Use of tacrolimus, a potent antifibrotic agent, in bleomycin-induced lung fibrosis

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ABSTRACT: Idiopathic pulmonary fibrosis has a poor prognosis and few efficacious treatments. The immunosuppressant cyclosporin A has been shown to inhibit tumour growth factor (TGF)-β-induced collagen deposition in vitro, and is widely used in Japan as a potent antifibrotic agent. Tacrolimus (FK506) is another attractive immunosuppressant, which may be useful in the treatment of pulmonary fibrosis. The aim of the present study was to elucidate the antifibrotic effect of FK506.

The inhibitory effect of FK506 on collagen synthesis in cultured lung fibroblastic cells, TIG-3-20, and its antifibrotic effect on bleomycin (BLM)-induced pulmonary fibrosis in mice was investigated.

FK506 inhibited TGF-β-induced collagen synthesis, and suppressed the expression of TGF-β type I receptor (TβR-I) in TIG-3-20 cells. Consistent with the in vitro findings, FK506 treatment starting on day 6 attenuated BLM-induced pulmonary fibrosis, in part, via reduced TβR-I expression. FK506 treatment in the acute BLM injury phase unexpectedly increased pro-inflammatory cytokine levels in bronchoalveolar lavage fluid and enhanced lung injury, resulting in poor survival.

In conclusion, the present results suggest that FK506 has a potent antifibrotic effect and may be useful for the treatment of pulmonary fibrosis, although its use in the acute inflammatory phase may exacerbate lung injury.

KEYWORDS: Bleomycin, fibroblast, FK506, pulmonary fibrosis, tacrolimus, transforming growth factor-β

Idiopathic pulmonary fibrosis is an incurable disorder with a poor prognosis. The molecular mechanisms that lead to pulmonary fibrosis are poorly understood. The resulting pulmonary histopathological changes can be diverse with overlapping features, characterised by varying degrees of inflammation and accumulation of extracellular matrix (ECM) molecules in the alveolar and interstitial spaces [1]. Corticosteroids, with or without cytotoxic agents, are currently used to treat pulmonary fibrosis [2]. However, this disease is generally progressive and irreversible, regardless of treatment.

Tacrolimus (FK506) is a calcineurin inhibitor and a specific inhibitor of T-lymphocyte function that has been used widely as an immunosuppressant in human organ transplantation. The inhibitory mechanism of FK506 is similar to that of cyclosporin A (CsA), although there are substantial differences in potency and chemical structure [3]. Recently, it was reported that CsA inhibits tumour growth factor (TGF)-β-induced signalling and collagen deposition in vitro via direct inhibition of AP-1/JunD activation [4]. Furthermore, in clinical reports CsA has been noted as an attractive drug for the treatment of interstitial pneumonia [5]. FK506, which is a higher-potency immunosuppressant, may be a useful antifibrotic agent for the treatment of pulmonary fibrosis.

TGF-β is a central fibrogenic factor in the development of pulmonary fibrosis [6]. This cytokine is mitogenic and chemotactic for fibroblasts and promotes the accumulation of ECM proteins by increasing collagen synthesis and inhibiting matrix degradation. Neutralisation of TGF-β with antibodies reduces experimental lung fibrosis [7] and exogenous over expression of Smad7, an inhibitor of the TGF-β signalling pathway, prevents bleomycin (BLM)-induced lung fibrosis in mice [8]. Therefore, blockage of the cellular effects of TGF-β may provide an effective therapy for pulmonary fibrosis. FKBP12, a cytoplasmic binding protein of FK506, has been shown to interact with the activation domain of the TGF-β receptor (TβR-I), which is responsible for initiating the downstream signalling pathway
However, it has not been fully elucidated if, or how, FK506 interacts with TβR-I in lung fibrosis. The present study examined the effect of FK506 on TGF-β-induced collagen synthesis in fibroblasts. The antiboftric effect of FK506 in a murine BLM-induced lung fibrosis model was also evaluated.

MATERIAL AND METHODS

Reagents

FK506 was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). The solution form of FK506 (5 mg/ampoule) was diluted in 4 mL sterile PBS to make a stock solution, which was then further diluted into an appropriate concentration with PBS for use in experiments. BLM hydrochloride (Nihonkayaku, Osaka, Japan) was dissolved in sterile PBS.

Cell culture

Human lung fibroblastic cell line TIG-3-20 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cells were cultured in tissue culture flasks (Falcon, Franklin Lakes, NJ, USA) in basal Eagle’s medium (BEM; Gibco Invitrogen, Carlsbad, CA, USA), supplemented with 10% foetal bovine serum (FBS; Serological Corp., Norcross, GA, USA) and 1% penicillin-streptomycin (Gibco Invitrogen). Treatments were performed in 5% CO2 at 37°C in a humidified atmosphere. Cells were used between the 25th and 30th passage.

Collagen assay

Collagen assays were performed with the Sircol Collagen Assay kit (Biocolor, Newtownabbey, UK) according to the manufacturer’s protocol. Briefly, 1 mL Sircol dye reagent was added to 100 μL culture supernatant or collagen standard and incubated at 25°C for 30 min. After centrifugation at 10,000 × g for 5 min, supernatants were discarded. To release the bound dye into solution, 1 mL of 0.5 NaOH was added to the collagen-bound dye pellet. The optical density of each sample at 540 nm was determined with a spectrophotometer.

Preparation of proteins and western blots

TIG-3-20 cells were grown to subconfluence in BEM plus 10% FBS. After 24-h serum starvation, cells were dissolved in radioimmunoprecipitation assay (RIPA) lysis buffer (sc-24948; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The lysates were stored at -80°C for further analysis.

Murine lung tissues were homogenised (1 g in 3 mL) in RIPA lysis buffer. The homogenates were incubated on ice for 1 h and clarified by centrifugation at 15,000 rpm for 10 min. The lower layer containing lipids was discarded and the samples were again centrifuged at 15,000 rpm for 10 min. After removal of the remaining top lipid layer, the supernatants were stored at -80°C until analysis.

For Western blot analysis, 15 μg of protein sample determined using a DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) was separated by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Invitrogen, Carlsbad, CA, USA). Blots were probed overnight with a 1:1000 dilution of primary antibody against TβR-I or TβR-II (rabbit polyclonal antibody to TβR-I (v-22) or TβR-II (c-16); Santa Cruz Biotechnology Inc.) in 0.5% milk/Tris-buffered saline plus 0.05% Tween 20. Blots were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (IgG). Protein levels were visualised with Western Lightning Plus Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA, USA) and quantified with a scanning spectrophotometer.

Animals and treatments

Six-week-old male C57BL/6 mice weighing 20–22 g were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained under specific pathogen-free conditions at a constant temperature with food and water ad libitum at the Kumamoto University animal facility (Kumamoto, Japan). All procedures involving animals were approved by the Animal Care and Use Committee of Kumamoto University.

Mice were randomly assigned to one of five groups each containing five mice. Mice in groups one and two were treated intratracheally with 50 μL sterile PBS alone or PBS containing FK506 (1 mg·kg⁻¹). To induce pulmonary fibrosis, mice in groups three, four and five were given BLM (3–5 U·kg⁻¹ body weight in 50 μL sterile PBS). Group three was then treated with PBS as a vehicle, and mice in groups four and five were treated daily with intraperitoneal injections of FK506 (1 mg·kg⁻¹) starting on day 0 or day 6. All procedures were performed under anaesthesia induced by intraperitoneal injection of chloral hydrate (400 mg·kg⁻¹ i.p.). At the designated time points (days 3, 7 or 14 after BLM administration) mice were killed, bronchoalveolar lavage (BAL) was performed and both lungs were removed and frozen immediately in liquid nitrogen. Tissue samples were stored at -80°C until further processing.

Histology

Animals were anaesthetised and perfused via the left ventricle with 2.5 mL PBS. Lungs were fixed by inflation with 0.7 mL buffered 10% formalin solution for 24 h, then processed according to standard procedures and embedded in paraffin. Sections 3 μm in size were cut, mounted on slides and stained with haematoxylin and eosin.

Hydroxyproline quantification

To quantify collagen deposition, hydroxyproline content was measured as described previously [10]. In brief, a minced lung was homogenised in 6 N HCl and hydrolysed at 110°C for 16 h. The pH was adjusted to 6.5–7.0 with NaOH, and the sample volume was adjusted to 30 mL with sterile water. A 2-mL aliquot of the sample solution was added to 1 mL 1.4% chloramine T and incubated at room temperature for 20 min, followed by the addition of 1 mL Erlich’s solution. After incubation at 60°C for 20 min, absorbance was measured at 560 nm with a spectrophotometer. The amount of hydroxyproline was determined by comparison with a standard curve prepared from known concentrations of hydroxyproline reagent.

Immunohistochemistry

Lung sections (3 μm thick) embedded in paraffin were deparaffinised in xylene and rehydrated in a graded ethanol series and PBS. Immunohistochemical staining for TβR-I was performed with a rabbit polyclonal antibody to TβR-I (v-22) diluted 1:200 overnight at 4°C. Immunoreactivity was visualised with a secondary antibody conjugated to
peroxidase-labelled dextran polymer (ENVISION+; DAKO Corp., Carpinteria, CA, USA) and diaminobenzidine as a substrate (DAKO), according to the manufacturer’s recommendations. The sections were then counterstained with haematoxylin.

**BAL and lung tissue preparation**

Following intraperitoneal chloral hydrate anaesthesia, a total of 3 mL PBS was injected intratracheally, in three aliquots of 1 mL each, and retrieved. The BAL fluid (BALF) was centrifuged at 1,500 rpm for 5 min, and cell-free supernatants were used for biochemical assays. For each animal, the cell pellet was resuspended in 1 mL PBS, and total white blood cell counts were performed with a haemocytometer. Cell differentials were determined by centrifuging in a cytospin and staining with DiffQuik (Sysmex, Kobe, Japan).

**Assessment of albumin leakage**

To examine pulmonary vascular permeability, BALF/serum albumin (B/S) ratios were determined by means of ELISA (Bethyl Laboratories Inc., Montgomery, TX, USA).

**Lung wet-to-dry weight ratios**

In another series of experiments, mice were anaesthetised and the chest opened via a midline incision. The lungs were gently infused with 2.5 mL sterile PBS from the heart for 30 s and carefully dissected from large airways, heart and mediastinal structures, and excess fluid was absorbed with soft tissue paper. The lungs were immediately weighed in dishes (wet weight) and again after being placed overnight at 80°C in a drying oven (dry weight) for calculation of the wet/dry (W/D) weight ratio.

**Determination of cytokine levels in BALF**

The level of active TGF-β1 was measured with a mouse immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s recommendations. The sensitivity of the assay was 3 pg·mL⁻¹. Levels of interleukin (IL)-1β, tumour necrosis factor (TNF)-α and interferon (IFN)γ in BALF were determined with mouse ELISA kits (BioSource International, Camarillo, CA) according to the manufacturer’s protocols.

**Statistical analysis**

Data are expressed as mean ± SEM for all analyses in which mean values were compared. A t-test or Mann–Whitney U-test was used for two-group comparisons. A p-value of <0.05 was considered statistically significant.

**RESULTS**

**FK506 inhibits TGF-β-induced collagen synthesis in TIG-3-20 cells**

To test the ability of FK506 to decrease TGF-β-induced collagen synthesis, human lung TIG-3-20 cells were stimulated with 10 ng·mL⁻¹ recombinant human TGF-β in the absence or presence of varying concentrations of FK506 (fig. 1a). Stimulation with 10 ng·mL⁻¹ TGF-β1 induced 1.5-fold enhancement of collagen production. Treatment with FK506 significantly reduced TGF-β1-induced collagen synthesis. However, FK506 showed no effect on baseline levels of collagen production in these cells. FKBP12, a cytoplasmic binding protein of FK506,
has been reported to interact with the activation domain of TβR-I [9]. Therefore, the present authors hypothesised that FK506 inhibits TGF-β-induced collagen synthesis by modulating TβR-I expression. Western blotting was used to analyse the expression level of TβR-I induced by BLM stimulation in the absence or presence of FK506 (fig. 1b). Compared with unstimulated cells, the cells stimulated with 100 ng·mL⁻¹ BLM showed increased levels of TβR-I protein. Treatment with FK506 (10 ng·mL⁻¹) significantly reduced BLM-induced expression of TβR-I. Expression of TβR-II was also determined. In contrast to TβR-I, TβR-II was expressed constitutively and was not significantly altered in response to BLM treatment.

**FK506 attenuates BLM-induced pulmonary fibrosis in mice**

The previously mentioned results encouraged investigation into the effect of FK506 in vivo. Therefore, a mouse model of BLM-induced pulmonary fibrosis was used to evaluate the antifibrotic effect of FK506. FK506 treatment was initiated on day 6 to avoid the anti-inflammatory effect of FK506 in the acute BLM-injury phase, from day 1 to day 5 [11]. The antifibrotic effect of FK506 was assessed by histological examination of haematoxylin and eosin stained slides on day 14 (fig. 2). No histological changes were observed in control lungs from PBS-treated mice (PBS/vehicle; fig. 2a) or from PBS/FK506-treated mice (PBS/FK506; fig. 2b). Lung sections from BLM-challenged mice in the absence of FK506 showed marked histological changes including: 1) large fibrous areas; 2) collapsed alveolar spaces; and 3) traction bronchiectasis in subpleural and peribronchial regions (BLM/vehicle; fig. 2c). However, compared with lungs from BLM/vehicle mice, lungs from BLM/FK506-treated mice (days 6–13) showed limited fibrotic lesions of attenuated intensity (BLM/FK506; fig. 2d).

Lung fibrosis was also examined by measuring the pulmonary hydroxyproline content as an index of collagen accumulation. Data are expressed as hydroxyproline content per left lung (fig. 3a). The upregulated hydroxyproline accumulation in response to BLM administration was significantly decreased in

**FIGURE 2.** Haematoxylin and eosin photomicrographs of lungs at day 14. a) PBS/vehicle: normal-lung architecture. b) PBS/FK506: normal-lung architecture. c) Bleomycin (BLM)/vehicle: extensive fibrosis with marked inflammatory cell infiltration. d) BLM/FK506: fibrotic lesions were observed in the peribronchial area, but the extent was limited and the intensity was attenuated compared with those in BLM/vehicle mice. Scale bar=500 μm.
response to FK506 compared with vehicle (BLM/vehicle, 61.8 ± 8.4 µg/ left lung versus BLM/FK506 76.8 ± 7.2 µg/ left lung; n=7; p<0.05). FK506 treatment alone did not alter hydroxyproline levels (PBS/FK506: 51.7 ± 5.5 µg/ left lung, n=3; PBS/vehicle: 55.1 ± 4.9 µg/ left lung; n=3).

It has been reported that TGF-β plays a crucial role in the pathogenesis of pulmonary fibrosis [6]. To determine whether the antifibrotic effect of FK506 depends on TGF-β activity, the levels of active TGF-β in BALF was measured on days 7 and 14. The TGF-β level was increased in response to BLM administration, and was higher on day 14 than on day 7. However, there was no significant difference in active TGF-β levels between BLM/vehicle mice and BLM/FK506 mice (day 7: 49.9 ± 17.5 pg/mL versus 40.1 ± 5.1 pg/mL; day 14: 87.6 ± 6.8 pg/mL versus 92.8 ± 8.6 pg/mL; fig. 3b).

To show a biological effect of active TGF-β, cells require the presence of TβR-I. Therefore, immunohistochemical examination of TβR-I expression was performed on lung sections from BLM-challenged mice in the absence or presence of FK506. Lung sections from control or BLM-challenged mice on day 14 were stained with an anti-TβR-I antibody. In normal lungs (PBS/vehicle), TβR-I staining was ubiquitous on bronchial cells (data not shown), alveolar epithelial cells and alveolar macrophages (fig. 4a). In lungs from BLM/vehicle mice, strong TβR-I staining was identified in epithelial cells and spindle-shaped interstitial cells in the area of fibrosis (fig. 4b). In contrast, lungs from BLM/FK506 mice showed reduced TβR-I staining in the cells throughout the fibrous area (fig. 4d). In particular, TβR-I staining of fibroblasts in the fibrous area was significantly decreased in BLM/FK506 mice compared with that in BLM/vehicle mice. Control lungs with rabbit IgG instead of anti-TβR-I showed no immunoreactivity (fig. 4f). Furthermore, Western blotting was used to analyse the TβR-I expression level in lung tissues (fig. 5). Compared with PBS/vehicle mice, BLM-challenged mice showed increased levels of TβR-I protein. Treatment with FK506 (days 6–13) significantly reduced the BLM-induced TβR-I expression. In contrast to TβR-I, TβR-II was expressed constitutively and was not significantly altered in response to BLM or FK506 treatment.

Effect of early FK506 treatment in BLM mice

It has been reported that calcineurin inhibitors inhibit the release of pro-inflammatory mediators in addition to possessing T-lymphocyte-associated immunosuppressive activity [12]. Therefore, continuous FK506 treatment initiated at the acute BLM injury phase would be expected to result in decreased fibrosis in the BLM model. BLM-challenged mice were given FK506 daily from the day before BLM administration until day 13, and survival was analysed by Kaplan–Meier curves (fig. 6). Only a few BLM/vehicle or BLM/FK506 (days 6–13) mice died by day 14. The survival of BLM/FK506 (days 0–13) mice was clearly decreased, and the survival rate was not improved even when FK506 treatment was limited to days 0–5 (data not shown). Early FK506 treatment in this group also decreased the survival.

To determine why early FK506 treatment decreased the survival of BLM-challenged mice, lung sections from day 7 were examined histologically (fig. 7). Sections from PBS/vehicle mice and PBS/FK506 (days 0–6) mice showed normal lung architecture (fig. 7a and 7b). Sections from BLM/vehicle (days 0–6) mice showed localised interstitial wall thickening and interstitial mononuclear cell infiltrates, predominantly in peribronchial areas (fig. 7c). Sections from BLM/FK506 (days 0–6) mice showed marked interstitial oedema with inflammatory cell infiltration in the alveolar space (fig. 7d).

To analyse the effect of FK506 on lung inflammation in BLM-challenged mice, total cell counts and differential cell analysis were assessed in BALF on days 3, 7 and 14. Compared with PBS-treated mice, BLM-challenged mice showed significantly
increased total cell numbers in BALF on days 3 and 7 (table 1). The major cell type in BALF was alveolar macrophages, often with signs of activation (enlargement with increased numbers of intracellular vacuoles). On day 7, BLM/FK506 (days 0–7) mice showed higher total cell counts than BLM/vehicle mice. There were no significant differences in differential cell analysis between these two groups.

**Effect of FK506 on pulmonary vascular permeability**

On the basis of the histological findings and results of BALF analysis, the current authors hypothesised that FK506 enhances pulmonary permeability in BLM mice, particularly in the acute BLM injury phase. Changes in lung permeability in response to BLM were analysed by comparing B/S ratios (fig. 8a). On day 7, the B/S ratios in BLM/vehicle mice and BLM/FK506 (days 0–6) mice were significantly increased compared with those of control mice, PBS/vehicle mice or BLM/FK506 (days 0–6) mice were significantly increased compared with those of control mice, PBS/vehicle mice or BLM/vehicle mice. There were no significant differences in differential cell analysis between these two groups.

**FIGURE 4.** Tumour growth factor (TGF)-β receptor (TβR)-I expression in lungs of control and bleomycin (BLM)-challenged mice on day 14. Immunohistochemical demonstration of TβR-I representing: a) normal control lungs (PBS/vehicle), showing ubiquitous TβR-I staining in alveolar epithelial cells; b and c) BLM/vehicle mice, showing strong TβR-I immunoreactivity in interstitial spindle-shaped cells in areas of fibrosis; d and e) BLM/FK506 mice showed decreased immunostaining for TβR-I in the interstitial cells compared to lungs from BLM/vehicle mice; f) control with rabbit immunoglobulin G instead of anti-TβR-I. Haematoxylin was used as a counterstain. Scale bar≈50 μm.

**FIGURE 5.** a) Western blot analysis on tumour growth factor-β receptor (TβR)-I and TβR-II expression in lungs of control and bleomycin (BLM)-challenged mice on day 14. Representative results of three different experiments are shown. b) TβR-I protein levels measured by scanning densitometry shown relative to control set at 100 densitometric units. Data are reported as the mean±SEM of triplicate measurements. *: p<0.05 compared with BLM stimulation alone; **: p<0.01 compared with control.

**FIGURE 6.** Kaplan–Meier survival curves of bleomycin (BLM)-challenged mice with or without FK506 treatment. Mice were followed for 14 days after administration 3 U·kg⁻¹ BLM. Mice were assigned to one of three groups (each n=12) and treated with PBS or with FK506 (days 0–13 or days 6–13, respectively). There was no difference in survival between BLM/vehicle mice and BLM/FK506 (days 6–13) mice. However, a significant reduction in survival of BLM/FK506 (−−−; days 0–13) mice was observed compared with BLM/vehicle mice or BLM/FK506 (−−−; days 6–13) mice (p<0.05). −−−: BLM/vehicle mice, days 0–13; −−−: PBS/vehicle mice, days 0–13.
FIGURE 7. Haematoxylin and eosin photomicrographs of lung tissues at day 7. a) PBS/vehicle: normal lung architecture. b) PBS/FK506 (days 0–6): normal lung architecture. c) Bleomycin (BLM)/vehicle: interstitial wall thickening with interstitial mononuclear cell infiltrate. d) BLM/FK506 (days 0–6): marked interstitial and alveolar oedema with extensive inflammatory cell infiltration. Scale bar=100 μm.

TABLE 1 Bronchoalveolar lavage fluid analysis: total cell count and differential cell counts

| Day   | Treatment       | Total \( \times 10^6 \text{ mL}^{-1} \) | AM %     | Neu. %   | Lym. %   |
|-------|-----------------|----------------------------------------|----------|----------|----------|
| Day 3 | BLM/vehicle     | 38.13 ± 18.73                          | 94.72 ± 48.47 | 2.31 ± 1.65 | 2.94 ± 1.52 |
|       | BLM/FK506 days 0-2 | 35.54 ± 16.92                          | 96.71 ± 45.08 | 1.32 ± 2.39 | 1.97 ± 1.77 |
| Day 7 | BLM/vehicle     | 57.70 ± 36.70                          | 87.16 ± 51.49 | 6.40 ± 6.46 | 6.43 ± 6.78 |
|       | BLM/FK506 days 0-6 | 120.00 ± 45.49*                        | 83.13 ± 20.81 | 8.83 ± 10.03 | 8.05 ± 7.18 |
| Day 14 | BLM/vehicle    | 20.70 ± 6.19                           | 68.26 ± 19.03 | 4.88 ± 7.10 | 26.67 ± 28.60 |
|       | BLM/FK506 days 0-13 | 26.10 ± 9.45                           | 83.03 ± 34.18 | 8.74 ± 9.00 | 7.93 ± 5.29  |
|       | control PBS     | 13.05 ± 2.30                           | 98.62 ± 16.63 | 0.0 ± 0.0  | 1.38 ± 1.15  |

Data are presented as mean±SD. BLM: bleomycin; AM: alveolar macrophage; Neu.: neutrophil; Lym.: lymphocyte. On day 7, FK506-treated mice showed higher total cell counts than BLM-alone mice, although there was no significant difference in differential cell counts between these two groups. *: p<0.05 versus BLM/vehicle at the same time points.
PBS/FK506 (days 0–6) mice. However, the increase on day 7 was significantly greater in BLM/FK506 (days 0–6) mice than in BLM/vehicle mice (16-fold increase versus 9-fold increase, respectively). No significant differences were observed in B/S ratios between these two groups on day 3 (data not shown). In addition, lung W/D weight ratios were determined and compared in each group (n=3) on day 7 (fig. 8b). The lung W/D ratio was significantly increased in BLM/FK506 (days 0–6) mice compared with that in BLM/vehicle mice (1.47-fold versus 1.22-fold, respectively).

**Pro-inflammatory cytokine levels in BALF**

To further elucidate the effect of FK506 on lung inflammation and vascular permeability, concentrations of the pro-inflammatory cytokines TNF-α, IL-1β and IFN-γ were measured in BALF on days 3 and 7 (fig. 8c). In BLM/vehicle and BLM/FK506 (days 0–6) mice, increased pro-inflammatory cytokine levels were detected on day 3 which returned to basal levels by day 7. However, on day 3, the levels were significantly higher in BLM/FK506 (days 0–6) mice than in BLM/vehicle mice (p<0.05). There were no significant differences in the levels on day 7.
DISCUSSION

The present study demonstrated the following. 1) FK506 inhibits TGF-β-induced collagen synthesis in human lung fibroblastic cells. 2) FK506 attenuates BLM-induced pulmonary fibrosis in mice when FK506 treatment is initiated on day 6 after BLM administration. 3) FK506 suppresses the BLM-induced expression of TβR-I receptor in vitro and in vivo. 4) Treatment with FK506 during the acute BLM injury phase increases pulmonary vascular permeability and exudation, and decreases survival of BLM-challenged mice.

Current therapeutic options for pulmonary fibrosis are ineffective and associated with severe morbidity and poor outcome [2, 13]. Current treatments for this disease focus on suppression of fibrosis with antifibrotic agents [14, 15]. Recently, it has been suggested that CsA inhibits TGF-β-induced signalling and collagen deposition in vitro [4]. Given that FK506 is a more potent immunosuppressant than CsA, the current authors were interested in whether FK506 is a more effective antifibrotic drug.

FK506 is known to exhibit its pharmacological effects on inflammatory cells, including T-cells, macrophages [12], eosinophils [16] and neutrophils [17]. Little is known regarding the effect of FK506 on fibroblasts. The present study showed that FK506 inhibits TGF-β-induced collagen synthesis in human lung fibroblastic cells.

TGF-β has been shown to play a pivotal role in tissue fibrosis, including pulmonary fibrosis [6, 7, 18]. TGF-β initiates signalling through the activation of the heterodimeric transmembrane complex of the TβR-I and TβR-II serine/threonine kinases. Upon binding TGF-β, constitutively active and autophosphorylated TβR-II phosphorylates and activates TβR-I. TGF-β sends signals from the receptor to the nucleus via a set of Smad proteins. Although the immunophilin FKBP12, which mediates the effects of FK506, has been shown to directly interfere with TβR-I signalling [9], its function in TGF-β-mediated signalling remains controversial [19, 20]. However, FK506 has been shown to compete effectively with TβR-I for FKBP12 binding [21]. To elucidate the possible role of FK506 in TGF-β signalling and TGF-β-induced collagen synthesis, the effect of FK506 on TIG-3-20 cells was evaluated. BLM has been reported to stimulate the expression of TβR-I in lung fibroblasts [22]. Therefore, BLM-induced upregulation of TβR-I expression was assessed in both the absence and presence of FK506. A significant inhibitory effect of FK506 on BLM-induced TβR-I expression was clearly shown. FK506 also completely inhibited TGF-β-induced collagen synthesis. This may also be due to inactivation of transcription factors [23] or to regulation of internalisation of TGF-β receptors, which is important for TGF-β signalling and for receptor turnover [24]. The precise mechanism remains unclear and requires further investigation.

It has been reported that the expression of TGF-β mRNA in the murine BLM-induced lung fibrosis model increases rapidly and peaks 5 days after BLM administration [25]. Therefore, in the present study, FK506 treatment was initiated on day 6 to evaluate its antifibrotic effect. In the current study, FK506 treatment starting on day 6 significantly attenuated BLM-induced lung fibrosis. Although no difference was observed in the level of active TGF-β in BALF between BLM/vehicle mice and BLM/FK506 mice, TβR-I expression in fibrotic lungs, assessed by immunostaining and Western blotting, was greatly decreased in BLM/FK506 mice, suggesting that FK506 inhibits TGF-β signalling via reduced TβR-I expression in fibroblasts, resulting in an amelioration of BLM-induced pulmonary fibrosis.

FK506 treatment during the acute BLM injury phase unexpectedly exacerbated BLM-induced exudative inflammation, resulting in a high mortality rate. The early administration of FK506 increased pro-inflammatory cytokine levels in BALF on day 3 and enhanced pulmonary permeability, resulting in severe lung injury. Pro-inflammatory cytokines, such as IL-1β, TNF-α and IFN-γ, have been shown to increase pulmonary vascular endothelial permeability and subsequent lung oedema [26]. Consistent with this, excessive pulmonary oedema, as assessed histopathologically, elevated B/S ratio and lung W/D weight ratio, was characteristically observed on day 7 in BLM/FK506 (days 0–6) mice. The mechanisms of the undesirable effect of FK506 are unknown. However, with respect to CsA, it has been shown to induce the production of pro-inflammatory cytokines in human airway epithelial cells in a dose-dependent manner [27], although other studies have indicated that CsA has inhibitory effects on the release of pro-inflammatory mediators and has T-lymphocyte-associated immunosuppressive actions [12]. These findings of acute injury phase could implicate that individuals who chronically receive FK506 as an immunosuppressive drug may be at greater risk for acute-lung injury and permeability-pulmonary oedema.

In conclusion, FK506 may have antifibrotic effects in vitro and in vivo. However, it may also have adverse pulmonary effects when administered under conditions of acute inflammation. Although FK506 is an attractive therapeutic candidate for human pulmonary fibrosis, additional investigation is necessary.

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REFERENCES

1. Katzenstein A-LA, Myers JL. Idiopathic Pulmonary Fibrosis. Clinical relevance of pathologic classification. Am J Respir Crit Care Med 1998; 157: 1301–1315.
2. Luppi F, Cerri S, Beghe B, Fabбри LM, Richeldi L. Corticosteroid and immunomodulatory agents in idiopathic pulmonary fibrosis. Respir Med 2004; 98: 1035–1044.
3. Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. Immunol Today 1992; 13: 136–142.
4. Eickelberg O, Pansky A, Koehler E, et al. Molecular mechanisms of TGF-beta antagonism by interferon gamma and cyclosporine A in lung fibroblasts. FASEB J 2001; 15: 797–806.
5 Homma S, Kawabata M, Kishi K, et al. [Cyclosporin A treatment of interstitial pneumonia]. Nihon Kokyuki Gakkai Zasshi 2003; 41: 427–433.

6 Bartram U, Speer CP. The role of transforming growth factor beta in lung development and disease. Chest 2004; 125: 754–765.

7 Giri S, Hyde D, Hollinger M. Effect of antibody to transforming growth factor beta on bleomycin induced accumulation of lung collagen in mice. Thorax 1993; 48: 959–966.

8 Nakao A, Fujii M, Matsumura R, et al. Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. J Clin Invest 1999; 104: 5–11.

9 Wang T, Donahoe PK, Zervos AS. Specific interaction of cyclosporin A on inflammatory cytokine production by human alveolar macrophages. Respir Med 1998; 92: 722–728.

10 Woessner JF Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 1961; 93: 440–447.

11 Izbicki G, Segel MJ, Christensen TG, Conner MW, Breuer R. Time course of bleomycin-induced lung fibrosis. Int J Exp Path 2002; 83: 111–119.

12 Garcia JEL, Rodriguez FM, Lopez AJ, et al. Effect of cyclosporin A on inflammatory cytokine production by human alveolar macrophages. Respir Med 1998; 92: 722–728.

13 Crystal RG, Bitterman PB, Mossman B, et al. Future research directions in idiopathic pulmonary fibrosis: summary of a National Heart, Lung, and Blood Institute working group. Am J Respir Crit Care Med 2002; 166: 236–246.

14 American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). Am J Respir Crit Care Med 2000; 161: 646–664.

15 American Thoracic Society/European Respiratory Society. International multidisciplinary consensus classification of the idiopathic interstitial pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS Board of Directors, June 2001 and by The ERS executive committee, June 2001. Am J Respir Crit Care Med 2002; 165: 277–304.

16 Hom JT, Estridge T. FK506 and rapamycin modulate the functional activities of human peripheral blood eosinophils. Clin Immunol Immunopathol 1993; 68: 293–300.

17 Sasakawa Y, Sakuma S, Higashi Y, Sasakawa T, Amaya T, Goto T. FK506 suppresses neutrophil chemoattractant production by peripheral blood mononuclear cells. Eur J Pharmacol 2000; 403: 281–288.

18 Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. N Engl J Med 1994; 331: 1286–1292.

19 Wang T, Li BY, Danielson PD, et al. The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. Cell 1996; 86: 435–444.

20 Bassing C, Shou W, Muir S, Heitman J, Matzuk M, Wang X. FKBP12 is not required for the modulation of transforming growth factor beta receptor I signaling activity in embryonic fibroblasts and thymocytes. Cell Growth Differ 1998; 9: 223–228.

21 Okadome T, Oeda E, Saitoh M, et al. Characterization of the interaction of FKBP12 with the transforming growth factor-beta type I receptor in vivo. J Biol Chem 1996; 271: 21687–21690.

22 Zhao Y, Shah DU. Expression of transforming growth factor-beta type I and type II receptors is altered in rat lungs undergoing bleomycin-induced pulmonary fibrosis. Exp Mol Pathol 2000; 69: 67–78.

23 Frantz B, Nordby EC, Bren G, et al. Calcineurin acts in synergy with PMA to inactivate I-kappa B/MAD3, an inhibitor of NF-kappa B. EMBO J 1994; 13: 861–870.

24 Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. Nat Cell Biol 2003; 5: 410–421.

25 Gurujeyalakshmi G, Giri SN. Molecular mechanisms of antifibrotic effect of interferon gamma in bleomycin-mouse model of lung fibrosis: downregulation of TGF-beta and procollagen I and III gene expression. Exp Lung Res 1995; 21: 791–808.

26 Orfanes SE, Mavrommati I, Korovessi I, Roussos C. Pulmonary endothelium in acute lung injury: from basic science to the critically ill. Intensive Care Med 2004; 30: 1702–1714.

27 Borger P, Kaufman HF, Timmerman JA, Scholma J, van den Berg JW, Koeter GH. Cyclosporine, FK506, mycophenolate mofetil, and prednisolone differentially modulate cytokine gene expression in human airway-derived epithelial cells. Transplantation 2000; 69: 1408–1413.