Expression of pulmonary aquaporin 1 is dramatically upregulated in mice with pulmonary fibrosis induced by bleomycin

Xiwen Gao1, Guifang Wang1, Wei Zhang1, Qing Peng1, Min Xue1, Hu Jinhong1

A b s t r a c t

Introduction: Pulmonary fibrosis occurs due to fibroblast proliferation and collagen production in the lung and begins with alveolar inflammatory edema. Aquaporins (AQPs) play pivotal roles in lung fluid transport. In this study we establish the experimental model for pulmonary fibrosis in C57BL/6 mice to investigate expressions of AQP1 and AQP5 in lung tissue.

Material and methods: Mice in model groups were treated intratracheally with bleomycin with the dose of 5 mg/kg body weight. The mice were sacrificed at 1 week, 2 weeks and 3 weeks respectively. The left upper lungs were harvested for histopathologic H-E and Masson’s staining. The mRNAs of AQP1 and AQP5 were analyzed by real-time polymerase chain reaction (real-time PCR) and the proteins of AQP1 and AQP5 were analyzed by western blotting.

Results: Real-time PCR showed that AQP1 mRNA in bleomycin 1 w, 2 w, and 3 w groups increased by 377%, 880% and 823% respectively compared to that in the control group (p < 0.01). Western blotting showed that the expression of AQP1 protein in bleomycin 1 w, 2 w, and 3 w groups increased by 53%, 144%, and 141%, respectively (p < 0.05). AQP5 mRNA in bleomycin 1 w and 2 w group decreased by 78% and 66%, respectively (p < 0.05). In bleomycin 2 w and 3 w groups it decreased by 69% and 80% (p < 0.05).

Conclusions: The expression of AQP1 dramatically increased in pulmonary fibrosis. AQP1 plays an important role in the progress of pulmonary fibrosis.

Key words: aquaporin 1, aquaporin 5, pulmonary fibrosis, bleomycin.
bers in acute lung injury and lung cancer [4, 5]. To different extents, it has been reported that AQPI and AQPS were correlated with lung injury [6], and decreased expression of AQPS in bleomycin-induced lung fibrosis was found [7]. But AQPI in pulmonary fibrosis has not been investigated. AQPI is expressed in the apical and basolateral membrane of the microvascular endothelium, as well as in the visceral pleura [8].

Pulmonary fibrosis is a parenchymal lung disease characterized by progressive interstitial fibrosis [9]. Predicting risk factors remain unknown though lung fibroblasts maybe in terms of proinflammatory mediators and pro-fibrotic pathways. The persistence of alveolitis causes progressive disruption and destruction of the alveolar architecture, which can result in end-stage fibrosis [10]. A well-characterized animal model of human lung fibrosis is induced by bleomycin. Administration of bleomycin in animals results in an acute pan-alveolitis with pulmonary edema in the first week, followed by a 2 to 3 week subacute phase characterized by proliferation of fibroblasts and synthesis of extracellular matrix proteins [11, 12]. It is necessary for the resolution of acute lung injury to remove the pulmonary edema fluid from the alveolar space. This will probably prevent its late fibrotic consequences. The importance of the resolution of alveolitis has recently been shown by the fact that the removal of alveolar edema fluid early is associated with a better clinical outcome, specifically with a lower mortality rate in lung injury [13, 14]. We can find new therapeutic targets for pulmonary fibrosis resulting from lung injury by evaluating changes in the expression of aquaporins involved in the clearance of pulmonary edema.

Pulmonary inflammation and edema in mice are associated with a marked reduction in the expression levels of AQPI and AQPS in the distal lung, suggesting that the AQPI and AQPS in abnormal fluid fluxes have a role during pulmonary inflammation [15]. In the present study, we assessed the expression of AQPI and AQPS in lung tissue with pulmonary fibrosis induced by bleomycin. It showed that AQPI dramatic up-regulation was positively related to the severity of pulmonary fibrosis. Furthermore, AQPS down-regulation is presented at some time. A better understanding of the expression of AQPI in pulmonary fibrosis could be critical in finding new treatments for this life-threatening disease.

Material and methods

Animals

Male eight-week-old C57BL/6 mice purchased from Fudan University Animal Breeding and Research Center (Shanghai, China) were housed and cared for using protocols approved by Research Animal Care of Shanghai Changhai Hospital. Mice were treated intratracheally with bleomycin (Nippon Kayaku Kabushiki Kaisha, Japan) with a dose of 5 mg/kg body weight on day 0. The mice were sacrificed at 1 week, 2 weeks and 3 weeks. The left upper lungs were harvested for histopathological H-E and Masson’s staining. Forty-eight mice were divided into 4 groups: a bleomycin 1 w group, a bleomycin 2 w group, a bleomycin 3 w group and a control group treated with the same volume of normal saline.

Histopathological evaluation

The left upper lung tissues dehydrated in graded ethanol were embedded in paraffin, for sectioning into 4 μm and staining with hematoxylin and eosin (H&E). The slides were reviewed by 3 pathologists. The overall severity of fibrosis was determined by the pattern and intensity of interstitial inflammation according to the Szapiel 1979 working classification scale [16].

RT-PCR of AQPI and AQPS

The samples homogenized with the appropriate volume of TRIzol (Invitrogen, USA) solution were placed in centrifuge tubes for mRNA extraction and RT-PCR. The AQPI upstream primer sequence was 5’CTGGCCTTTGTGTTGAGCAT3’ and the downstream primer sequence was 5’CCAACACTGGCGATTGAT3’, a total of 150 bp. The AQPS upstream primer sequence was 5’GGCCTCAGCAACACAC3’ and the downstream primer sequence was 5’GTGTAACGCCAAGCCAATG3’, a total of 150 bp. The β-actin upstream primer sequence was 5’ACGGCCAGTCATCATTTG3’ and the downstream primer sequence was 5’TGGATGCCACAGGTCCAT3’. The 150 bp PCR products from each sample including AQPI, AQPS and β-actin were analyzed on 1% agarose gels (Promega, USA). The final results visualized on an ultraviolet light box were photographed on Kodak film. The density of each band was compared with a 150-bp band that expressed β-actin. The intensity ratio of the difference was later used to compare the results after fibrosis.

Immunohistochemical analysis of AQPI and AQPS

Anti-AQPI specificity antibody (AB3065) and anti-AQPS specificity antibody (AB3069) were purchased from Chemicon International Group (Canada). The sections were washed with phosphate buffered saline (PBS) (pH 7.4) thrice for 3 min each time. The sections were fixed with 3% H2O2 for 15 min at room temperature, and washed with PBS thrice, each time for 5 min. After PBS washing 3 more times, we added anti-AQPI or anti-AQPS antibody at 4°C for 12 h, and washed with PBS thrice further.
Results

Histopathological expression

After 1 week, the mice were treated with bleomycin, we observed the fact that some mice died. There was one in the 1 w group, two in the 2 w group and four in the 3 w group. The bleomycin groups showed pulmonary fibrosis at days 7, 14 and 21 and displayed higher scores compared with normal controls \((p < 0.05)\). The PF score increased gradually week by week. The PF score was higher in the 3 w group compared with the 1 w group \((p < 0.05, \text{Table I})\).

AQP1 and AQP5 mRNA Expression

To evaluate serial changes in AQP1 and AQP5 associated with pulmonary fibrosis, RT-PCR was used to analyze mRNA expression at days 7, 14 and 21 after the mice were treated with bleomycin. Real-time PCR showed that AQP1 mRNA expression in bleomycin 1 w, 2 w, and 3 w groups increased by 3.77, 8.8 and 8.23 times respectively compared to that in the control group \((p < 0.05)\) (Figure 1). AQP5 mRNA expression in the bleomycin 1 w and 2 w group decreased by 78% and 66% respectively \((p < 0.05)\), while there was no significant difference between the bleomycin 3 w group and the control group \((p > 0.05)\) (Figure 2).

AQP1 and AQP5 protein expression

Immunohistochemistry results showed that AQP1 was expressed on microvascular endothelial cells. Staining of AQP1 showed increased immunoreactivity in bleomycin groups compared to that in the control group, especially in the severe fibrotic areas (Figure 3), while AQP5 was expressed on type I pneumocytes in the alveolus. Staining of AQP5 showed decreased immunoreactivity in bleomycin groups compared to that in the control group (Figure 4).

Western blotting showed that AQP1 protein expression in bleomycin groups was significantly upregulated at days 7, 14 and 21 compared with the normal control group \((p < 0.05)\). Expression of AQP1 protein in bleomycin 1 w, 2 w, and 3 w groups increased by 53%, 144%, and 141% respectively of the control group value. There were no differences in AQP1 protein expression among the bleomycin groups (Table II, Figure 5). AQP5 protein expression in the bleomycin 1 w group decreased by 13% compared to that in the control group \((p > 0.05)\) and in the bleomycin 2 w and 3 w groups it decreased by 69% and 80% respectively \((p < 0.05)\) (Table III, Figure 6).

Discussion

The pathogenesis of pulmonary fibrosis is not clear but it appears to be the consequence of acute, persistent, or recurrent lung injury and inflammation [17]. Following this inflammatory response, increased vascular permeability and pulmonary

### Table 1: Semi-quantitative scores of pulmonary fibrosis (X ± s)

| Group          | n | Scores   |
|----------------|---|----------|
| Control        | 12| 1.00 ±0.00|
| Bleomycin 1 w  | 11| 1.60 ±0.89*|
| Bleomycin 2 w  | 10| 2.00 ±0.71*|
| Bleomycin 3 w  | 8 | 3.60 ±0.55**|

*\(p < 0.05\) vs. control group, \*\(p < 0.05\) vs. bleomycin 1 w group.

![Figure 1](image1.png)

**Figure 1.** Determine AQP1 mRNA expression by real-time PCR in mice with pulmonary fibrosis induced by bleomycin

![Figure 2](image2.png)

**Figure 2.** Determine AQP5 mRNA expression by real-time PCR in mice with pulmonary fibrosis induced by bleomycin

Then the second antibody was added and the section maintained at 37°C for 12 h, washed with PBS 3 more times and counterstained. The sections were then examined under a microscope. The epithelial cell membranes IN Collection system which has a brown staining WAS positive cells. Image analysis of the gray value of positive cells (IOD values) yielded the relative expression of AQP1 and AQP5 proteins.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 for Windows and Student’s \(t\)-test and ANOVA for repeated measures considering values of \(p < 0.05\) to be significant.
edema ensue [18]. The pathway for the flow of water across the endothelial and epithelial lung barrier is not completely elucidated, but recently AQP5 has been described as playing a critical role in water removal from the lung extracellular space [19]. Decreased expression of AQP1 and AQP5 has also been described in pathological conditions of the lung, such as acute lung injury [8]. However, alteration of AQP1 and AQP5 expression in chronic inflammatory processes such as that observed in

Figure 3. AQP1 protein expression is showed in immunohistochemistry. A – Control group, B – bleomycin 3 w group

Figure 4. AQP5 protein expression is showed in immunohistochemistry. A – Control group, B – bleomycin 3 w group
trapping water in the alveolar and interstitial spaces. Although AQPs may be differentially expressed according to tissue injury type and that epithelial cells’ response to treatment. But in our study the decrease was less at 21 days, indicating that the return of AQPS expression to baseline levels was coincident with a lessening of the inflammatory response. Previous studies [23] have shown that treatment of murine lung epithelial cells with tumor necrosis factor-α (TNF-α) results in a dependent decrease of concentration and time in AQPS mRNA and protein expression, suggesting that TNF-α may play an important role in AQPS down-regulation in the inflamed lung. Interestingly, the loss of AQPS in epithelial cells was not also observed in the fibrotic lung of mice that had received intratracheal instillation of bleomycin but in those receiving continuous infusion of bleomycin through a subcutaneous mini-pump, suggesting that both acute and chronic lung injuries induce reduction in AQPS expression. In our study AQPS mRNA expression started to return but protein expression decreased further. It showed that pulmonary fibrosis impaired the cellular membrane not damaged to genetic transcription. The biological significance of abnormal extracellular accumulation of fluid in the lung from patients with lung fibrosis is unclear. Pulmonary edema may play an active role in the development of lung fibrosis. Impairment of clearance of edema in patients with acute lung injury and interstitial

| Table II. AQPI protein expression determined by Western blotting (X ± s) |
|-----------------------------|---|------------------|
| Group | n | Gray scale ratio |
| Control | 8 | 0.34 ±0.05* |
| Bleomycin 1 w | 8 | 0.52 ±0.14* |
| Bleomycin 2 w | 6 | 0.83 ±0.17* |
| Bleomycin 3 w | 6 | 0.82 ±0.11* |

*p < 0.05 vs. control group. Gray scale ratio – AQPI protein/GAPDH

| Table III. AQPS protein expression determined by Western blotting (X ± s) |
|-----------------------------|---|------------------|
| Group | n | Gray scale ratio |
| Control | 8 | 3.32 ±0.80 |
| Bleomycin 1 w | 8 | 2.88 ±0.72 |
| Bleomycin 2 w | 6 | 1.04 ±0.25* |
| Bleomycin 3 w | 6 | 0.65 ±0.21* |

*p < 0.05 vs. control group. Gray scale ratio – AQPS protein/GAPDH

increased dramatically. AQPI may be more important in edema development following bleomycin inhalation, and the inability of pulmonary vascular endothelial cells to clear the air space may be an important factor in the inflammatory process of pulmonary fibrosis. Increased expression of AQP-1 has been reported in the frontal cortex of patients with Creutzfeldt-Jacob disease [21] and on the pleura of rats with a tuberculous pleural effusion [22]. The different types of injury and the different time points of the same injury might cause different alterations in AQP-1.

It was found by Gabazza et al. [7] that the expression of AQPS protein was significantly decreased in pulmonary edema resulting from bleomycin treatment. But in our study the decrease was less at 21 days, indicating that the return of AQPS expression to baseline levels was coincident with a lessening of the inflammatory response. Previous studies [23] have shown that treatment of murine lung epithelial cells with tumor necrosis factor-α (TNF-α) results in a dependent decrease of concentration and time in AQPS mRNA and protein expression, suggesting that TNF-α may play an important role in AQPS down-regulation in the inflamed lung. Interestingly, the loss of AQPS in epithelial cells was not only observed in the fibrotic lung of mice that had received intratracheal instillation of bleomycin but in those receiving continuous infusion of bleomycin through a subcutaneous mini-pump, suggesting that both acute and chronic lung injuries induce reduction in AQPS expression. In our study AQPS mRNA expression started to return but protein expression decreased further. It showed that pulmonary fibrosis impaired the cellular membrane not damaged to genetic transcription.

The biological significance of abnormal extracellular accumulation of fluid in the lung from patients with lung fibrosis is unclear. Pulmonary edema may play an active role in the development of lung fibrosis. Impairment of clearance of edema in patients with acute lung injury and interstitial...
lung disease is associated with poor clinical outcome [24]. Some previous studies have shown the development of lung fibrosis with the presence of chronic pulmonary edema after lung injury [25, 26]. In our model of lung fibrosis, we found a dramatic increase in the expression of AQP1. The chronic lung injury induced by bleomycin was probably the result of injected bleomycin entering into the systemic circulation at slow pace.

In conclusion, the results of the present study show that chronic lung injury and lung fibrosis is associated with up-regulated expression of AQP1 in the lung. Further studies will be necessary to clarify the cause-effect relationship between AQP1 up-regulation and the development of pulmonary fibrosis.

References
1. Dibas AI, Mia AI, Yorio T. Aquaporins (water channels): role in vasopressin-activated water transport. Proc Soc Exp Biol Med 1998; 219: 183-99.
2. Itoh T, Rai T, Kuwahara M, et al. Identification of a novel aquaporin, AQP12, expressed in pancreatic acinar cells. Biochem Biophys Res Commun 2005; 330: 832-8.
3. Walz T, Fujiyoshi Y, Engel A. The AQP structure and functional implications. Handb Exp Pharmacol 2009; 190: 31-56.
4. Su X, Song Y, Liang J, Bai C. The role of aquaporin-1 (AQP1) expression in a murine model of lipopolysaccharide-induced acute lung injury. Respir Physiol Neurobiol 2004; 142: 1-11.
5. Hoque MO, Soria JC, Woo J, et al. Aquaporin 1 is overexpressed in lung cancer and stimulates NIH-3T3 cell proliferation and anchorage-independent growth. Am J Pathol 2006; 168: 1345-53.
6. Towne JE, Harrod KS, Bachurski CJ, Menon AG. Tumor necrosis factor-alpha inhibits aquaporin 5 expression in mouse lung epithelial cells. J Biol Chem 2001; 276: 18657-64.
7. Blivet S, Philip F, Sab JM, et al. Outcome of patients with idiopathic pulmonary fibrosis admitted to the ICU for respiratory failure. Chest 2003; 120: 209-12.
8. Koike K, Chida M, Suzuki S, et al. Pulmonary fibrosis after acute viral infection. Am J Respir Cell Mol Biol 2000; 22: 34-44.
9. Gabazza EC, Kasper M, Ohta K, et al. Decreased expression of aquaporin-5 in bleomycin-induced lung fibrosis in the mouse. Pathol Int 2004; 54: 774-80.
10. King LS, Nielsen S, Agre P. Man is not a rodent: aquaporins in the airways. In: Fishman AP, Elias JA, Fishman JA, Grippi MA, Kaiser LR, Senior RM (eds.). Fishman's pulmonary diseases and disorders. 3rd ed.; McGraw-Hill, New York 2002; 573-85.
11. Rodriguez A, Pérez-Gracia E, Espinosa JC, Pumarola M, Torres JM, Ferrer I. Increased expression of water channel aquaporin 1 and aquaporin 4 in Creutzfeldt-Jakob disease and in bovine spongiform encephalopathy-infected bovine-PrP transgenic mice. Acta Neuropathol 2006; 112: 573-85.
12. Du H, Xie C, He Q, Deng X. Increased expression of aquaporin-1 on the pleura of rats with a tuberculous pleural effusion. Lung 2007; 185: 325-36.
13. Towne JE, Krane CM, Bachurski CJ, Menon AG. Tumor necrosis factor-alpha inhibits aquaporin 5 expression in mouse lung epithelial cells. J Biol Chem 2001; 276: 18657-64.
14. Selman M, King TE, Pardo A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann Intern Med 2001; 134: 136-51.