Effects of doxycycline on production of growth factors and matrix metalloproteinases in pulmonary fibrosis

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Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is characterized by progressive fibrosis and a poor prognosis. Alveolar epithelial cells (AECs) are considered to play important roles by releasing growth factors and matrix metalloproteinases (MMPs), and by being involved in epithelial mesenchymal transition in IPF. Doxycycline hydrochloride (DOXY), an inhibitor of MMPs, attenuates pulmonary fibrosis in models and in patients with IPF; however, the mechanism of this action remains obscure. Objectives: The present study investigated the effect of DOXY upon growth factors and MMP production in AECs. Methods: Bleomycin (BL)-induced murine pulmonary fibrosis was treated with DOXY, and examined by pathological and immunohistochemical staining. The human alveolar epithelial cell line A549 was stimulated with transforming growth factor (TGF)-β1 and incubated with DOXY, and then the expression of growth factors, MMPs and collagen type I was evaluated at the mRNA and protein levels. We also evaluated the effects of DOXY on the TGF-β1-induced Smad signaling pathway. Results: DOXY reduced fibrosis scores and the production of collagen type I, connective tissue growth factor (CTGF) and TGF-β1 in BL models. DOXY inhibited the mRNA expression of MMP-2, -9, CTGF and collagen type I as well as the production of MMP-2 and platelet derived growth

factor-AA protein induced in A549 cells by TGF-β1, but not by Smad2 and Smad3 phosphorylation. We did not find a similar effect of DOXY in normal lung fibroblasts. Conclusions: Our results suggested that DOXY could be useful for attenuating pulmonary fibrosis through the inhibition of growth factors and MMP production in AECs.

Introduction

  Idiopathic pulmonary fibrosis (IPF) is the most common progressive and lethal

pulmonary fibrotic disorder, but its etiology is unknown. The pathogenesis of IPF was

originally thought to involve lung damage of unknown origin that produced an

inflammatory response leading to fibrosis. However, recent findings suggest that

epithelial cell damage is the initial event that triggers a series of repair pathways that are

in some way aberrant and lead to inappropriate fibrosis [1]. During this process of

pulmonary fibrosis, epithelial damage causes the consecutive release of several fibrotic

mediators including growth factors such as transforming growth factor (TGF)-β, platelet

derived-growth factor (PDGF), connective tissue growth factor (CTGF) and hepatocyte

growth factor (HGF), as well as metalloproteinases (MMPs). These mediators provoke

the migration, proliferation, and activation of mesenchymal cells with the formation of

active fibroblastic/myofibroblastic foci, leading to the exaggerated accumulation of

extracellular matrix (ECM) that mirrors abnormal wound repair [2]. Alveolar epithelial

cells (AECs) apparently serve as progenitor cells that can undergo differentiation to

fibroblasts/myofibroblasts in the presence of TGF-β [3,4]. This process is called

epithelial mesenchymal transition (EMT) and could regulate pulmonary fibrosis.

  Doxycycline hydrochloride (DOXY) is a tetracycline antibiotic that inhibits bacterial

protein synthesis and exerts anti-inflammatory effects as well as apoptosis [5] [6] [7].

DOXY also inhibits MMPs and thus has been widely applied to treat aneurysms [8],

periodontal disease [9], cancer, bone metastasis [10] and pleurodesis. The mechanisms

of MMP inhibition by DOXY are not yet completely understood, but DOXY binds

directly to the active target site of MMPs and induces degradation of the pro-MMP

zymogen or provokes the inhibition of MMP mRNA transcription [11]. In the

bleomycin (BL)-induced model of pulmonary fibrosis, DOXY attenuates fibrosis

through MMP inhibition [12], which led to the notion that preventing MMP activity

could be of therapeutic benefit to patients with IPF. The results of a recent small open

trial of such patients indicate that long-term DOXY administration might be a candidate

therapeutic regimen [13]. However, the definitive mechanism of DOXY with respect to

pulmonary fibrosis and its specific effects on alveolar epithelial cells in pulmonary

fibrosis remain obscure.

We evaluated the effect of DOXY on the BL-induced pulmonary fibrosis model *in vivo*, and on TGF-β1-induced growth factors and ECM production in the AECs and

fibroblasts that are relevant to the progression of lung fibrosis *in vitro*. We also

examined the Smad signalling pathway to clarify the mechanism of DOXY in the

production of TGF-β1-induced mediators.

Materials and methods

Animals

Nine-week-old male Institute for Cancer Research (ICR)-specific pathogen-free mice (Charles River Japan, Inc., Yokohama, Japan) weighing about 35 g were maintained under standard conditions with free access to drinking water and pelleted food at the Animal Centre of Biomedical Research, Nagasaki University School of Medicine, Nagasaki, Japan. The mice were intravenously injected with 10 mg/kg/day of BL (Nippon Kayaku, Tokyo, Japan) dissolved (2 mg/ml) in isotonic saline (SA) or with SA (5 ml/kg/day) alone for 5 consecutive days [14]. The animals were then orally dosed with either 2 mg/kg/day of DOXY (Wako Pure Medical Industries, Ltd., Osaka, Japan) in distilled water (0.35 mg/ml) or with distilled water alone as a control once daily, starting on the day before or 14 days after being injected i.v. with BL or SA throughout the study. The mice were randomly divided into the following groups: SA with distilled water (control), BL with distilled water (BL), DOXY 1 day before (pre-DOXY) and 14 days after (post-DOXY) BL injection. The mice were sacrificed and evaluated at 14, 42, and 56 days after the star of BL or SA administration. The Ethics Review Committees for Animal Experimentation of Nagasaki University School of Medicine approved the experimental protocol.

Histology and fibrosis scores

At 14, 42 and 56 days after starting BL or SA injections, the mice (n = 3 - 5 per group at each time point) were sacrificed by exsanguination under deep anaesthesia (sodium pentobarbital, 50 mg/kg, i.p.). The left lungs were then removed via a midline incision, fixed in 10% formaldehyde/neutral buffer, and embedded in paraffin and then 4-mm sections of embedded tissues were stained with haematoxylin-eosin (HE) and immunohistochemically analyzed. The degree of cellular infiltration and fibrosis was evaluated from 0 (normal) to 8 (most severe) based on mean Ashcroft scores [15] obtained from 25 consecutive microscopic fields that were randomly assessed along the pleura of each section.

Immunohistochemistry

Sections were conventionally analyzed by avidin-biotin-peroxidase histochemical staining using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) for TGF-β1, PDGF-A, PDGF-B, CTGF, MMP-2, MMP-9 and collagen type I. Antigen was retrieved in 10 mM sodium citrate at pH 6.0 using the steamer protocol for TGF-β1 or the standard protocol for PDGF-B, CTGF, MMP-2 and MMP-9. Antigen retrieval for PDGF-A and for collagen type I proceeded in 10 mM sodium citrate at pH 5.5 and with

protease K using the standard protocol, respectively. Endogenous peroxidase activity was quenched by incubation for 20 min in 0.3% H_2O_2 in absolute methanol. Non-specific binding was blocked with 1.5% goat or rabbit serum for 20 min and then sections were incubated overnight in rabbit TGF-β1, PDGF-A, CTGF or collagen type I antibody (Abcam Inc., Cambridge, MA, USA), rabbit PDGF-B antibody (Gene Tex, Irvine, CA, USA) or goat MMP-2 or MMP-9 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), respectively. The sections were washed with PBS and further processed using the kit according to the manufacturer's instructions. Areas of interest were visualized using 3, 3-diaminobenzidine and H_2O_2, and then the sections were counterstained with Mayer's haematoxylin.

Measurement of lung hydroxyproline content

We measured the hydroxyproline content in the right lungs of the mice at 14 and 56 days after BL or SA injection (n = 3 to 5 in each group) to estimate total collagen deposition in the lungs as an indicator of pulmonary fibrosis. Wet lungs were weighed, dried, homogenized and hydrolysed in 6 N hydrochloric acid. Hydroxyproline was then analyzed in the supernatant at 348 and 450 nm using high-performance liquid chromatography (HPLC 800 series; Nippon Bunko, Tokyo, Japan).

Cell culture and stimulation

Human type II alveolar epithelial cells (A549; ATCC, Manassas, VA, USA) and normal human lung fibroblasts (Lonza Japan Ltd., Tokyo, Japan) were maintained in low glucose-F-12 containing 10% fetal bovine serum (FBS; Life Technologies Corporation, Carlsbad, CA, USA) or in fibroblast growth medium (FGM)-2 (Lonza Japan Ltd.), respectively, at 37°C in a humidified 5% CO_2 atmosphere. Confluent cells were incubated in serum-free F-12 medium containing 0.1% bovine serum albumin (BSA) or in FGM-2 for 24 h before stimulation with recombinant human TGF-β1 (R&D systems, Minneapolis, MN, USA). A549 cells or fibroblasts were incubated with either various concentrations of TGF-β1 activated with BSA and HCl or equal amounts of vehicle. A549 cells were lysed and harvested 3 h later for reverse transcription (RT) PCR and the supernatants were extracted at 24 h for ELISA and MMP activity assays. A549 cells were incubated with several concentrations of DOXY from 3 h before stimulation with 1 ng/ml TGF-β1 until the end of the study. Fibroblasts were stimulated with 5 ng/ml of TGF-β1 with or without DOXY, and then lysed and harvested 48 h later for RT PCR.

Reverse transcription and real-time PCR

Primers for PDGF-A, PDGF-B, CTGF, HGF, collagen type I, MMP-2, MMP-9 and

β-actin mRNA were included in the TaqMan® Gene Expression Assays (Applied

Biosystems, Foster City, CA, USA). Total RNA was isolated using RNeasy Plus Mini

kit (Qiagen K.K., Tokyo, Japan) and RT-PCR proceeded using the SuperScript III First

Strand Synthesis System (Life Technologies Japan Ltd., Tokyo, Japan). The target was

reverse-transcribed with initial denaturation at 65°C/5 min followed by 40 cycles of

PCR amplification at 50°C for 50 min, 85°C for 5 min, and 37°C for 20 min using the

GeneAmp PCR System 9700 (Applied Biosystems). The inner control primer was

β-actin. The PCR products were analyzed with 7500 system SDS software (Applied

Biosystems).

ELISA and MMP activity assays

Concentrations of PDGF-AA, PDGF-AB, PDGF-BB, HGF (R&D systems), and CTGF

(Antigenix America, Inc., Huntington Station, NY, USA) were analyzed in culture

media using the appropriate ELISA kit and those of MMP-2 and MMP-9 were

measured using MMP activity assay systems (GE Healthcare Bio-Science K.K., Tokyo,

Japan) according to the manufacturer's protocol. Culture media were incubated in the

wells of plates pre-coated with primary antibodies. Enzyme-linked antibodies specific to each target were added, the plates were washed and chromogenic substrate was added. The amount of color generated was measured at 405 nm using a micro-plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Immunocytochemistry

A549 cells (1×10^4/well) were seeded onto Lab-TeK® chamber slides (Sigma-Aldrich, St. Louis, MO, USA) and maintained until they reached about 80% confluence. The cells were stimulated with 1 ng/ml TGF-β1 for 24 h with or without DOXY as described above and then examined by immunocytochemistry using the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) with goat anti-human collagen type I polyclonal antibody (Abcam Inc.) diluted 1:1000 in F-12 medium.

SDS-PAGE and western blotting

A549 cells were incubated with or without several concentrations of DOXY from 1 h before stimulation with 1 ng/ml TGF-β1 until the end of the study. The cells were scraped and lysed in cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing a protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan) at 60 min

after TGF-β1 stimulation. The lysates were centrifuged at 14,000 g for 10 min at 4°C.

Proteins were separated using 12% reducing SDS-PAGE and transferred to a

polyvinylidene difluoride membrane (Bio-Rad) in 20% methanol/25 mM Tris–HCl/0.2

M glycine. Non-specific binding was blocked by incubating the membranes with 5%

skimmed milk in tris buffer saline with 0.1% Tween 20 for 1 h at room temperature.

Immunoreactive proteins were detected by incubating the membranes with rabbit

anti-human Smad2 and Smad3, and phospho-Smad2 and -Smad3 antibodies (Cell

Signaling Technology) and GAPDH (Santa Cruz Biotechnology, Inc.) diluted at 1:500,

1:250 and 1:1000, respectively, overnight at 4°C. Subsequently, the membranes were

incubated for 1 h with anti-rabbit IgG conjugated to HRP (1:10 000), washed and then

developed using enhanced chemiluminescence reagents (GE Healthcare,

Buckinghamshire, UK). Western blot images were scanned and analyzed using NIH

Image J software. Results were expressed as a ratio of band density of phospho-Smad to

the respective total Smad.

Statistical analysis

Data were statistically analyzed by an unpaired t-test for pairwise comparisons, and by

ANOVA followed by Dunnett's post hoc test for multiple comparisons using StatView

J5.0 (SAS Institute, Inc., Cary, NC). All results are presented as the means $\pm$ SD of at

least three independent experiments. P values of <0.05 were considered statistically

significant.

Results

Effect of DOXY on histological findings in the BL-induced fibrosis model

A histological evaluation revealed no significant changes in the lungs of the control group at 14, 42 and 56 days (Fig. 1A, B, C, respectively), whereas interstitial fibrosis was obvious in the lungs of the BL group (Fig. 1D, E, F, respectively) and fibrotic areas in the lungs of the pre-DOXY group were reduced (Fig. 1G, H, I, respectively), compared with the BL groups. Fibrotic areas were also reduced in the lungs of the post-DOXY group at 42 and 56 days (not shown). The mean fibrosis scores in the BL group at 14, 42 and 56 days (4.80 ± 1.61, 2.33 ± 0.59, 3.28 ± 0.56, respectively) were significantly higher than those in the control group (1.05 ± 0.09, 1.11 ± 0.31, 0.83 ± 0.18, respectively; Fig. 2). Furthermore, a prior incubation with DOXY significantly reduced mean fibrosis scores at 14, 42 and 56 days (3.01 ± 0.35, 1.48 ± 0.34, 1.99 ± 0.43, respectively; Fig. 2). Post-DOXY also inhibited the score (2.08 ± 0.70; Fig. 2) at 56 days compared with the BL group.

Effect of DOXY on lung hydroxyproline content in BL-induced fibrosis model

Figure 3 shows the effect of DOXY on the hydroxyproline content in the right lungs of mice at various times. The hydroxyproline contents were increased by BL at day 14

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compared with the control group (195.2 ± 31.8 vs. 148.8 ± 11.0 μg/lung) and DOXY

significantly reduced the level increased by BL (147.0 ± 13.2 μg/lung). The

hydroxyproline levels induced by BL at 56 days were also significantly elevated and the

increase was similarly diminished by pre- and post-DOXY.

Effect of DOXY on immunohistochemical expression of fibrotic mediators in a model of

BL-induced fibrosis

Figure 4 shows representative immunohistochemically-stained mediators in the model

of BL-induced fibrosis. Compared with the control group (A, B, C, respectively), the

immunoreactivity of TGF-β1 (D) was increased mainly in macrophages and AECs, and

numbers of CTGF (E)-positive cells were obviously increased among enlarged type II

AECs and fibroblasts in the active fibrotic area in the BL group. The amount of collagen

type I (F) at ECM was also obviously increased. DOXY reduced the number of cells

that were positive for TGF-β1 (G), CTGF (H) and collagen type I (I) in the fibrotic area

in the pre-DOXY group. On the other hand, PDGF-A and PDGF-B was mainly stained

in macrophages that accumulated in the active fibrotic area of the BL model but not in

AECs, and DOXY reduced macrophage immunoreactivity (data not shown).

Macrophages and AECs in the BL group were stained for MMP-2 compared with

controls, and MMP-2 immunoreactivity was slightly decreased in the pre-DOXY group (data not shown). Macrophages in the BL group were mainly stained for MMP-9, whereas MMP-9 immunoreactivity in the pre-DOXY group did not change (data not shown).

Effect of DOXY on growth factor mRNA expression and ECM components induced by TGF-β1 in AECs

We assessed the production of TGF-β1-induced profibrotic factors in the alveolar epithelial cell line A549 *in vitro* to determine how DOXY inhibits pulmonary fibrosis in the BL model. A preliminary experiment showed that stimulation with TGF-β1 (1 ng/ml for 3 h) significantly up-regulated the mRNA expression of PDGF-A, CTGF, collagen type I, MMP-2 and MMP-9 and inhibited that of PDGF-B, but did not affect that of HGF. Based on these findings, we examined the effect of DOXY on the expression of profibrotic factors stimulated with 1 ng/ml of TGF-β1 for 3 h in A549 cells. The mRNA expression of PDGF-A, CTGF, collagen type I, MMP-2 and MMP-9 induced by TGF-β1 (Fig. 5) was attenuated by DOXY at 15 or 30 μg/ml. Furthermore, DOXY up-regulated the expression of PDGF-B that was down-regulated by TGF-β1, but had no effect on HGF expression.

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DOXY attenuated the protein production of PDGF-AA, active MMP-2 and collagen type I in AEC

The secretion of PDGF-AA (Fig. 6A) and active MMP-2 (Fig. 6B) induced by TGF-β1 was attenuated by DOXY. However, DOXY did not significantly affect PDGF-AB, PDGF-BB, HGF and MMP-9 production (data not shown) and CTGF was undetectable by ELISA in the supernatant. The production of collagen type I enhanced by 1 ng/ml TGF-β1 (Fig. 6C-2) in A549 cells at 24 h compared with control (Fig. 6C-1) was attenuated by DOXY (Fig. 6C-3).

Effect of DOXY on TGF-β1-induced Smad signalling pathway

The phosphorylation of Smad2 and Smad3 was examined in A549 cells at 60 min after stimulation with TGF-β1 (1 ng/ml). TGF-β1 induced the phosphorylation of both Smad2 and Smad3 without affecting the expression of total Smad2 and Smad3 (Fig. 7A, B). DOXY did not affect the TGF-β1-mediated phosphorylation of Smad2 and Smad3.

Effect of TGF-β1 and DOXY on fibroblasts

We evaluated the effect of DOXY *in vitro* on fibroblasts, another important target of therapy for IPF. The mRNA expression of CTGF and collagen type I was upregulated in

fibroblasts at 48 h after TGF-β1 (5 ng/ml) administration. The mRNA expression of MMP-2 and -9, and HGF was not affected by TGF-β1, and neither PDGF-A nor PDGF-B were detectable in fibroblasts. Various concentrations of DOXY up to 30 μg/ml did not affect the CTGF and collagen type I mRNA expression induced by TGF-β1 (data not shown).

Discussion

The present study demonstrated that DOXY attenuated BL-induced pulmonary fibrosis. The production of TGF-β1-induced mediators that are considered to play important roles in the progression of pulmonary fibrosis was also attenuated by DOXY in AECs *in vitro*. Considering that multiple cycles of epithelial cell damage and activation might cause IPF, AECs are considered to play various roles including provoking the migration, proliferation and activation of mesenchymal cells along with the formation of fibroblastic/myofibroblastic foci and excessive ECM accumulation, thus mirroring abnormal wound repair [2].

Here, we focused on the effect of DOXY on AECs that release various types of growth factors and ECM components that can induce the profibrotic role of fibroblasts as described above and conclusively progress pulmonary fibrosis [2]. Among these, PDGF, CTGF, HGF and MMPs are considered to play major roles in pulmonary fibrosis. The present study showed that DOXY attenuated the TGF-β1-induced mRNA expression of PDGF-A, CTGF, MMP-2 and MMP-9, as well as the protein production of PGDF-AA and MMP-2 in AECs *in vitro*. We also showed that DOXY reduced the protein production of CTGF in AECs *in vivo*.

The potent mitogen PDGF is also a chemoattractant factor for mesenchymal cells and

it has profibrotic activities [16]. The dimeric isoforms PDGF-A and PDGF-B are up-regulated in AECs and in bronchoalveolar lavage fluid from the lungs of patients with IPF [17] [18]. Although its role is now debatable, the PDGF/PDGF receptor system remains a promising target for treating fibrotic diseases [19]. Although we did not identify PDGF expression in AECs in our BL model and the reason for the different responses of PDGF-A and -B to both TGF-β1 and DOXY remains unknown, our findings suggests that DOXY attenuates lung fibrosis by reducing PDGF expression from AECs.

Furthermore, CTGF is considered to act in concert with TGF-β1 downstream and to play a significant role in promoting and maintaining fibrogenesis induced by proliferating AECs and activated fibroblasts in IPF [20]. Thus, CTGF is also a target in IPF and a CTGF-specific antibody has been tested in a clinical trial of patients with IPF [1]. Our present findings that DOXY attenuated CTGF production in the BL-model and TGF-β-induced-CTGF expression in AECs suggest that DOXY could attenuate pulmonary fibrosis by inhibiting CTGF production from AECs.

We also showed that DOXY in AECs affected the production of MMP-2 and MMP-9, which play roles in cell proliferation, adhesion, migration and differentiation through proteolytic effects on ECM components and basement membrane that progress fibrosis

[21]. The gene and protein expression of MMP-2 and MMP-9 is elevated in tissues and in bronchoalveolar lavage fluid from patients with IPF [22]. Fujita et al. demonstrated that DOXY decreases the production and activity of MMP-9 protein but not the expression of MMP-9 mRNA from inflammatory cells in BAL fluid from a model of BL-induced pulmonary fibrosis [12]. In addition, the synthetic MMP inhibitor batimastat prevents BL-induced pulmonary fibrosis by reducing MMP-2 and MMP-9 activity [23]. These reports indicate that MMP is a potential therapeutic target of pulmonary fibrosis. Although our results in vivo did not uncover a significant association between MMP inhibition and pulmonary fibrosis, our in vitro study indicates that DOXY reduces pulmonary fibrosis by not only inhibiting MMPs activity but also reducing the production of MMP-2 induced by TGF-β1 from AECs.

The origin of the expanded populations of lung fibroblasts and myofibroblasts in lungs with pulmonary fibrosis is of substantial interest. Reports indicate that AECs can serve as progenitor cells that can undergo differentiation to fibroblasts/myofibroblasts in response to TGF-β with the generation of ECM [3,4]. The process is called epithelial mesenchymal transition (EMT), a process by which epithelial cells lose cell-cell attachment, polarity and epithelial-specific markers, undergo cytoskeletal remodeling, and acquire a mesenchymal phenotype [24]. Epithelial mesenchymal transition can

occur in the lungs and contribute to fibrosis in patients with IPF [25]. Mediators such as collagen type I, CTGF and MMP-2 that are down-regulated by DOXY in AECs are also considered to be markers of EMT [3,26]. These results indicate that DOXY attenuates the production of TGF-β-induced mediators by inhibiting EMT. TGF-β-mediated EMT is considered to be partly dependent on Smad2 and Smad3 phosphorylation [3,26]. Activated Smads mediate transcriptional regulation through other families of transcription factors resulting in activation of mesenchymal gene expression including MMPs and collagen [27]. DOXY did not reduce TGF-β-induced-Smad phosphorylation in the present study. Activation of the Ras-Erk MAP kinase pathway, p38 MAP kinase and JNK signaling, as well as Rho GTPase signaling and the PI3 kinase/Akt pathway, also contribute to TGF-β-induced EMT [27]. DOXY inhibits TGF-β-induced activation of Smad2, JNK, ERK, and p38 MAP kinases in human corneal epithelial cells [28]. DOXY also decreases p38 MAP kinase in the TNF-α-, IL-1β- and IFN-γ-treated murine lung alveolar epithelial cell line LA4 [6]. Although further studies are required, our findings indicated that DOXY attenuates the production of TGF-β-induced mediators in alveolar epithelial cells via a non-Smad pathway.

We also examined the effect of DOXY in lung fibroblasts *in vitro*. We could not assess the effect of DOXY on the expression of mediators (PDGF-A, PDGF-B, HGF, MMP-2,

and MMP-9) in fibroblasts, because TGF-β1 stimulation did not affect their expression.

The RNA expression of CTGF and collagen type I upregulated by TGF-β1 stimulation

was not affected by DOXY. We investigated the effect of DOXY in lung fibroblasts in

plastic dishes in vitro, and others have reported the influence of collagen type I on

fibroblast function and the effect of MMP inhibitors such as DOXY [29] [30]. The

presence of collagen type I in the medium might affect the present results.

Although one limitation of this study is that only A549 cells and primary human lung

fibroblasts were investigated, the inhibitory effect of DOXY on pulmonary fibrosis

might be partly explained as reduced production of profibrotic factors from AECs but

not from lung fibroblasts.

We used TGF-β1 to stimulate AECs and lung fibroblasts *in vitro*. TGF-β1 plays the

most pivotal role in the development and progress of fibrosis. The present study showed

that DOXY reduced TGF-β1 expression in a model of pulmonary fibrosis induced by

BL. Although the precise mechanism of the reduced TGF-β1 expression in BL-induced

pulmonary fibrosis remains unknown, our results *in vitro* and *in vivo* suggest that

DOXY attenuates pulmonary fibrosis by reducing both TGF-β1 production and the

TGF-β1-induced fibrotic response. Additionally, DOXY attenuated fibrosis in both pre-

and post-treatment groups *in vivo*. One study has found that BL-induced pulmonary

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fibrosis is attenuated by earlier, but not by later, DOXY administration, which is somewhat controversial. Although the two studies are similar, the species of mouse and period of BL administration differ. The most obvious difference is in the administration routes of BL, which was intratracheally in the previous report and intravenously in the present study. Intratracheal BL reportedly induces lung fibrosis by day 14, whereas intravenous administration induces fibrosis more slowly [31]. These factors might explain the different results. DOXY inhibits inflammation not only during the earlier phase, but also fibrosis at the later phase of pulmonary fibrosis.

Advances in elucidating disease pathogenesis and identifying new targets for treating IPF have recently led to several clinical trials of many agents. However, none has unequivocally caused a survival benefit. Consequently, the search for safe and effective agents for treating IPF continues [32]. The therapeutic mechanisms of DOXY remain unknown, and evidence of its effectiveness in patients with IPF is inadequate. On the other hand, prolonged therapy with DOXY is well tolerated for several months under various conditions [13]. This could be favorable for treating IPF. Our data support the notion that DOXY could become a therapeutic option for the treatment of IPF.

In conclusion, DOXY appears to attenuate pulmonary fibrosis through the inhibition of growth factors and MMP production in AECs.

Acknowledgments

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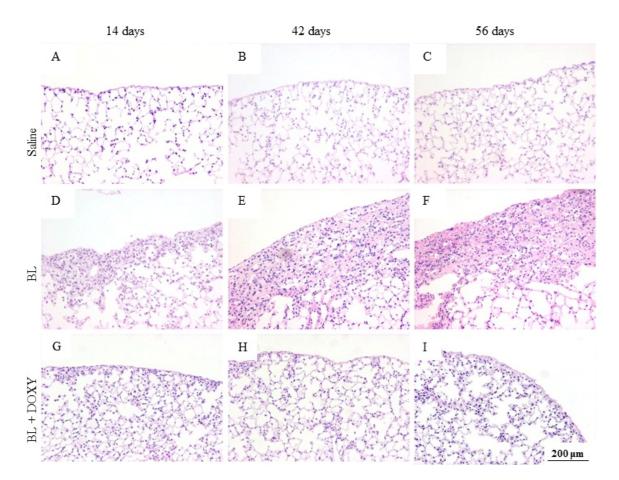
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Figure legends

Fig. 1. Representative photographs of lungs from each group at various times (H&E stain).

At days 14, 42 and 56, interstitial fibrosis is evident in lungs from BL group (D, E, F, respectively) compared with control lungs (A, B, C, respectively) and fibrotic areas are obviously reduced in lungs of pre-DOXY group (G, H, I, respectively), relative to those of BL groups at corresponding times. Scale bar, 200 μm.
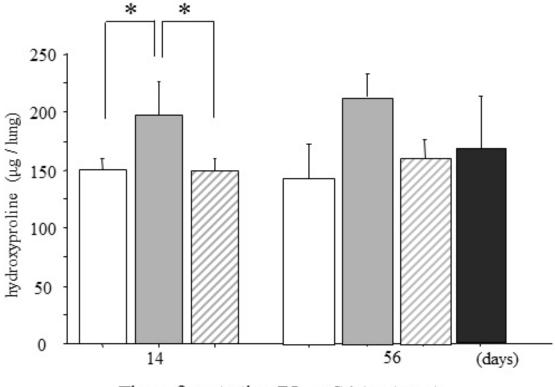
Fig. 2. Effects of DOXY on fibrosis scores of BL-induced fibrosis model at various times.

Pre-DOXY incubation reduced Ashcroft scores of BL-induced fibrosis model at days 14, 42 and 56 compared with the BL group. Post-DOXY incubation also reduced this score at 56 days compared with the BL group.

☐Control, ▨ BL, ▨ pre-DOXY, ■ post-DOXY. The results are expressed as mean ± SD of data from 3 to 5 animals. $*p < 0.05$ compared with other groups at corresponding times.
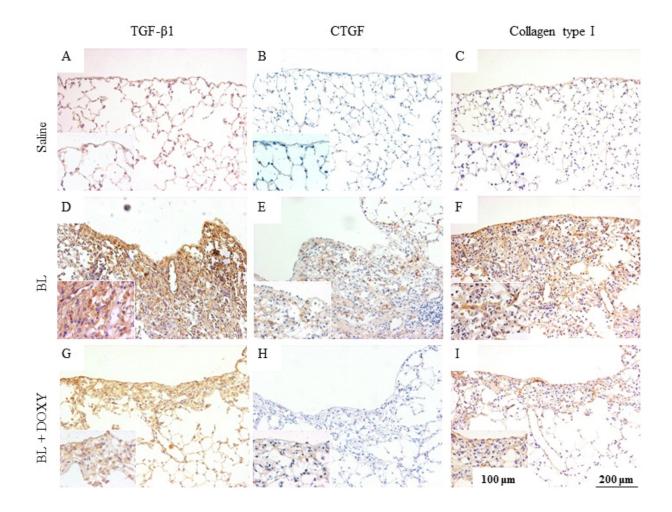
Fig. 3. Effects of DOXY on hydroxyproline content in BL-induced fibrosis model at various times.

Incubation with DOXY at day 14 significantly reduced hydroxyproline levels relative to corresponding values of BL-treated group. Similar tendency at day 56 did not reach significance.

☐ Control , ◼ BL , ▨ pre-DOXY, ◼ post-DOXY. The results are expressed as mean ± SD of data from 3 to 5 animals. *$p < 0.05$ compared with other groups at corresponding time intervals.

Fig. 4. Immunohistochemical evaluation of control, BL and pre-DOXY groups at day 56. Type-II AECs and macrophages are positive for TGF-β1 (A) and type-II AECs are positive for CTGF (B). Collagen type I is essentially undetectable (C) in control lung. Proliferative, TGF-β1- (D) and CTGF- (E) positive cells in active fibrotic area and collagen type I positive ECM (F) are increased in lungs from BL group. Numbers of TGF-β1- (G) and CTGF H)-positive cells as well as collagen type I production (I) are reduced in pre- and post-DOXY groups (not shown).
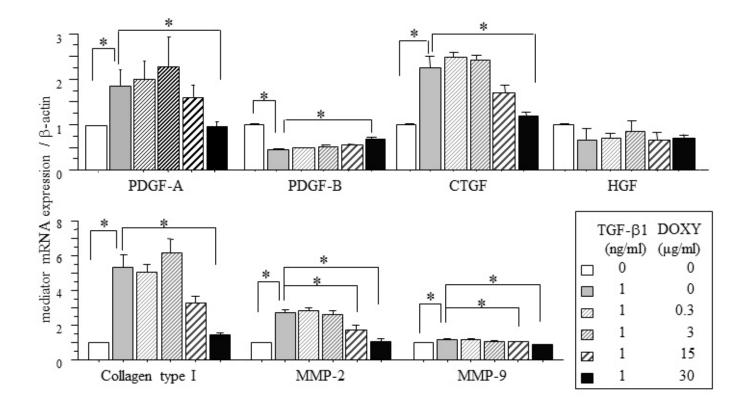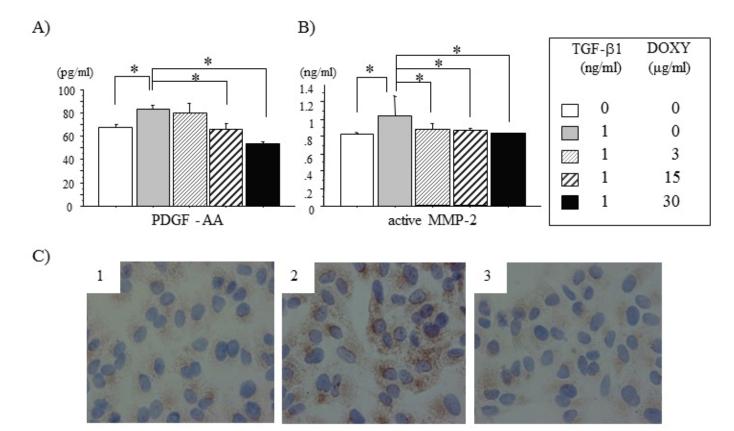
Fig. 5. Effects of DOXY on mRNA expression of growth factors, collagen type I and MMPs induced by TGF-β1 in A549 cells.

DOXY attenuated TGF-β1-induced mRNA expression of PDGF-A, CTGF, collagen type I, MMP-2 and MMP-9, but recovered PDGF-B mRNA expression inhibited by TGF-β1. Data are presented as means ± SD. $*p < 0.05$ compared with expression after 3 h TGF-β1 stimulation.

Fig. 6. Effects of DOXY on production of PDGF-AA, collagen type I and active

MMP-2 induced by TGF-β1 in A549 cells.

ELISA and MMP activity assays show that DOXY attenuated production of PDGF-AA

(A) and active MMP-2 (B) induced by TGF-β1 in supernatant. DOXY attenuated

production of collagen type I enhanced by 1 ng/ml TGF-β1 (Fig. 6C-2) in A549 cells at

24 h compared with control (C-1, C-3) (immunocytochemical staining). Data are shown

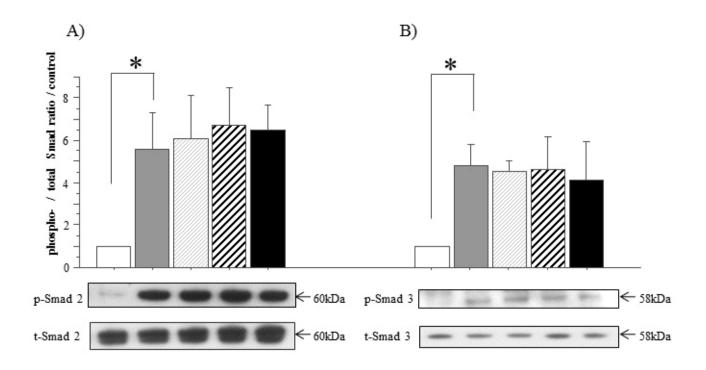as means ± SD. *p < 0.05 compared with expression after TGF-β1 stimulation.

Fig. 7. Effects of DOXY on TGF-β1-induced Smad signalling pathway.

TGF-β1 induced expression of phospho-Smad2 (A) and phospho-Smad3 (B) but did not affect total Smad2 and Smad3 values. DOXY did not affect Smad2 and Smad3 phosphorylation. Data are presented as means ± SD. $*p < 0.05$.