CN. Therefore, the mechanisms underlying the anti-FMD effects of CsA

were elucidated.

*HIF-1*α *was involved in TGF-β1-induced FMD.* TGF-β1 responses are mediated by the Smad pathway in various cells including lung fibroblasts (Rahimi and Leof, 2007). TGF-β receptor type I-phosphorylated Smad3 forms a complex with Smad4, translocates into the nucleus as a transcription factor and binds to the *Acta2* (αSMA gene) promoter containing CAGA motifs, termed the Smad3-binding element, where the TGF-β1/Smad3 signaling axis regulates the expression of αSMA at the transcriptional level in myofibroblast differentiation (Hu *et al.,* 2003). Consistent with this finding, SIS3, a specific Smad3 inhibitor, significantly inhibited the TGF-β1-induced up-regulated expression of αSMA in our FMD assay with WI-38 cells (in my publication, Fig. 2A). TGF-β1 augmented the phosphorylation of Smad3 in WI-38 cells within 15 min of the stimulation, which was insensitive to the treatment with CsA (in my publication, Fig. 2B). These results indicate that the anti-FMD effects of CsA are not accompanied by the modulation of Smad3 dephosphorylation.

In lung fibroblasts, it has been demonstrated that TGF-β1 responses are regulated by another transcription factor, HIF-1 (Abdul-Hafez *et al.,* 2009; Zhou *et al.,* 2011; Kottmann *et al.,* 2012) and also that TGF-β1 regulates HIF-1α levels (Bardos and Ashcroft, 2005; D'Angelo *et al.,* 2003). We investigated the contribution of HIF-1α to TGF-β1-induced FMD in WI-38 cells. Expression levels of HIF-1α markedly increased 3 and 6 h after the treatment of cells with TGF-β1 (in my publication, Fig. 3A). The inhibition of HIF-1α by its chemical inhibitor (HIFi) significantly decreased the TGF-β1-induced expression of αSMA and fibronectin (in my publication, Fig. 3B). The inhibitory effects of HIFi on TGF-β1-induced αSMA expression were replicated by the introduction of siHIF-1α into cells (in my publication, Fig. 3C). On the other hand, neither the TGF-β1-induced phosphorylation of Smad3 nor the TGF-β1-induced translocation of active Smad3 were affected by the treatment

of WI-38 cells with HIFi (in my publication, Fig. 3, D and E). Considering the function of HIF-1α as a transcriptional factor, the inhibitory effects of HIFi on TGF-β1-induced protein expression of αSMA and fibronectin could result from the changes in mRNA levels of the two key molecules in FMD. To evaluate this point and investigate whether CsA treatment also suppresses the TGF-β1-induced mRNA expression of αSMA (*Acta2*) and fibronectin (*FN1*), qPCR was performed with monitoring the inhibitory effects of SIS3 as indices. As shown in Fig. 3F in my publication, either treatment with HIFi or CsA significantly suppressed the TGF-β1-induced mRNA expression of *Acta2* and *FN1*.

In C6 glioma cells, a previous study showed that CsA stimulates PHD activity, specifically modifying Pro-564 within the oxygen-dependent degradation domain of HIF-1α, and triggered the VHL-dependent ubiquitin-proteasome pathway, leading to the destabilization of HIF-1α (D'Angelo *et al.,* 2003). In the same study, FK506 did not function as a direct stimulator of PHD. In conjunction with our present results that only CsA was effective against TGF-β1/HIF-1α signaling-mediated FMD, we hypothesized that CsA may lower HIF-1α protein stability. Considering their established high transfection efficiency and endogenous PHD2 activity, HEK293T cells were used in a degradation assay of HIF-1α (Berchner-Pfannschmidt *et al.,* 2008). As shown in Fig. 4A in my publication, HIF-1α-introduced cells showed a strong HIF-1α signal in immunoblotting. This HIF-1α expression was significantly decreased by the 30-min treatment of cells with 10 μM CsA, but not 10 μM FK506. The CsA-induced down-regulated expression of HIF-1α was canceled by the application of 10 μM MG-132, a proteasome inhibitor, to cells. The expressions of PHD2 and VHL protein were not affected in this experimental condition (in my publication, Fig. 4B). These results indicate that CsA exerts anti-FMD effects via the proteasome-mediated degradation of HIF-1α.

CsA and inhibition of HIF-1α-induced dedifferentiation of MyoLCs. In addition to the inhibition of FMD, the induction of the dedifferentiation of established myofibroblasts may be a highly relevant strategy in therapies for progressive fibrotic disorders (Hecker *et al.,* 2011). Therefore, we investigated whether CsA induced the dedifferentiation of MyoLCs derived from the lungs of the patient with IPF. Primary cultured cells at passage 3 clearly showed immunoreactivity for αSMA, but not S100A4, a fibroblast marker, and expressed a markedly higher level of fibronectin than that in WI-38 cells (in my publication, Fig. 5, A and B), indicating that primary cells possessed the characteristics of myofibroblasts. In MyoLCs, the treatment with CsA induced a marked down-regulation in HIF-1α expression (in my publication, Fig. 5C). Immunoblotting and immunofluorescent studies clearly showed that CsA down-regulated the expression of αSMA and fibronectin in MyoLCs, which was replicated by the inhibition of HIF-1α (in my publication, Fig. 5, D and E).

Therefore, the possibility of CsA exerting anti-FMD effects and myofibroblast dedifferentiation-promoting activity that are beneficial for pulmonary fibrosis was proven. This was also supported by additional *in vitro* observations that CsA, but not FK506 significantly inhibited TGF-β1-induced epithelial-mesenchymal transition (EMT) associated with a decrease in E-cadherin expression in A549 cells (in my publication, Fig. S2). EMT is recognized as one of the mechanisms responsible for pulmonary fibrogenesis (Kasai *et al.,* 2005). Due to the suppressive effects of CsA on fibrosis, the anti-fibrotic effects of CsA were then examined in a BLM-induced lung injury model.

CsA and inhibition of HIF-1α prevented BLM-induced lung injury. In a mouse model with the single intratracheal administration of BLM, acute alveolitis and interstitial inflammation accompanied by the accumulation of leukocytes and pulmonary edema were induced within one week, and fibrotic lung lesions were clearly observed in patches at 7 dpi, which is termed

the early fibrotic stage. BLM-induced fibrotic responses including the synthesis of pro-fibrotic proteins such as αSMA and collagen subsequently progressed, and large parts of alveoli were obliterated with fibrous masses at 14 dpi (Kobayashi *et al.,* 2015; Yamauchi *et al.,* 2011). Consistent with previous findings, the up-regulation of αSMA in the lung was typically detected at 7 dpi, the level of which increased at least until 14 dpi (in my publication, Fig. S3A). At 7 dpi, but not 3 dpi, when the BLM-induced up-regulated expression of αSMA was detected, small fibrotic foci were sparsely observed in lung sections stained with HE (in my publication, Fig. S3B), indicating that this time point represents the plausible onset of the fibrotic stage. Prior to the BLM-induced up-regulated expression of αSMA, the expression of HIF-1α in the lung was induced at 3 dpi and sustained at least until 14 dpi (in my publication, Fig. S3A). The sustained induction of TGF-β1 mRNA was occasionally detected on 3 dpi (Gurujeyalakshmi *et al.,* 1995), indicating that the TGF-β1/HIF-1α signaling pathway contributes to *in vivo* FMD. This was supported by an immunofluorescent study showing that HIF-1α-like immunoreactivity was mainly localized in αSMA^+ myofibroblasts and $S100A4^+$ fibroblasts in the lung at 7 dpi (in my publication, Fig. S3C, a and b). HIF-1α-like immunoreactivity was weakly observed in ionized calcium-binding adaptor molecule (Iba)-1^+ macrophages, as previously reported (Ueno *et al.,* 2011), but not in $gp36^+$ type I or pro-surfactant protein (SP)-C^+ type II alveolar epithelial cells (in my publication, Fig. S3C, c-e). Based on these results, BLM-treated mice received an intratracheal injection of CsA or HIFi at 7, 8, and 9 dpi when in the early fibrotic state (in my publication, Fig. 6A).

At 14 dpi, the expression level of αSMA in the lung was significantly higher in the BLM group than in the control and CsA-control groups, and was lower in the BLM-CsA group (in my publication, Fig. 6B). In parallel with these phenomena, the BLM-induced up-regulated expression of HIF-1α was fully sensitive to the administration of CsA (in my publication, Fig. 6C). Furthermore, the inhibition of BLM-induced αSMA expression by CsA was clearly

replicated by HIFi at 14 dpi. As shown in Fig. 6D in my publication, the expression level of αSMA in the lung was significantly higher in the BLM group than in the control and HIFi-control groups, and was significantly lower in the BLM-HIFi group. In order to elucidate whether changes in αSMA expression among the groups correlated with pathological outputs in the lung, lung sections from each group at 14 dpi were subjected to HE and MT (in my publication, Fig. 6E and F, respectively) staining. Fibrotic lesions including robust collagen deposition, thickening of the alveolar walls, and the accumulation of inflammatory cells were observed in lungs from the BLM group, and were markedly decreased in lungs from the BLM-CsA and BLM-HIFi groups. In contrast, no pathological changes were observed in the three control groups (control, CsA-control, and HIFi-control). These histopathological results were supported by both the modified Ashcroft scale analysis and the soluble collagen assay. BLM markedly and significantly increased the Ashcroft score and the collagen content over those in the three control groups, and they were significantly decreased by the application of CsA or HIFi (in my publication, Fig. 6G and H). Similarly, the numbers of total inflammatory cells in BALF at 14 dpi were significantly elevated by BLM, and this was significantly inhibited by the application of CsA or HIFi (in my publication, Fig. 6I). These results indicate that the CsA-sensitive HIF-1α pathway functions at least at the early fibrotic stage and contributes to the establishment of fibrosis in the BLM-induced lung injury model. In order to rule out the possibility that the CsA-sensitive HIFi pathway simply delays BLM-induced lung fibrotic formation, fibrosis read-outs were extended to 21 dpi. As shown in Fig. S4 in my publication, the BLM-induced up-regulated expression of αSMA was significantly attenuated by the application of HIFi, showing good parallelism with HIFi markedly ameliorating BLM-induced lung fibrosis at 21 dpi.

5. DISCUSSION

Some patients with pulmonary fibrosis are treated with immunosuppressive drugs. However, their effects on FMD have not yet been studied in detail. Furthermore, treatments with immunosuppressive drugs occasionally lead to serious clinical issues such as renal dysfunction, which are in part due to the administration route and dosage. In the present study, we showed that CsA inhibited TGF-β1-induced FMD in human lung fibroblasts and reduced the established expression of αSMA and fibronectin in lung MyoLCs from a patient with IPF. These effects of CsA were mediated via the down-regulated expression of HIF-1α. We also demonstrated that the intratracheal administration of CsA or HIFi ameliorated pulmonary fibrosis in a BLM-induced lung injury model.

CsA targets HIF-1α, which plays an important role in the TGF-β1 signaling pathway.

Considering the mode of action of CsA as a CN inhibitor, we speculated that TGF-β1-induced FMD in human lung fibroblasts may be mediated in a CN-dependent manner. We confirmed the marked inhibition of CN activity in WI-38 cell lysates treated with CsA or FK506. However, CsA, but not FK506 exerted anti-FMD effects in HFL-1 and WI-38 cells, suggesting that CN activity does not function in TGF-β1-induced FMD in human lung fibroblasts. We then focused on HIF-1α in the TGF-β1-evoked signaling pathway and pulmonary fibrosis. *In vitro,* TGF-β1 has been shown to inhibit the expression of PHD2 to increase HIF-1α (Abdul-Hafez *et al.,* 2009; Ueno *et al.*, 2011; McMahon *et al.*, 2006; Han *et al.,* 2013). Furthermore, under our experimental conditions, TGF-β1 and BLM induced the expression of HIF-1α *in vitro* and *in vivo*, respectively. In both cases, an increase in HIF-1α was achieved prior to the induction of αSMA and formation of fibrotic foci, suggesting that HIF-1α is a cause of FMD. Similar to the CsA treatment, the inhibition of HIF-1α by HIFi and its siRNA markedly attenuated the TGF-β1-induced expression of αSMA in WI-38 cells. These results were supported by previous findings showing that HIF-1α interacts with

hypoxia response elements in the αSMA promoter as a positive regulator (Han *et al.*, 2013; Watanabe *et al.*, 2014).

Consistent with previous findings (D'Angelo *et al.*, 2003; Kim *et al.*, 2008), we observed that transiently overexpressed HIF-1α in HEK293 cells was significantly decreased by the application of CsA, and this was canceled when cells were treated with CsA concomitantly with MG132. Therefore, the anti-FMD effects of CsA may be mediated by promoting the proteasomal degradation of HIF-1α; although we did not investigate whether CsA changed PHD2 activity under our experimental conditions, at least the protein levels of PHD2 and VHL were not changed. Moreover, HIF-1α positively regulates EMT in various cells including A549 human adenocarcinomic alveolar epithelial cells (Zhu *et al.*, 2016). The number of pulmonary epithelial cells in the lungs of IPF patients was shown to decrease due to EMT and apoptosis (Thannickal and Horowitz, 2006). In the present study, the CsA treatment inhibited TGFβ1-induced reductions in E-cadherin, a marker of epithelial cells that decreases when EMT occurs.

CsA and HIFi may induce the dedifferentiation of MyoLCs.

In recent studies, two types of myofibroblasts have been proposed: proto-myofibroblasts (an intermediate state between fibroblasts and differentiated myofibroblasts) and differentiated myofibroblasts. The former expresses less αSMA and is capable of proliferating, whereas the latter expresses αSMA more strongly in extensively developed actin stress fibers and is nearly incapable of proliferating (Tomasek *et al.*, 2002; Driesen *et al.*, 2014). In the MyoLCs used in the present study, we observed the established expression of αSMA and fibronectin, similar to other reported primary (myo-) fibroblasts derived from the lungs of patients with pulmonary fibrosis (Reddy et al., 2014). Although the αSMA was not incorporated into actin stress fibers, when MyoLCs were cultivated with TGF-β1, most of

αSMA in MyoLCs co-localized with actin stress fibers (data not shown). In addition, cells exhibited proliferative ability with serum (cell doubling time: 72-84 h, n=9), similar to normal fibroblasts. Based on these results, MyoLCs are likely characterized as proto-myofibroblasts. Even if MyoLCs are not fully differentiated myofibroblasts, they were sufficiently aggressive to cause severe fibrosis in the patient's lungs. The expression of myofibroblast markers was significantly decreased by the treatment of MyoLCs with CsA or HIFi. Recent studies have demonstrated that the dedifferentiation of myofibroblasts associated with the reduced expression of αSMA and ECM proteins may be induced in lung fibroblasts under certain experimental conditions (Reddy *et al.*, 2014; Garrison *et al.*, 2013; Penke *et al.*, 2014). Hence, in addition to anti-FMD effects, CsA and HIFi may also promote the dedifferentiation of myofibroblasts, at least *in vitro*. Basically, blocking an autocrine loop with the positive feedback of TGF-β1 appears to be one of the necessary, but not sufficient steps for inducing the dedifferentiation of myofibroblasts (Hecker *et al.*, 2011; Kosla *et al.*, 2013). However, the detailed molecular mechanisms remain unclear.

Anti-fibrotic effects of CsA and HIFi in BLM-induced pulmonary fibrosis model mice.

We found that HIF-1α was induced the latest from 3 dpi and then clearly localized in fibrotic foci expressing αSMA at 7 dpi. Based on our *in vitro* results, since CsA may lead to the loss of function of HIF-1α, the administration of CsA or HIFi at the fibrotic stage of BLM-induced pulmonary injury (from 7 dpi) may exert therapeutic effects *in vivo*. In addition, we considered this administration protocol to be reasonable and practical. Most IPF patients presenting to clinicians for the first time with subjective symptoms show a reduced forced vital capacity, suggesting the early fibrotic stage (King *et al.*, 2001b). As expected, BLM-induced lung fibrosis was significantly inhibited by the application of CsA or HIFi. We have investigated that the concomitant administration of CsA and HIFi did not show any

synergistic inhibitory effect on BLM-induced lung fibrosis (data not shown), supporting the notion that CsA may exert anti-FMD effects via the proteasome-mediated degradation of HIF-1α.

Although the administration of PGE2 was previously reported to be capable of dedifferentiating myofibroblasts *in vitro* (Garrison *et al.*, 2013; Wettlaufer *et al.*, 2016), it had no therapeutic effect on pulmonary fibrosis when administered at the fibrotic stage of BLM-induced lung injury (Dackor *et al.*, 2011). Similar to the case described in the present study, the endogenous agonist of peroxisome proliferator-activated receptor γ (PPARγ), nitrated fatty acid, which exhibits anti-FMD and myofibroblastic dedifferentiation activities, may inhibit BLM-induced pulmonary fibrosis following its intratracheal administration at the fibrotic stage (Reddy *et al.*, 2014). Therefore, the amelioration of BLM-induced lung fibrosis by CsA or HIFi may be achieved in part by dedifferentiating myofibroblasts; however, more detailed studies are warranted.

In our studies, the intratracheal administration of a low dose of CsA (0.5 mg/kg/day) succeeded in ameliorating BLM-induced pulmonary fibrosis. On the other hand, the administration of 50 mg/kg/day CsA through an unknown route also suppressed BLM-induced lung fibrosis by blocking CD147 in macrophages (Geng *et al.*, 2014). Therefore, the effects of CsA on various types of cells in lung tissues need to be examined more.

Although various drug candidates have been developed, there is still no effective drug for the treatment of pulmonary fibrosis that prolongs survival. Greater effectiveness may be achieved if the drug exerts anti-FMD effects and the ability to promote the dedifferentiation of myofibroblasts. Therefore, the results of the present study will be useful for developing novel therapeutic drugs.

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7. EXAMINERS

This thesis was reviewed by the following examiners authorized by the Graduate School of Pharmaceutical Sciences, Chiba University.

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9. LIST OF PUBLICATIONS

[Main thesis publication]

Yamazaki R, Kasuya Y, Fujita T, Umezawa H, Yanagihara M, Nakamura H, Yoshino I, Tatsumi K, Murayama T: Anti-fibrotic Effects of Cyclosporine A on TGF-β1-treated Lung Fibroblasts and Lungs from Bleomycin-treated Mice: Role of Hypoxia-inducible Factor-1α. FASEB J. (2017) in press, doi: 10.1096/fj.201601357R

10. TABLE AND FIGURE

Antibody	Type	Clone	Provider
α-smooth muscle actin (αSMA)	mouse monoclonal	1A4	Sigma-Aldrich
β-tubulin	mouse monoclonal	TUB2.1	Sigma-Aldrich
β-actin	mouse monoclonal	AC-15	Sigma-Aldrich
fibronectin	mouse monoclonal	IST-3	Sigma-Aldrich
ED-A fibronectin	mouse monoclonal	IST-9	Santacruz
phospho-Ser$^{423/425}$-Smad3 (pSmad3)	rabbit monoclonal	N.A.	Cell Signaling Technology
Smad3	rabbit monoclonal	N.A.	Cell Signaling Technology
E-cadherin	mouse monoclonal	36	BD Biosciences
hypoxia-inducible factor 1α (HIF-1α)	mouse monoclonal	H1alpha67	NOVUS biologicals
prolyl hydroxylase 2 (PHD2)	mouse monoclonal	H-8	Santa Cruz
von Hippel-Lindau (VHL)	mouse monoclonal	VHL40	Santa Cruz
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	mouse monoclonal	5A12	Wako
S100 calcium-binding protein A4 (S100A4)	rabbit polyclonal	N.A.	Abcam
ionized calcium-binding adaptor molecule (Iba)-1	rabbit polyclonal	N.A.	Wako
podoplanin/gp36	hamster monoclonal	RTD4E10	Abcam
pro-surfactant protein (pro-SP)-C	rabbit polyclonal	N.A.	provided by Dr. Kasuya Y

Table 1. The list of primary antibodies used in this study

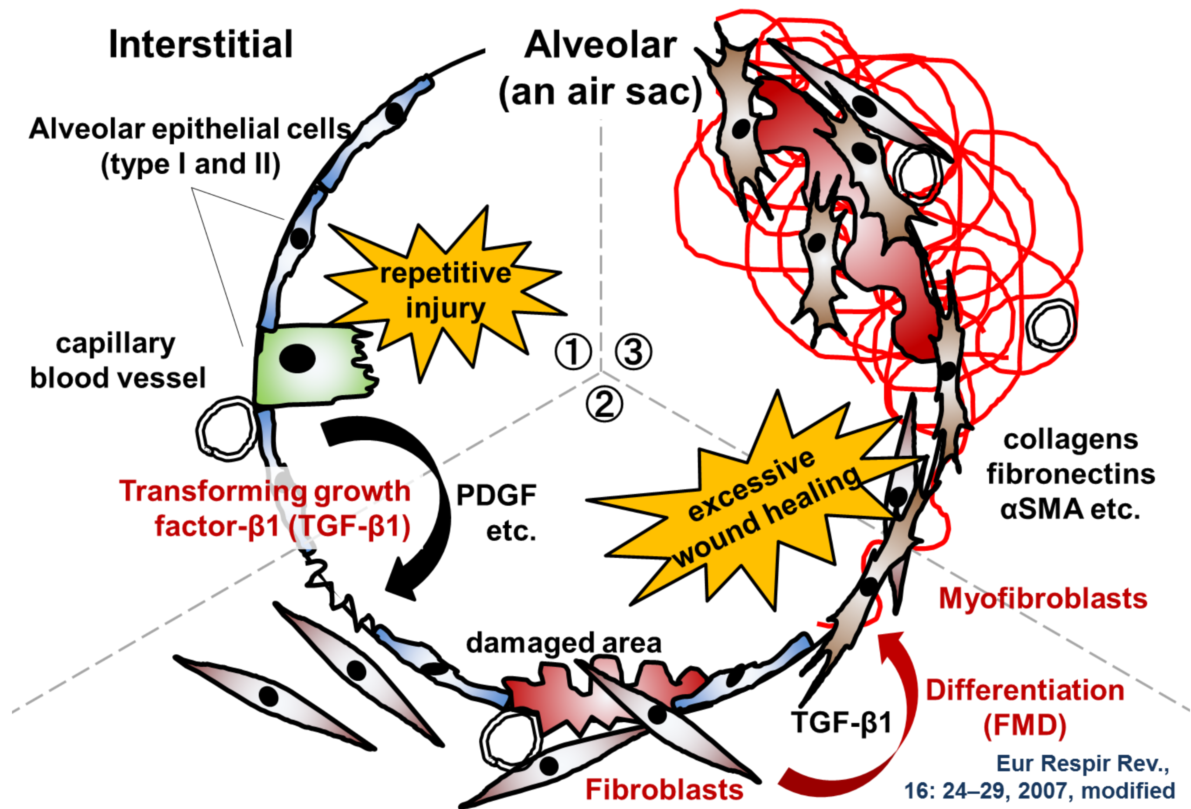

Figure 1. Current model for pathogenesis of IPF

11. POSTSCRIPT

1. Mode of action of CsA in promoting the activity of PHD2

CsA needs to form complex with an immunophilin, cyclophilin A, when it inhibits calcineurin. However, it is not known if CsA needs cyclophilin A as well in the case of promoting the activity of PHD2.

To answer the question, the following experiments would be needed: ① treating WI-38 cells with DEBIO-025, an analogue of CsA, which cannot bind to cyclophilin A (Paeshuyse et al., 2006), and then test if DEBIO-025 can inhibit TGF-β1-induced FMD, ② examining the effect of CsA in WI-38 cells that carry much less amount of cyclophilin A by RNA interference, or ③ *in vitro* PHD2 activity assay using recombinant human PHD2 protein, its substrates, and cyclosporine A. At least, *in vitro* PHD2 activity was increased by CsA in homogenized cell lysates (D'Angelo et al., 2003, listed in "6 References".

2. Dedifferentiation from MyoLCs

The MyoLCs treated with HIFi for 48 h expressed S100A4, a marker protein for fibroblasts, supporting the possibility of dedifferentiation by inhibiting HIF-1α (unpublished data). To further examining what is occurred during the dedifferentiation, gene expression in the cells treated with or without CsA and HIFi analysis should be performed. It was reported that PGE2 reversed the expression of genes up-regulated by TGF-β1 (Wettlaufer et al., 2016, listed in "6. Reference"). However, concerning that inhibition of TGF-β1 signaling is not enough for inducing dedifferentiation (Kosla et al., 2016, listed in "6. Reference"), inhibition of HIF-1α pathway might affect other gene expression besides TGF-β1 signaling.

It is also required to have other maker proteins for fibroblasts. In my study, S100A4 was used as a marker protein for fibroblasts. In general, it is preferable to use plural markers when identifying the character of the cells, however there is not many specific marker proteins

available for fibroblasts. Comparing gene expressions between fibroblasts and myofibroblasts would be helpful to find appropriate specific genes (proteins) as marker proteins for fibroblasts. It is important to identify the origin of myofibroblasts in order to understand the pathogenesis of IPF because recently it is thought that myofibroblasts can be differentiated from various types of cells as described in "5. Discussion" (also, Stempien-Otero et al., 2016).

3. Roles of inflammation/inflammatory cells

Inflammation was thought to be important in development of IPF. Currently, it is widely accepted that inflammation and fibrosis should be considered as different phenomena. Still, emerging numbers of reports suggesting the positive correlation between some populations of macrophages and fibrosis (Sato T *et al.,* 2017). Among them, SatM (Segregated Nucleus atypical monocytes) were shown to have strong ability to promote BLM-induced pulmonary fibrosis in mice (Sato T *et al.,* 2017). CCAAT/enhancer-binding protein (C/EBP) β is essential for differentiate the progenitor cells of SatM into SatM. However, if CsA and HIFi inhibit C/EBPβ is unknown.

On 14 dpi, the total cell number in BALF (bronchoalveolar lavage fluid) was increased in BLM group compared with control. Those were decreased in BLM/CsA group and BLM/HIFi group (data not shown). The results indicated that treatment with CsA or HIFi suppressed inflammation on 14 dpi. Hence, it is difficult to exclude the possibility that CsA or HIFi restored fibrosis by inhibiting inflammatory response. However, according to the results in vitro and concerning that inflammation mainly occurs within a week, it is more reasonable to think that anti-inflammatory effects were not their main effect in my experimental condition.

4. Which would be better treatment agent for IPF, CsA or inhibitor of HIF-1α?

CsA is clinically used drug for decades, thus its metabolic pathway and safety in human use has been confirmed. Concerning the long process of drug approval, CsA has slightly less barrier than inhibitors of HIF-1αbefore they would become treatments for IPF. However, in this case, the change in formulation will be necessary. In the case of inhalant, to let it reach the alveoli, the particle size should be around 5 μm. Larger or smaller particles will be trapped before it reaches alveoli or come out with exhaled breath.

Although the current disadvantage of inhibitor of HIF-1α is that there is no clinically used drug as a HIF-1α inhibitor, recently, some compounds are in clinical trials for cancer: 2-Methoxyestradiol (Phase 2), BAY 87-2243 (Phase 1), PX-478 2HCl (Phase 1). If one (some) of them will be approved, the drug could be extended its application to IPF. In addition, blocking HIF-1α seems preferable than using CsA in terms of side-effects. HIF-1α is essential for the embryo development and HIF-1α knock out mouse dies by E11 mainly due to cardinal abnormalities and failure of neural tube closure (Iyer *et al.,* 1998). On the other hand, in adult, it usually degraded and only accumulates in where hypoxia occurs (i.g. ischemic region, cancer tissues, fibrotic region) or where HIF-1α-stabilizing signal is running. Therefore, inhibition of HIF-1α seems less harmful and more favorable than CsA.

5. The results could be applied to fibrosis in other tissues?

TGF-β1 is involved in not only pulmonary fibrosis but also other fibrosis such as liver fibrosis, kidney fibrosis, and skin fibrosis (Klass *et al.,* 2009). In kidney fibrosis, the expression level of HIF-1α is upregulated (Liu *et al.,* 2017). It is reasonable that HIF-1α is upregulated in fibrotic region where the cell density is increased. Therefore, given that TGF-β1/HIF-1α axis plays an important role in other kinds of fibrosis as well as pulmonary fibrosis, treatment with CsA or HIFi would restore the fibrosis. However, for other fibrosis,

the optimization of the administration route of the drug for each tissues will be required.

One concern in the case of kidney fibrosis is that CsA itself might cause renal fibrosis. Although the risk will be high when the patient uses CsA more than 2 years, the continuous monitoring of renal function is necessary. Probably, for the treatment for kidney fibrosis, HIF-1α inhibitor will be a better choice.

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