Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 536 (2006) 287–295
www.elsevier.com/locate/ejphar

Short courses of low dose dexamethasone delay bleomycin-induced lung fibrosis in rats

Fangping Chen, Likun Gong, Ling Zhang, Hui Wang, Xinming Qi, Xiongfei Wu, Ying Xiao, Yan Cai, Linlin Liu, Xianghong Li, J. Ren *

Received 23 November 2005; received in revised form 2 March 2006; accepted 6 March 2006
Available online 14 March 2006

Abstract

After comparing mortality and clinical signs in rats receiving different dexamethasone treatments, we investigated whether 0.5 mg/kg/d dexamethasone could delay pulmonary fibrosis induced by bleomycin and its time course (1, 3, 7, 14, 21 and 28 days). Tissue injury was assessed by apoptosis, lactate dehydrogenase (LDH) release, malondialdehyde content, and protein content; and inflammation was measured in terms of myeloperoxidase (MPO) activity, inflammatory cell count, and the mRNA expression of pro/inflammatory cytokines. Fibrogenic activity was analyzed by measuring the mRNA expression of fibrotic cytokines in tissue, and the promotion of fibroproliferation and synthesis of collagen type I by bronchoalveolar lavage fluids in vitro; and fibrosis was assessed by measuring the hydroxyproline content and collagen-I mRNA expression, and by histology. Bleomycin treatment induced tissue injury, inflammation and fibrogenic activity in lung, and led to fibrosis. Treatment with dexamethasone diminished the extent of fibrosis by strongly reducing inflammation, lung damage, and fibrogenic activity. These results demonstrate that the progression of bleomycin-induced pulmonary fibrosis in rats can be delayed by dexamethasone treatment, which appeared to alleviate not only inflammation but also lung damage and fibrogenic activity, indicating a possible new role for dexamethasone in the treatment of fibrosis.

Keywords: Leomycin; Dexamethasone; Inflammation; Pulmonary fibrosis

1. Introduction

Lung fibrosis is a pathological process characterized by the replacement of normal alveolar space by mesenchymal cells and extracellular matrix. The sequence of events leading to lung fibrosis involves injury with inflammation and disruption of the normal tissue architecture, followed by tissue repair with accumulation of mesenchymal cells in the area of derangement (Martinet et al., 1996). Risk factors associated with pulmonary fibrosis include smoking, environmental exposure, gastro-esophageal reflux disease, genetic factors, diabetes mellitus, infectious agents, and commonly prescribed drugs, such as bleomycin (Zisman et al., 2005).

Evaluation of several human fibrotic lung diseases reveals evidence of inflammation, a disordering of lung parenchymal cells, and fibrosis (Martinet et al., 1996; Reynolds, 2005). Recent studies support the notion that apoptosis of parenchymal cells, especially in the alveolar epithelium and capillary endothelial cells, contributes to the proliferation and accumulation of fibroblasts and myofibroblasts, and increased collagen deposition (Li et al., 2004; Selman et al., 2001). The inflammatory phase is also initiated by epithelial and/or endothelial injury, followed by the invasion of inflammatory cells into the alveolar interstitium (Teder and Noble, 2000). The finding that a variety of cytokines appear to be involved is intriguing, and significant progress has been made in many aspects of pulmonary fibrosis research, such as inflammation, fibroblast proliferation, and excessive extracellular matrix deposition (Zhang and Phan, 1996). There is growing evidence that the situation is complex and that no one factor is solely responsible for lung fibrosis (Elias et al., 1990).

Current therapeutic strategies are primarily aimed at controlling the inflammatory processes, often through the oral administration of glucocorticosteroids, which have been the first line of therapy for pulmonary fibrosis since the 1950s

* Corresponding author.
E-mail address: fancychen_78@hotmail.com (J. Ren).

0014-2999/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.03.011

(Hamman and Rich, 1944). In general, the use of anti-inflammatory agents to improve pneumonitis complications may have been hindered by a lack of knowledge of the importance of the timing and dose used, and by the absence of a molecular correlate to show their effectiveness. In a previous clinical report, short-term treatment with the corticosteroid dexamethasone started 12–48 h after birth in infants with neonatal respiratory distress syndrome, increased survival without causing bronchopulmonary dysplasia and reduced the requirement for subsequent late dexamethasone treatment (Garland et al., 1999). This suggests that a short course of corticosteroids initiated early after injury is able to prevent lung fibrosis. Studies of patients who developed clinically evident bleomycin-induced pneumonitis showed that alterations in lung function and chest radiography abnormalities are reversible with time, suggesting that the pulmonary changes due to bleomycin may be reversible, probably before the process of fibrosis starts (Stefan, 2001). Furthermore, one study examining possibly safer therapies for pulmonary fibrosis revealed that low doses of dexamethasone delivered constantly by autologous erythrocytes slowed the progression of lung disease (Rossi et al., 2004). We also found that the survival and body weight of rats treated with 0.5 mg/kg/d dexamethasone for 7 days was higher than that of rats treated with the same dose for 28 days or that of rats treated with 5 mg/kg/d dexamethasone for 7 days. Therefore, if short early treatment with a low dose of dexamethasone indeed prevents the development of fibrosis, it may be helpful in the treatment of patients at risk of developing pulmonary fibrosis.

2. Materials and methods

2.1. Chemicals and reagents

Dexamethasone acetate was purchased from Shanghai Sine (Shanghai, China). Bleomycin A_5 hydrochloride was purchased from Tianjin Taihe (Tianjin, China). The RT-PCR kit was obtained from Promega (WI, USA) and the PCR primers were synthesized by Bioasia Biologic Technology (Shanghai, China). The Cell-counting Kit-8 and human type I collagen detection kit were obtained from Dojindo Laboratories (Tokyo, Japan) and Chondrex (Redmond, WA, USA) respectively. In situ detection of DNA fragmentation was determined using a kit came from Roche (Mannheim, Germany). The kits for assaying myeloperoxidase (MPO) activity, lactate dehydrogenase (LDH) activity, protein content, and malondialdehyde were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were of the highest commercial grade available.

2.2. Animals

Male Sprague–Dawley (SD) rats (200–220 g; Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were maintained in a controlled environment and provided with water and standard rodent food. All rats were acclimatized to their new surroundings for 1 week prior to the animal experiments, which were performed at the Animal Department (Shanghai Institute of Material Medica) and approved by the Shanghai Animal Care and Use Committee.

2.3. Comparison of different dexamethasone treatments

The 50 SD rats were randomly divided into the following five groups ($n=10$ each): saline–water; bleomycin–water; bleomycin plus 7 days of 0.5 mg/kg/d dexamethasone; bleomycin plus 28 days of 0.5 mg/kg/d dexamethasone; bleomycin plus 7 days of 5 mg/kg/d dexamethasone. Briefly, the trachea was exposed and punctured with a 27-gauge needle via a small cervical skin incision and separation of the strap muscles under light chloral hydrate (40 mg/kg, 5 ml/kg, intraperitoneal) anesthesia. Rats in the saline group were injected intratracheally with 2 ml/kg saline; the others were injected intratracheally with bleomycin (5 mg/kg, 2 ml/kg in saline). Twenty-four hours after bleomycin treatment, rats were given by gavage 0.5 or 5 mg/kg/d dexamethasone for 7 consecutive days or 0.5 mg/kg/d dexamethasone for 28 consecutive days. Dose volume was 5 ml/kg. The day of intratracheal injection with bleomycin or saline was designated day 0. Rats were given drugs and weighed at 9:00 AM every day.

2.4. Experimental protocol for a short course of low-dose dexamethasone

The 180 SD rats were randomly divided into three groups ($n=60$ each): saline–water; bleomycin–water; bleomycin plus 7 days of 0.5 mg/kg/d dexamethasone. Rats were treated and drugs were given as described in Section 2.3. Rats were anesthetized with sodium barbital (80 mg/kg, intraperitoneal) at 15:00 PM on days 1, 3, 7, 14, 21 and 28. The lung was perfused free of blood, and then the left lungs were removed from the trachea and hilar nodes. The left lungs of half of the animals were fixed in 4% phosphate-buffered paraformaldehyde for histopathologic preparation, while the other samples were frozen in liquid nitrogen for the measurement of hydroxyproline, MPO, malondialdehyde and mRNA expression. Bronchoalveolar lavage fluid samples were collected from the right lung of each animal. Total and differential cell counts were performed, and total protein content and LDH activity were measured. For all animals, recovery of bronchoalveolar lavage fluid was about 80% of the utilized lavage volume. Recovery did not differ among the treatment and control groups or across the examined time points.

2.5. Lung tissue homogenate examination

Lung tissue samples were suspended at a w/v ratio of 1:10 in Tris–HCl buffered saline (pH 7.4, 10 mM Tris–HCl, 0.1 mM EDTA–2Na, 10 mM saccharose, 0.8% sodium chloride solution), and homogenized three times at 4 °C. A portion of each homogenate (0.1 ml) was assayed for MPO activity, using the MPO test kit and measuring absorbance at 460 nm. The MPO activity is expressed as units per gram of tissue (U/g); 1 U/

g of tissue is defined as the MPO activity in one gram of tissue capable of degrading 1 mM peroxide at 37 °C. The remainder of each homogenate was centrifuged at 3000 ×*g* for 10 min at 4 °C, and the supernatant was used for measurement malondialdehyde levels and collagen content. The malondialdehyde content was determined using a malondialdehyde test kit (Jiancheng, Nanjing, China) based on the thiobarbituric acid method; the results are expressed as nM malondialdehyde per gram of protein (nM/g). Collagen content was examined in terms of hydroxyproline, an amino acid common to all collagens. Hydroxyproline levels were quantified by chloramine T in duplicate lung tissue samples, as previously described (Brown et al., 2001). The data are expressed as micrograms of hydroxyproline per gram of tissue protein (μg/g).

2.6. Fibroblast proliferation and collagen type I content in vitro

Human fetal lung fibroblasts (WI-38 cells) obtained from ATCC (MD, USA) were seeded at 5×10^4 cells/ml into 96-well plates in Dulbecco's modified Eagle's medium (DMEM; Gibco, Renfrewshire, UK) supplemented with 10% fetal calf serum, and allowed to adhere. When cells reached sub-confluence, the medium was replaced with DMEM containing 0.4% FBS and 50 μg/ml of ascorbate. Twenty-four hours later, the cells were cultured for another 24 h in a 20% dilution of bronchoalveolar lavage fluid from rats in DMEM/0.4% fetal calf serum ($n=2$, each rat). Proliferation was assessed using the cell-counting Kit-8, according to the manufacturer's instructions. Human type I collagen was measured by ELISA with a human type I collagen detection kit and standardized according to the total protein content of cells.

2.7. Histopathological evaluation

Rat lung tissues were processed for routine paraffin embedding, and serial sections (5 μm) were stained with hematoxylin and eosin. The extent of alveolitis and fibrosis was blindly assessed using the previously described semi-quantitative criteria (Szapiel et al., 1979; Ashcroft et al., 1988).

2.8. In situ detection apoptosis

Briefly, paraffin-embedded tissues were sectioned (5 μ), and antigen retrieval was performed using citrate buffer. In situ detection of DNA fragmentation was done with the TUNEL assay according to the manufacturer's instructions. The slides were then analyzed by confocal microscopy (Leica, Heidelberg, Germany).

2.9. RT-PCR analysis for cellular factors

Total RNA was extracted from 100-mg frozen lung tissue using the TRI Reagent (Sigma, MO, USA), and 2 μg of total RNA were reverse transcribed into cDNA at 42 °C for 1 h with Moloney murine leukemia virus reverse transcriptase (Promega, WI, USA). The following primers were used for PCR: TGF-β1 (forward, 5′-CAA AGA CAT CAC ACA CAG TA-3′; reverse, 5′-GGT GTT GAG CCC TTT CCA GG-3′), TNF-α (forward, 5′-GTA GCC CAC GTC GTA GCA AA-3′; reverse, 5′-CCC TTC TCC AGC TGG GAG ACC-3′), macrophage chemoattractant protein-1 (MCP-1) (forward, 5′-ATG CAG GTC TCT GTC ACG-3′; reverse, 5′-TCA TAC TGT CTC TTG ATC-3′), endothelin-1 (forward, 5′-ACT TCT GCC ACC TGG ACA TC-3′; reverse, 5′-CAG ACA AAG AAC TCC GAG CC-3′), platelet-derived growth factor-β (PDGF-β) (forward, 5′-CTG AGC TGG ACT TGA ACA TG-3′; reverse, 5′-TTA AAC TTT CGG TGC TTG CC-3′), collagen type I α1 chain (forward, 5′-CCA ATCT GGT TCC CTC CCA CC-3′; reverse, 5′-GTA AGG TTG AAT GCA CTT TTG-3′), glyceraldehyde 3′-phosphate dehydrogenase (GAPDH) (forward, 5′-AAT GCA TCC TGC ACC ACC AA-3′; reverse, 5′-ATA CTG TTA CTT ATA CCG ATG-3′). Amplifications were for 28–40 cycles of 60 s at 95 °C, 45 s at 49–62 °C, and 75 s at 72 °C, and the PCR products were visualized on a 2% agarose gel using a Molecular Analyst Densitometer (Bio-Rad, Hercules, CA).

2.10. Statistical methods

Results are given as means ± S.D. Chi-square tests were used to compare mortality. Homogeneity of variance was evaluated using Levene's test. For homogeneous data, the mean difference between each treated group was assessed using LSD test. Otherwise, it was assessed using Dunn's test. Significance level was set at the 0.05.

3. Results

3.1. Comparison of mortality and body weight of rats after different dexamethasone treatments

Table 1 shows that the mortality of rats in the 7 days of 0.5 mg/kg/d dexamethasone group was lower than that of the other groups. By the end of the study, 60% of rats receiving 7 days of 5 mg/kg/d and 50% of rats receiving 28 days of 0.5 mg/kg/d dexamethasone had died. This mortality rate was significantly higher than that in the bleomycin group [30% (3/10)]. In addition, the body weight of bleomycin-treated rats and 7-day 0.5 mg/kg/d dexamethasone-treated rats decreased significantly from day 1 to day 7, and increased thereafter, but the body weight gain of the 7-day 0.5 mg/kg/d dexamethasone-treated animals was greater than that of the

Table 1
The mortality of rats in different dexamethasone treatment groups

	Total number of rats	The number of dead rats		Mortality (%)
		Day 1–7[a]	Day 8–28	
Bleomycin	10	3	0	30
7-day of 0.5 mg/kg/d dexamethasone	10	1	0	10
7-day of 5 mg/kg/d dexamethasone	10	4	2	60
28-day of 0.5 mg/kg/d dexamethasone	10	1	4	50

[a] Time interval.

bleomycin-treated animals at the end of the experiment. However, in the 7-day 5 mg/kg/d and 28-day 0.5 mg/kg/d dexamethasone groups, body weight gain was lower than in the bleomycin-treated group (data not shown). Because mortality was lower and body weight was higher in the 7-day 5 mg/kg/d dexamethasone group than in the other two treatment groups, we used the 7-day 5 mg/kg/d dexamethasone treatment group for further investigations.

3.2. Effects of dexamethasone treatment on markers of lung injury

The LDH activity in bronchoalveolar lavage fluid, which represents the extent of lung cell damage, was significantly increased in rats treated with bleomycin alone (Fig. 1A). In contrast, bleomycin-treated rats receiving a 7-day course of low-dose dexamethasone showed a significantly smaller increase in bronchoalveolar lavage fluid LDH activity, with basal activity being seen by day 28. Malondialdehyde induction in tissue samples and increased protein content in bronchoalveolar lavage fluid are two other important markers of lung injury, with the latter indicating alveolar edema. Bleomycin-treated rats showed significant increases in malondialdehyde and protein content levels peaked on day 7 after bleomycin treatment and declined thereafter. Rats receiving a short course of low-dose dexamethasone showed a significantly smaller increase in malondialdehyde and protein content on days 1, 3 and 7 ($P<0.01$) (Fig. 1B, C). Interestingly, the protein content in bleomycin rats decreased significantly from day 7, and there were no statistically significant differences on day 28 between rats receiving bleomycin and saline. These data collectively indicate that dexamethasone treatment protects lung cells from bleomycin-induced damage in rats.

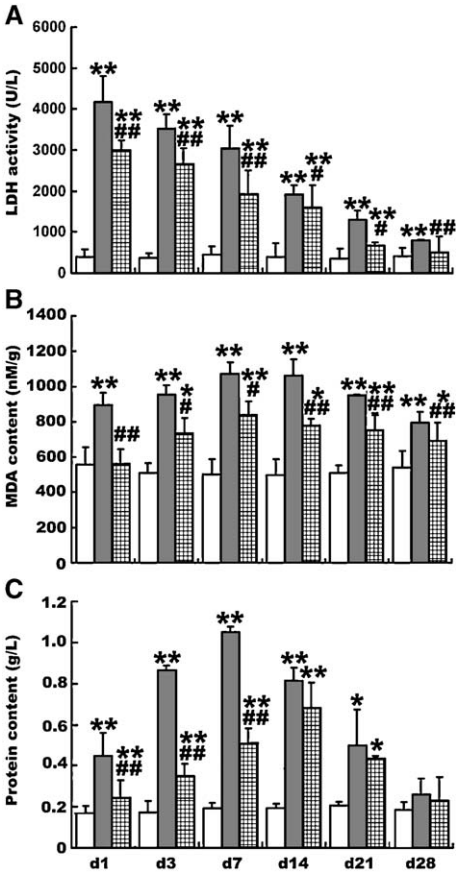

Fig. 1. Effect of saline (□), bleomycin (▩) and bleomycin plus dexamethasone (▦) on the LDH activity (A) and protein content (B) of bronchoalveolar lavage fluid samples, and the MDA content (C) of rat lung tissue. Data are presented as means±S.D. ($n=10$ for bronchoalveolar lavage fluid sample, $n=5$ for lung tissue, per group). Treatment with dexamethasone significantly reduced the bleomycin-induced LDH activity, and the content of protein and MDA on days 1, 3, 7, 14 and 21. **$P<0.01$, *$P<0.01$ vs. saline control group; $^{\#\#}P<0.01$, $^{\#}P<0.05$ vs. bleomycin group.

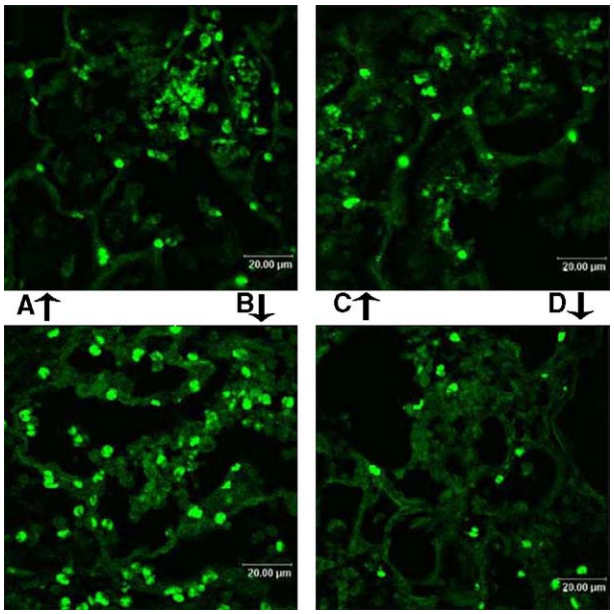

Fig. 2. Effect of bleomycin and bleomycin plus dexamethasone on apoptosis of lung tissue sections (5 μm) by In situ TUNEL detection. Internal scale bars=20 μm. A) bleomycin-treated rats on day 1, showing major apoptosis in alveolar space and alveolar septum. B) bleomycin-treated rats on day 7, showing more apoptotic nuclei than A). C) dexamethasone/bleomycin-treated rats on day 1, similar to A). D) dexamethasone/bleomycin-treated rats on day 7, showing significantly fewer apoptotic less apoptosis nuclei in both alveolar space and alveolar septum.

In bleomycin-treated rats, the first event noted was endothelial damage of the lung vasculature and apoptosis of alveolar epithelial type II cells (Adamson and Bowden, 1974). On the basis of this, apoptosis in lung tissue was analyzed day 1 and 7 by in situ TUNEL assay. On day 1, TUNEL-positive nuclei were observed mainly in the alveolar septum in both bleomycin-treated rats and 7-day 0.5 mg/kg/d dexamethasone-treated rats (Fig. 2A, C). However, on day 7, there were clearly fewer apoptotic nuclei in lung tissue from dexamethasone-treated rats than from bleomycin-treated rats (Fig. 2B, D). These results suggest that the dexamethasone treatment effectively inhibited apoptosis of mesenchymal cells in the alveolar septum in an early phase.

3.3. Effects of dexamethasone on the inflammatory response

Bleomycin treatment significantly increased the total number of cells in bronchoalveolar lavage fluid samples ($P<0.01$), with

numbers peaking on day 7. Administration of a short course of low-dose dexamethasone significantly reduced the bleomycin-induced increase in total cell number in bronchoalveolar lavage fluid samples over the entire experimental period (Fig. 3A). Similar results were found when specific counts of macrophages, neutrophils and lymphocytes were examined on days 1, 3, 7, 14, 21 and 28 (Fig. 3B, C, D).

The MPO activity of lung tissue samples was measured to determine neutrophil sequestration, which reflects the degree of lung inflammation. As shown in Fig. 4, bleomycin treatment induced rapid, high-level increases in MPO activity. Rats receiving a short course of low-dose dexamethasone showed significantly lower bleomycin-induced increases on days 1, 3 and 7 ($P<0.01$), with near-basal levels noted on days 1 and 3. These results indicate that dexamethasone treatment ameliorates the early stages of bleomycin-induced lung inflammation.

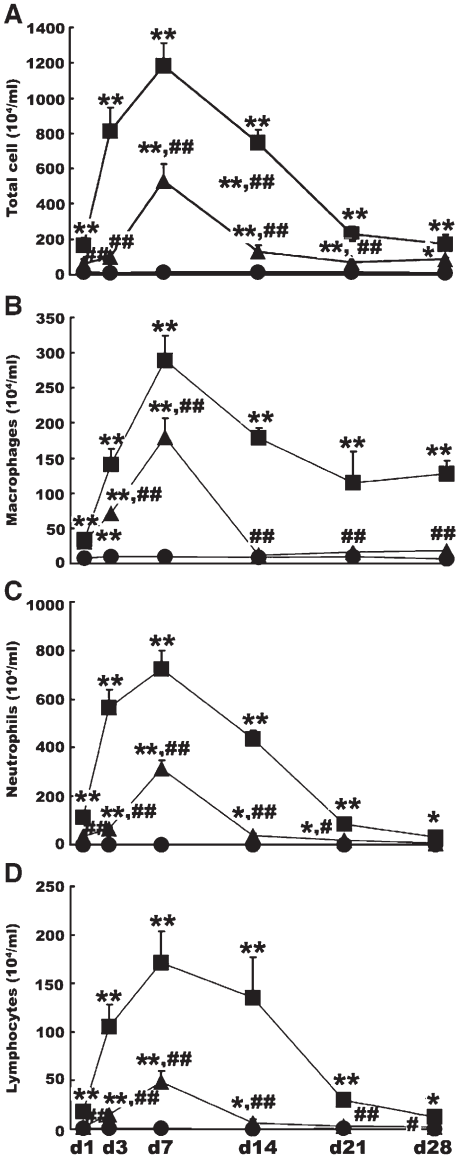

Fig. 3. Effect of saline (–●–), bleomycin (–■–) and bleomycin plus dexamethasone (–▲–) on total cell, macrophage, neutrophil and lymphocyte counts in bronchoalveolar lavage fluid. Data are presented as means±S.D. ($n=10$ per group). Treatment with dexamethasone significantly reduced the bleomycin-induced increase in total and specific inflammatory cells on days 3, 7, 14 and 21. $^{**}P<0.01$, $^{*}P<0.01$ vs. saline control group; $^{\#\#}P<0.01$, $^{\#}P<0.05$ vs. bleomycin group.

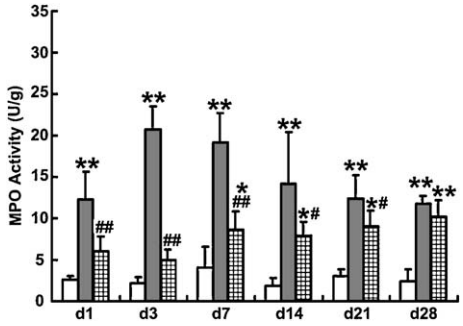

Fig. 4. Effect of saline (□), bleomycin (▩) and bleomycin plus dexamethasone (▦) on MPO in homogenates. Data are presented as means±S.D. ($n=5$ per group). Treatment with dexamethasone significantly reduced the bleomycin-induced MPO activity on days 1, 3, 7, 14 and 21. $^{**}P<0.01$, $^{*}P<0.01$ vs. saline control group; $^{\#\#}P<0.01$, $^{\#}P<0.05$ vs. bleomycin group.

3.4. Effects of the dexamethasone treatment on fibroblast proliferation and collagen type I protein content in vitro

Bronchoalveolar lavage fluid samples from animals in each group were added to cultured fibroblasts, and the mitogenic effects were monitored over 24 h. As shown in Fig. 5, cultures exposed to bronchoalveolar lavage fluid samples from bleomycin-treated rats showed significantly increased fibroblast proliferation and collagen type I protein content in cells on days 1, 3 and 7, with levels returning to control levels on days

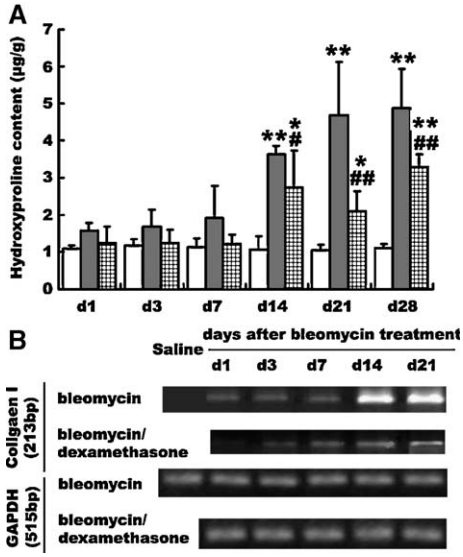

Fig. 5. Effect of bronchoalveolar lavage fluid from saline-treated (□), bleomycin-treated (▩) and bleomycin/dexamethasone-treated (▦) rats on fibroblast proliferation and collagen type I protein content in vitro. Data are presented as means±S.D. ($n=10$ per group). Bleomycin-induced fibroblast proliferation and collagen type I protein content were significantly less in cultures treated with bronchoalveolar lavage fluid samples taken from dexamethasone-treated rats on days 1, 3 and 7 versus those from rats treated with bleomycin alone. $^{**}P<0.01$, $^{*}P<0.01$ vs. saline control group; $^{\#\#}P<0.01$, $^{\#}P<0.05$ vs. bleomycin group.

14, 21 and 28. Cultures exposed to bronchoalveolar lavage fluid from bleomycin-treated rats receiving a 7-day course of 0.5 mg/kg/d dexamethasone on days 1, 3, and 7 showed significantly lower increases in fibroblast proliferation and collagen type I protein content $P<0.05$).

3.5. Effects of dexamethasone treatment on transcription of cytokines in lung tissue

Bleomycin-treated rats showed significant increases in the mRNA expression levels of pro-inflammatory mediators (TGF-β1 and TNF-α), inflammatory mediators (MCP-1) and fibroblast/myofibroblast proliferation mediators (TGF-β1, PDGF-β and endothelin-1) at all time points compared with the control group (Fig. 6). Rats receiving a 7-day course of 0.5 mg/kg/d dexamethasone showed significantly smaller increases in expression, providing additional evidence that dexamethasone counteracts the early bleomycin-induced inflammatory and fibroproliferative response.

3.6. Effects of dexamethasone treatment on collagen deposition and collagen-I mRNA expression levels

Fibrosis was examined in terms of collagen deposition. The lung tissue of bleomycin-treated rats had a significantly

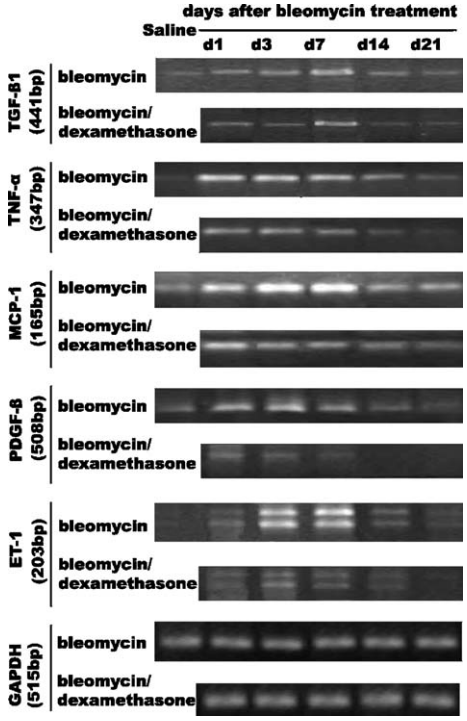

Fig. 6. Effect of dexamethasone on mRNA expression of inflammatory and mitogenic mediators in lung tissue following bleomycin-induced lung injury. The mRNA expression of TNF-α, TGF-β1, MCP-1, PDGF-β, endothelin-1 and GAPDH were examined on days 1, 3, 7, 14, and 21 after intratracheal instillation of bleomycin. On days 1, 3, and 7, the expression of TNF-α, TGF-β1, MCP-1, PDGF-β and endothelin-1 was significantly induced by bleomycin treatment, but this increase was blocked by treatment with dexamethasone. Data are representative for three independent experiments.

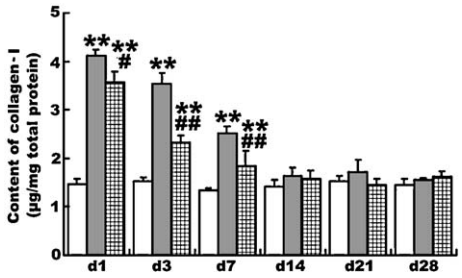

Fig. 7. A) Effect of saline (□), bleomycin (▩) and bleomycin plus dexamethasone (▦) on fibrosis assessed in rat lung homogenates. Data are presented as means ± S.D. ($n=5$ per group). Hydroxyproline content was significantly induced on days 14, 21 and 28 after intratracheal instillation of bleomycin, but this increase was reduced by dexamethasone treatment. $^{**}P<0.01$, $^{*}P<0.01$ *vs.* saline control groups; $^{\#\#}P<0.01$, $^{\#}P<0.05$ vs. bleomycin group. B) Effect of dexamethasone on mRNA expression of collagen-I in lung tissue following bleomycin-induced lung injury. These findings were consistent with the hydroxyproline content. Data of RT-PCR are representative of three independent experiments.

increased hydroxyproline content (used to assess collagen deposition) on days 14, 21 and 28 ($P<0.01$) (Fig. 7A). On day 28, the hydroxyproline content of lung tissues from bleomycin-treated rats was more than four-fold higher than from control animals. Bleomycin-treated rats receiving a short, low-dose course of dexamethasone showed significantly smaller increases in hydroxyproline content on days 14, 21 and 28 ($P<0.05$). Furthermore, RT-PCR revealed that transcription of the gene for collagen-I was significantly higher on days 14 and 21 in bleomycin-treated rats as compared to saline-treated controls (Fig. 7B), but bleomycin-treated rats receiving dexamethasone showed significantly smaller increases in collagen-I mRNA expression. These findings provide additional evidence that a 7-day course of low-dose dexamethasone protects rats against bleomycin-induced pulmonary fibrosis.

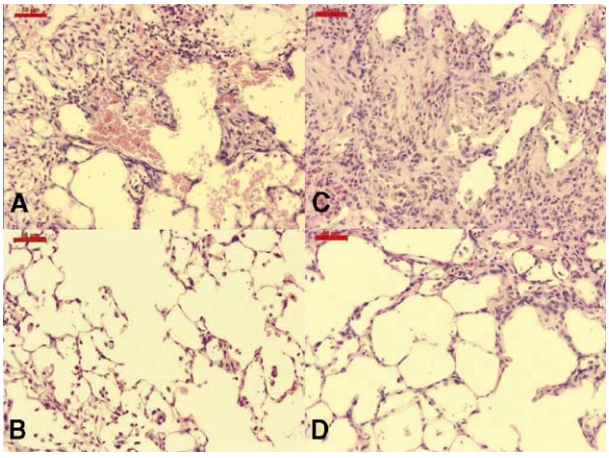

Fig. 8. Effect of dexamethasone on bleomycin-induced lung injury assessed by comparison of hematoxylin and eosin-stained lung tissue. Images were selected according to the alveolitis and fibrosis scores. Internal scale bars = 50 μm. A) bleomycin-treated rat on day 7, with major inflammation in the lung interstitium. B) bleomycin/dexamethasone-treated rat on day 7, showing mild infiltration of inflammatory cells. C) bleomycin-treated rat on day 28, with major fibrosis in the lung interstitium. D) bleomycin/dexamethasone-treated rat on day 28 showing less fibrosis.

Table 2
Effect of dexamethasone on the severity of bleomycin-induced lung alveolitis in rats

	Alveolitis Score					
	1 d	3 d	7 d	14 d	21 d	28 d
Saline	0.17±0.01	0.16±0.04	0.17±0.03	0.14±0.08	0.13±0.05	0.16±0.10
Bleomycin	0.74±0.14[a]	1.89±0.08[a]	2.69±0.22[a]	1.73±0.19[a]	0.77±0.31[b]	0.35±0.20
Dexamethasone	0.31±0.15[c]	0.9±0.13[a,c]	1.14±0.27[a,c]	1.02±0.09[a,d]	0.51±0.40[a]	0.25±0.10

[a]$P<0.01$, [b]$P<0.05$ vs. saline control group; [c]$P<0.01$, [d]$P<0.05$ vs. bleomycin group.

3.7. Histopathological findings

Lung tissue sections from the control (saline-treated) group had a normal structure (data not shown). On day 7 post-treatment, bleomycin-treated rats showed severe edema and large numbers of inflammatory cells in the alveoli and interstitium (Fig. 8A), while bleomycin/dexamethasone-treated rats showed less inflammation at this time point (Fig. 8B). On day 28, the bleomycin-treated rats showed marked histopathological changes, such as large fibrous areas and collapsed alveolar spaces (Fig. 8C). Although fibrotic lesions were observed in the bleomycin/dexamethasone group at this time point (Fig. 8D), the fibrosis was less severe.

To confirm the effects of dexamethasone on the histopathology of bleomycin-induced lung injury and fibrosis, the overall grade of lung inflammatory and fibrotic changes was scored on days 1, 3, 7, 14, 21 and 28. The alveolitis scores of the lung sections from bleomycin-treated rats peaked on day 7 and decreased thereafter. The bleomycin/dexamethasone-treated rats had significantly lower scores than the bleomycin-treated animals on days 1, 3, 7 and 14 ($P<0.05$), but there were no significant differences between the treatment groups on days 21 and 28 (Table 2). In contrast, the fibrosis scores of the bleomycin/dexamethasone group were significantly lower than those of the bleomycin group at all tested time points ($P<0.05$; Table 3).

4. Discussion

Comparison of mortality and body weight showed that a 7-day course of 0.5 mg/kg/d dexamethasone is better for treating pulmonary fibrosis induced by bleomycin in rats than the other two treatments, namely a 7-day course of 5 mg/kg/d and a 28-day course of 0.5 mg/kg/d dexamethasone. Our study also showed that the short course of low-dose dexamethasone delayed pulmonary fibrosis in rats, as determined by the decrease in lung hydroxyproline content and histopathologic lung fibrosis scores on day 28 after bleomycin, accompanied by a significant amelioration of bleomycin-induced body weight loss, cell apoptosis, lung edema, inflammatory response, fibroproliferation, collagen type I synthesis and cytokines during the development of lung injury in the acute phase.

Morphologic evidence has clearly demonstrated that most cases of pulmonary fibrosis exhibit an inflammatory response with scant, if any, fibrosis. This diffuse or multifocal inflammatory reaction is observed in diseases of unknown (i.e., sarcoidosis, desquamative interstitial pneumonia) or known (i.e., hypersensitivity pneumonitis, drugs) etiology (Ward and Hunninghake, 1998). At present, one of the theories on the pathogenesis of pulmonary fibrosis is that the inflammatory response is the primary response, followed by injury, and the release of mediators by inflammatory cells and resident lung cells as well as blood-borne mediators induce fibroblasts to proliferate and/or to produce excess collagen (Zisman et al., 2005; Walsh et al., 1993). Indeed, an increased MPO content in tissue and increased numbers of inflammation-related neutrophils, macrophages, lymphocytes, and total cells in bronchoalveolar lavage fluid samples have been found before day 7 in bleomycin-treated rats in previous studies and our study (Tarnell et al., 1992), accompanied by a high expression of (pro) inflammatory cytokines such as TNF-α, TGF-β1, macrophage inflammatory protein (MIP), MCP, interferon (IFN), interleukin (IL) and others, which are primarily secreted by multiple cells and peak on day 7 in rats suffering from bleomycin-induced pulmonary fibrosis (Izbicki et al., 2002). These results suggest that depression of the acute inflammation in the early phase may be a major target for drug treatment. Fortunately, bleomycin-treated rats receiving a 7-day course of low-dose dexamethasone showed significant decreases in the bleomycin-induced inflammatory response ($P<0.01$) and a significantly lower induction of the expression of inflammatory mediators before day 7. As noted, we were able to withdraw gluococorticosteroid treatment on day 7 after bleomycin treatment and still saw alleviation of inflammation. So we suggest that the serious inflammation

Table 3
Effect of dexamethasone on the severity of bleomycin-induced lung fibrosis in rats

	Fibrosis Score					
	1d	3d	7d	14d	21d	28d
Saline	0	0	0	0	0	0
Bleomycin	0.33±0.15[a]	1.33±0.05[a]	5.00±0.53[a]	5.5±0.84[a]	6.29±1.53[a]	7.42±0.38[a]
Dexamethasone	0	0.78±0.21[a,d]	3.06±1.33[a,c]	2.38±0.15[a,c]	2.89±0.65[a,c]	2.75±1.59[a,c]

[a]$P<0.01$ vs. saline control group; [c]$P<0.01$, [d]$P<0.05$ vs. bleomycin group.

arising as the disease progresses could be inhibited potently by a low dose of gluococorticosteroid, and once the inflammation is under control, it is better to withdraw the drug to prevent more serious inflammation because of immunosuppression.

Numerous studies have demonstrated that injury/activated alveolar epithelial cells and capillary endothelial cells in the early stage of alveolitis are important factors in the progression of lung fibrosis (Pardo and Selman, 2002). Consistent with previous findings of pulmonary injury in bleomycin-treated rats, we observed that bleomycin treatment caused lung injury, as shown by a significant increase in cell apoptosis, LDH activity, malondialdehyde content and protein content from day 0 to day 7, when most levels peaked and decreased thereafter. The short course of low-dose dexamethasone effectively inhibited these increases in the first 7 days, and levels remained low in the later stage, which implies that depression of lung injury is another target of this treatment.

The development of pulmonary fibrosis is mainly dependent on the proliferation of fibroblasts together with the synthesis and deposition of extracellular matrix (Snider, 1983). The most valuable indicator of pulmonary fibrosis is the deposition of extracellular matrix (Raghow, 1994). In our study, bronchoalveolar lavage fluid samples taken from bleomycin-treated rats on days 1, 3 and 7 demonstrated significant mitogenic activity toward WI-38 cultured fibroblasts in vitro ($P<0.05$), while samples taken from bleomycin/dexamethasone-treated rats on days 1, 3, and 7 induced far lower levels of proliferation ($P<0.05$). Similar results were observed for collagen type I protein content. These results showed that dexamethasone could inhibit fibroproliferation and the synthesis of collagen type I induced by bronchoalveolar lavage fluid from bleomycin-treated rats, indicating the activity of fibrotic mediators in bronchoalveolar lavage fluid from bleomycin-treated rats was depressed by dexamethasone. In fact, mesenchymal cells, such as myofibroblasts and epithelial cells, have been found to be another important source of cytokines to promote fibroproliferation and extracellular matrix deposition (Zhang and Phan, 1996). Furthermore, cytokines such as TGF-β1, TNF-α, PGDF-β and endothelin-1 from bronchoalveolar lavage fluid and mesenchymal cell, play a major role in stimulating the replication, survival, and migration of myofibroblasts during the pathogenesis of fibrotic diseases (Naidu et al., 2004; Craig et al., 2004; Bonner, 2004). Growing evidence indicates that the situation is complex and that no one single cytokine is solely responsible for the fibrotic response, though TGF-β is probably the most important cytokine in terms of the direct stimulation of lung matrix expression (Elias et al., 1990). Based on this, we examined the mRNA expression patterns of a number of related molecules in bleomycin- and bleomycin/dexamethasone-treated rats. Our results revealed that bleomycin-treated rats showed significant overexpression of TGF-β1, TNF-α, PDGF-β and endothelin-1 in lung tissues during the early stage of the pulmonary response. In contrast, bleomycin-treated rats receiving a short course of low-dose dexamethasone showed significantly lower bleomycin-induced expression of these molecules. These findings suggest that dexamethasone causes remission of pulmonary fibrosis by down-regulating induced fibroproliferative activity, synthesis of extracellular matrix, and cytokines including, but not limited to, clearly revealed that collagen deposition and fibrosis were less in bleomycin/dexamethasone-treated rat lungs on days 14, 21 and 28, as compared to samples from rats treated with bleomycin alone. Collectively, these findings indicate that dexamethasone treatment alleviates the bleomycin-induced overdeposition of extracellular matrix, leading to improvement of pulmonary fibrosis.

Orally administered corticosteroids are used for conventional therapy of pulmonary fibrosis, but clinically significant adverse effects have been seen in as many as 50% of the patients receiving accepted pharmacological doses (Flagel et al., 2002), and corticosteroids are usually tapered off within 2 to 3 months in a few patients showing a good response (International Consensus Statement, 2000). Low doses of dexamethasone delivered constantly by autologous erythrocytes slow the progression of lung disease (Rossi et al., 2004), but no previous study has tested whether withdrawal of low doses of dexamethasone also could delay the pulmonary fibrosis when inflammation begins to weaken. Previous studies also have shown that prolonged administration of corticosteroids, initiated before or at the same time as bleomycin administration, inhibits the development of lung fibrosis in rats (Phan and Kunkel, 1992; Grunze et al., 1988), but few studies have investigated whether corticosteroid administration, started after lung injury has occurred, are effective. Consensus statements indicate that the best time for therapy to reverse lung function is before the process of fibrosis starts (International Consensus Statement, 2000), and this is why bronchiolitis obliterans with organizing pneumonia (BOOP) and eosinophilic hypersensitivity could respond to treatment with corticosteroids. Our results suggest that a short course of corticosteroid treatment initiated early after injury is able to prevent lung fibrosis. Accumulating evidence has shown cell toxicity and lung damage to occur as soon as 1 day after bleomycin treatment (Aoshiba et al., 2003). In the present study, on day 1, we also observed that LDH, malondialdehyde, protein content, and number of apoptosis-positive nuclei had increased significantly in lung tissue and that clinical signs, such as dyspnea, cyanopathy, peaky sign and weight loss, had occurred, that the expression of mRNA for cytokines involved in inflammation and fibrosis was elevated, and that the activity of fibrotic mediators in bronchoalveolar lavage fluid was enhanced in bleomycin-treated rats. Here, we found that a 7-day course of 0.5 mg/kg/d dexamethasone given to rats 1 day after bleomycin treatment induced remission of the progression of bleomycin-induced pulmonary fibrosis.

In summary, we found that a short course of low-dose dexamethasone could delay pulmonary fibrosis induced by bleomycin by decreasing inflammation, lung damage and fibrogenic activity in lung tissue. These results throw some light on the role of corticosteroids in treating pulmonary fibrosis, pneumonitis induced by harmful agents, BOOP, hypersensitivity pneumonia, and most commonly, interstitial pneumonitis, which ultimately may progress into fibrosis, but further research is required and more commonly used glucocorticosteroids should be tested.

References

Adamson, I.Y.R., Bowden, D.H., 1974. The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. Am. J. Pathol. 77, 185–191.

Aoshiba, K., Tsuji, T., Nagai, A., 2003. Bleomycin induces cellular senescence in alveolar epithelial cells. Eur. Respir. J. 22, 436–443.

Ashcroft, T., Simpson, J.M., Timbrell, V., 1988. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. J. Clin. Pathol. 41, 467–470.

Bonner, J.C., 2004. Regulation of PDGF and its receptors in fibrotic diseases. Cytokine Growth Factor Rev. 15, 255–273.

Brown, S., Worsfold, M., Sharp, C., 2001. Microplate assay for the measurement of hydroxyproline in acid-hydrolyzed tissue samples. Biotechniques 30 (38–40), 42.

Craig, E.D., Mark, C.W., Maryanne, E., Ted, J.K., Stephen, J.M., Andrew, H.L., Edward, B.L., 2004. Imatinib mesylate inhibits the profibrogenic activity of TGF-β and prevents bleomycin mediated lung fibrosis. J. Clin. Invest. 114, 1308–1316.

Elias, J.A., Freundlich, B., Kern, J.A., Rosenbloom, J., 1990. Cytokine networks in the regulation of inflammation and fibrosis in the lung. Chest 97, 1439–1445.

Flagel, S.B., Vazquez, D.M., Watson, S.J., Neal, C.R., 2002. Effects of tapering neonatal dexamethasone on rat growth, neurodevelopment, and stress response. Am. J. Physiol., Regul. Integr. Comp. Physiol. 282, R55–R63.

Garland, J.S., Alex, C.P., Pauly, T.H., Whitehead, V.L., Brand, J., Winston, J.F., Samuels, D.P., McAuliffe, T.L., 1999. A three-day course of dexamethasone therapy to prevent chronic lung disease in ventilated neonates: a randomized trial. Pediatrics 104, 91–99.

Grunze, M.F., Parkinson, D., Sulavik, S.B., Thrall, R.S., 1988. Effect of corticosteroids on lung volume–pressure curves in bleomycin-induced lung injury in the rat. Exp. Lung Res. 14, 183–195.

Hamman, L., Rich, A.R., 1944. Acute diffuse fibrosis of the lungs. Bull. Johns Hopkins Hosp. 74, 177–212.

International Consensus Statement, 2000. Idiopathic pulmonary fibrosis: diagnosis and treatment. Am. J. Respir. Crit. Care Med. 161, 646–664.

Izbicki, G., Segel, M.J., Christensen, T.G., Conner, M.W., Breuer, R., 2002. Time course of bleomycin-induced lung fibrosis. Int. J. Exp. Pathol. 83, 111–119.

Li, X., Shu, R., Filippatos, G., Uhal, B.D., 2004. Apoptosis in lung injury and remodeling. J. Appl. Physiol. 97, 1535–1542.

Martinet, Y., Menard, O., Vaillant, P., Vignaud, J.M., Martinet, N., 1996. Cytokines in human lung fibrosis. Arch. Toxicol., Suppl. 18, 127–139.

Naidu, B.V., Woolley, S.M., Farivar, A.S., Thomas, R., Fraga, C.H., Goss, C.H., Mulligan, M.S., 2004. Early tumor necrosis factor-alpha release from the pulmonary macrophage in lung ischemia-reperfusion injury. J. Thorac. Cardiovasc. Surg. 127, 1502–1508.

Pardo, A., Selman, M., 2002. Idiopathic pulmonary fibrosis: new insights in its pathogenesis. Int. J. Biochem. Cell Biol. 34, 1534–1538.

Phan, S.H., Kunkel, S.L., 1992. Lung cytokine production in bleomycin-induced pulmonary fibrosis. Exp. Lung Res. 18, 29–43.

Raghow, R., 1994. The role of extracellular matrix in postinflammatory wound healing and fibrosis. FASEB J. 8, 823–831.

Reynolds, H.Y., 2005. Lung inflammation and fibrosis: an alveolar macrophage-centered perspective from the 1970s to 1980s. Am. J. Respir. Crit. Care Med. 171, 98–102.

Rossi, L., Castro, M., D'Orio, F., Damonte, G., Serafini, S., Bigi, L., Panzani, I., Novelli, G., Dallapiccola, B., Panunzi, S., Di, C.P., Bella, S., Magnani, M., 2004. Low doses of dexamethasone constantly delivered by autologous erythrocytes slow the progression of lung disease in cystic fibrosis patients. Blood Cells Mol. Diseases 33, 57–63.

Selman, M., King, T.E., Pardo, A., 2001. American Thoracic Society; European Respiratory Society; American College of Chest Physicians. Idiopathic PF: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann. Intern. Med. 134, 136–151.

Snider, G.L., 1983. Interstitial pulmonary fibrosis—which cell is the culprit? Am. Rev. Respir. Dis. 127, 535–539.

Stefan, S., 2001. Bleomycin-induced pneumonitis. Chest 20, 617–624.

Szapiel, S.V., Elson, N.A., Fulmer, J.D., Hunninghake, G.W., Crystal, R.G., 1979. Bleomycin-induced interstitial pulmonary disease in the nude, athymic mouse. Am. Rev. Respir. Dis. 120, 893–899.

Tarnell, E.B., Oliver, B.L., Johnson, G.M., Watts, F.L., Thrall, R.S., 1992. Superoxide production by rat neutrophils in the bleomycin model of lung injury. Lung 170, 41–50.

Teder, P., Noble, P.W., 2000. A cytokine reborn? Endothelin-1 in pulmonary inflammation and fibrosis. Am. J. Respir. Cell Mol. Biol. 23, 7–10.

Walsh, J., Absher, M., Kelley, J., 1993. Variable expression of platelet-derived growth factor family proteins in acute lung injury. Am. J. Respir. Cell Mol. Biol. 9, 637–644.

Ward, P.A., Hunninghake, G.W., 1998. Lung inflammation and fibrosis. Am. J. Respir. Crit. Care Med. 157, S123–S129.

Zhang, K., Phan, S.H., 1996. Cytokines and pulmonary fibrosis. Biol. Signals 5, 232–239.

Zisman, D.A., Keane, M.P., Belperio, J.A., Strieter, R.M., Lynch III, J.P., 2005. Pulmonary fibrosis. Methods Mol. Med. 117, 3–44.