Article

Aerosolized STAT1 Antisense Oligodeoxynucleotides Decrease the Concentrations of Inflammatory Mediators in Bronchoalveolar Lavage Fluid in Bleomycin-Induced Rat Pulmonary Fibrosis

Ming Zeng[1], Bin Liao[2], Chen Zhu[1], Wenjun Wang[1], Xiaoqin Zhan[1] and Xianming Fan[1, 3]

It has been demonstrated that alveolar macrophages (AMs) play a key role in the pathogenesis of pulmonary fibrosis by releasing a variety of cytokines and inflammatory mediators. In addition, abnormal signal transducer and activator of transcription-1 (STAT1) activation in AMs may play a pivotal role in the process of alveolitis and pulmonary fibrosis. In this study, we transfected STAT1 antisense oligodeoxynucleotide (ASON) into rats by aerosolization, and then investigated the effect of STAT1 ASON on inflammatory mediators such as TGF-β, PDGF and TNF-α in bronchoalveolar lavage fluid (BALF) from rats with bleomycin (BLM)-induced rat pulmonary fibrosis. Our results showed that STAT1 ASON by aerosolization could enter into lung tissues and AMs. STAT1 ASON could inhibit mRNA and protein expressions of STAT1 and ICAM-1 in AMs of rat with pulmonary fibrosis, and had no toxic side effect on liver and kidney. Aerosolized STAT1 ASON could ameliorate the alveolitis through inhibiting the secretion of inflammatory mediators in BLM-induced rat pulmonary fibrosis. These results suggest that aerosolized STAT1 ASON might be considered as a promising new strategy in the treatment of pulmonary fibrosis. *Cellular & Molecular Immunology*. 2008;5(3):219-224.

Key Words: pulmonary fibrosis, STAT1, ASON, aerosolization, inflammatory mediator

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive interstitial lung disease. It is characterized by injury with loss of lung epithelial cells and abnormal repair of tissue, resulting in abnormal accumulation of fibroblasts and myofibroblasts, deposition of extracellular matrix (ECM) distortion of lung architecture, leading to respiratory failure (1). It is an insidious, progressive disease with a median survival of only 2 to 3 years following diagnosis (2). It has been demonstrated in many studies that inflammatory mechanisms are a major component of the pathogenesis of IPF and alveolar macrophages (AMs) play a key role in the pathogenesis of IPF by releasing a variety of cytokines and inflammatory mediators (3, 4). Among these cytokines and inflammatory mediators, TGF-β, PDGF and TNF-α are considered as potential participants in the inflammatory and fibrotic process of IPF.

Signal transducer and activator of transcription (STAT) proteins play a critical role in signal-transduction pathways triggered by cytokines. To date, seven mammalian STAT-encoding genes have been identified (5). These proteins are latent in the cytoplasm and become activated through tyrosine phosphorylation, which typically occurs through cytokine receptor associated Janus kinases (JAKs) (6). In normal mammalian cells, ligand-dependent activation of the STATs is a transient process, lasting for only several minutes to several hours. In contrast, in many diseases, the STAT proteins are persistently activated. Many studies supported that abnormal STAT activation was involved in many diseases such as tumors, inflammatory diseases and so on (7, 8).

The first discovered STAT protein was STAT1, which correlated with the occurrence and development of inflammatory and immune diseases (7-9). In previous study, we found that there was abnormal STAT1 activation in AMs of rats with bleomycin (BLM)-induced IPF based on nuclear transportation of STAT1, which led to upregulation of STAT1-dependent immune response gene intercellular adhesion molecule (ICAM-1) expression. The latter gave rise to the accumulation and activation of several types of inflammatory

[1]The Department of Respiratory Medicine, Affiliated Hospital of Luzhou Medical College, Luzhou 646000, China;

[2]The Department of Cardiothoracic Surgery, Affiliated Hospital of Luzhou Medical College, Luzhou 646000, China;

[3]Corresponding to: Dr. Xianming Fan, the Department of Respiratory Medicine, Affiliated Hospital of Luzhou Medical College, Luzhou 646000, China. Tel: +86-830-316-5321, Fax: +86-830-239-2753, E-mail: fxm129@163.com

Received Jan 14, 2008. Accepted Apr 3, 2008.

cells in lung tissue. These inflammatory cells released some cytokines, which in turn activated STAT1 and increased further releasing of the cytokines. Such positive feedback effect might lead to alveolitis and pulmonary fibrosis. Hence, abnormal STAT1 activation in AMs might play a pivotal role in this process. Furthermore, it has been demonstrated that STAT1 antisense oligodeoxynucleotide (ASON) aiming at AMs *in vitro* inhibited the expression of STAT1 mRNA and protein of AMs in BLM-induced rat pulmonary fibrosis (9-11). However, it is well known that studies *in vitro* are not always consistent with studies *in vivo*. In this study, we further investigated the *in vivo* effects of STAT1 ASON liposome complexes delivered by aerosolization on inflammatory mediators in bronchoalveolar lavage fluid (BALF) in a rat model of pulmonary fibrosis.

Materials and Methods

Animals and BLM-administration

Twenty adult female Wistar rats (purchased from the Laboratory Animal Department of Luzhou Medical College, China), weighing 180-200 g, were used in this study. They were intratracheally instilled with BLM, and then randomly divided into 4 groups: normal saline (NS) group, liposome (LP) group, ASON group, and ASON labeled at the 5'-end with fluorescein isothiocyanate (FITC-ASON) group. Briefly, after being anaesthetized and fixed, tracheostomies were performed on the rats to facilitate the intratracheal instillation of BLM-A5 (0.5 mg/100 g body weight) in 0.2-0.3 ml of 0.9% NaCl solution while rotating uprightly the rats to make the drug distribution in the lungs thorough and even. FITC-ASON group were aerosolized with phosphorothioated STAT1 ASON labeled at the 5'-end with FITC at day 7 after rats were intratracheally instilled with BLM, and then rats were sacrificed 6 h later. Moreover, NS group, LP group and ASON group were respectively aerosolized with NS, liposome (1,2-Dioleoyl-3-trimethyl-ammonium-propane, DOTAP) and phosphorothioated STAT1 ASON/liposome complexes at days 7, 9, 11 and 13 after rats were intratracheally instilled with BLM, and then they were sacrificed by exsanguination from right ventricle of heart under ketamine anaesthesia at day 14. The serum was stored at -20°C for the analysis of liver and kidney function.

Design and synthesis of STAT1 ASON

According to the theory of designing ASON and the analysis of STAT1 functional domain, the phosphorothioated STAT1 ASON was designed, and then synthesized by Sangon Biotechnology Engineering Company of Shanghai (Shanghai, China). The sequence for STAT1 ASON is 5'-GAA GCT CGT ACC ACT GCG ACA TCC-3'. The synthesized oligodeoxynucleotide was purified by HPLC and stored at -20°C. FITC-labeled ASON was also prepared.

Incubation of liposome with STAT1 ASON

Complexes were formed by incubating at 1:1 ratio of the liposome (400 μg) with STAT1 ASON (400 μg) for 30 min at room temperature in sterile saline (final volume, 3 ml).

Aerosolization

The mode of aerosolization was executed as previously described (12), and was ameliorated in this study. Briefly, to restrict rat movement, animals were confined in plastic bottle with the head extending from the top of the bottle. The open of ultrasonic nebulizer tube was aimed at the nose of rat. In this manner, all STAT1 ASON/liposome particles were forced down the tube past the rat nose for optimal effect of inhalation during respiration. Three milliliters of liposome, STAT1 ASON/liposome complexes, FITC-STAT1 ASON/ liposome complexes, or NS were respectively administered by nebulization for about 45 min using an ultrasonic nebulizer, model WH-802 (Yue Hua, China), every 3-5 min aerosolization with interval of 1-2 min.

Collection of BALF, isolation and purification of AMs

Rats were sacrificed by exsanguination from right ventricle of of heart under ketamine anaesthesia and then bronchoalveolar lavage (BAL) was performed. The left lung was ligated at the hilus and a small plastic tube was inserted into the trachea and placed in the right main bronchus. The tube was attached to a 10 ml syringe and 40 ml (in 5 ml, aliquots) of sterile phosphate-buffered saline (PBS) at 37°C was instilled. The fluid was retrieved by gentle aspiration after each infusion, then filtered through a double-layered sterile gauze on crushed ice, and then centrifuged at 4°C at 400 g for 15 min. Supernatants were stored at -20°C for the analysis of the concentration of TGF-β, PDGF and TNF-α by enzyme linked immunosorbent assay (ELISA). The cell pellet was resuspended in PBS and the total number of cells in BALF was counted in Neubauer haemocytometer chamber. After PBS was removed by centrifugation, the cell pellet was resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated for 2 h at 37°C in incubator having 5% CO_2 and 100% humidity. The medium was then removed and AMs were collected by washing with PBS to remove non-adherent cells. The isolated AMs were further studied for the analysis of STAT1 and ICAM-1 mRNA and protein expression.

At the same time, to localize ASON *in vivo*, BAL was performed in FITC-ASON group to obtain cells. The lung tissues were frozen, sectioned into 8 μm thick slices. Cells and the lung tissues were observed under fluorescence microscope.

RNA isolation and real-time fluorescent quantitative polymerase chain reaction (RT-PCR)

Total RNA was isolated from AMs using Trizol reagent and quantified using an $OD_{260/280}$ ratio, frozen at -70°C until the STAT1 and ICAM-1 mRNA expression were analysed by RT-PCR. Using QuantiTect SYBR Green RT-PCR Kit and the LightCycler Systems apparatus (Roche, Germany), RT-PCR was performed by the fluorescent dye SYBR Green methodology. QuantiTect Primer Assay: Rn_STAT1_1_SG QuantiTect Primer Assay (200) (QT00186291), Rn_ICAM1_ 1_SG QuantiTect Primer Assay (200) (QT00174447), and

Rn_Actb_1_SG QuantiTect Primer Assay (200) (QT00193473). Briefly, standard curves of STAT1, ICAM-1 and β-actin were firstly generated. Thirty ng RNA was serial 10-fold diluted. RNA of same volume was mixed with 10 μl 2× QuantiTect SYBR Green Master Mix, 1 μM of each primer, 2 μl RT/Taq Mix and 0.2 μl QuantiTect RT Mix in a final volume of 20 μl. The PCR reactions were as follows: cDNA synthesis at 50°C for 20 min, predenaturation at 95°C for 2 min, 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 30 s. Then the LightCycler Systems software provided a standard curve.

For each sample, the LightCycler Systems software provided an amplification curve constructed by relating the fluorescence signal intensity (normalized to the fluorescence of ROX internal passive reference) to the cycle number. The relative quantification of the steady-state of the target mRNA levels was calculated after normalization of the total amount of RNA tested by an active reference, β-actin. Fluorescence data from each sample were analyzed with the $2^{[-\Delta\Delta ct]}$ method with the mean of the NS group as the calibrator: relative copy number of GI RNA = $2^{[-\Delta\Delta ct]}$; $\Delta\Delta Ct = [Ct_{(GI)}$ (Unknown sample) - Ctβ-actin (Unknown sample)] - [($Ct_{(GI)}$ (NS group) - Ctβ-actin (NS group)]; GI is the gene of interest, and Ct is the cycle threshold that was defined as the cycle number at which a significant increase in the fluorescence signal crosses an arbitrary intensity threshold.

Western blotting analysis of STAT1 and ICAM-1 in AMs

AMs were lysed for 1 h at 4°C with 1% Nonidet P-40 in the presence of the following protease inhibitors: 100 μg/ml PMSF, 5 μg/ml aprotinin, 5 μg/ml p-tosyl arginine methyl lester, and 5 μg/ml of leupeptin. Equivalent quantities (50 μg) protein extracted from AMs was separated on 10% SDS-PAGE and blotted onto nitrocellulose. The membrane was blocked for 2 h in 5% nonfat milk and respectively incubated with mouse anti-rat STAT1 monoclonal antibody (dilution, 1:500), goat anti-rat ICAM-1/CD54 monoclonal antibody (dilution, 1:1,000) and rabbit anti-rat β-actin antibody (dilution, 1:1,000) in 3% nonfat milk in PBS for overnight at 4°C, washed three times with the same solution and respectively incubated with alkaline phosphatase-conjugated goat anti-mouse IgG-HRP, mouse anti-goat IgG-HRP and goat anti-rabbit IgG-HRP (dilution 1:2,000) in 3% nonfat milk in PBS for 2 h. After extensive washing, bands on the membrane were visualized with chemilumine-scence reagent according to the manufacturer's protocol.

Analysis of liver and kidney functional parameters

Blood was collected at day 14 from the right ventricle of the rats in heperinized tube. Serum was separated and stored at -70°C until analysis. The concentrations of alanine amino-transferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and serum creatinine (Scr) in serum of rats was measured by Olympus AU-2700.

Analysis of total number of cells in BALF

Animals were sacrificed and cells from the bronchoalveolar spaces were harvested by BAL as described above. The total number of cells in BALF was counted in Neubauer haemocytometer chamber.

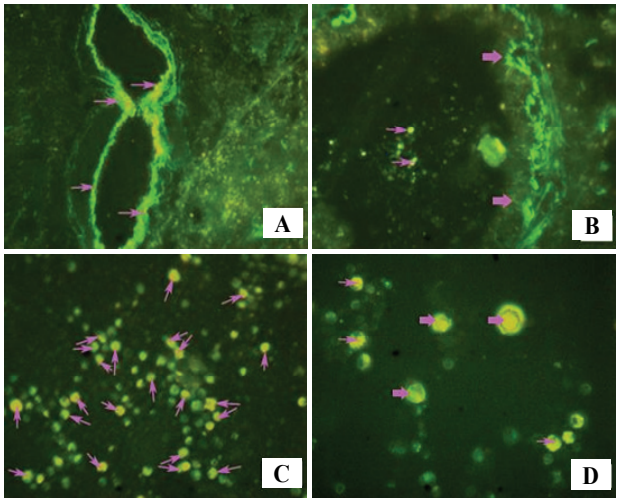

Figure 1. The induced fluorescence examination of lung tissue and cells in BALF. After rats were aerosolized with phospho-rothioated STAT1 ASON labeled at the 5'-end with fluorescein isothiocyanate, fluorescence microscope was used to observe the lung tissue (A, B) and cells (C, D) in BALF. The induced fluorescence was detected in (A) trachea epithelia (200×), (B) cells in alveolar space (thinner arrow) and epithelia (bolder arrow) (200×), (C) cells in BALF, mainly in AMs (200×), (D) cytoplasm (thinner arrow) and nuclei (bolder arrow) (400×).

ELISA for determining TGF-β, PDGF and TNF-α in BALF

BALF was thawed at room temperature and condensed 10 times by lyophilization, and then added to wells of rigid flat bottom microtiter plates coated with murine anti-rat TNF-α monclonal antibody. After incubation of the samples and thorough washing of the wells, HRP-conjugated anti-TNF-α antibody was added to the test wells. After a second incubation, the excess HRP-conjugated antibody was removed by washing. The HRP substrate was then added, and the color intensity measured with a microtiter plate reader. Assay for PDGF and TGF-β was prepared in a similar way following the manufacturer's instructions.

Statistical analysis

The data were presented as means ± SEM. One-way analysis of variance followed by a Dunnett test was used to determine significant difference between two groups. Probability value $p < 0.05$ was considered statistically significant.

Results

Visualization of FITC-positive cells and lung tissue

In order to visualize distribution of fluorescence by fluorescence microscope, rats were aerosolized with phosphorothioated FITC-STAT1 ASON, then sacrificed 6 h later. BAL was performed to obtain cells. At the same time, the lung tissues

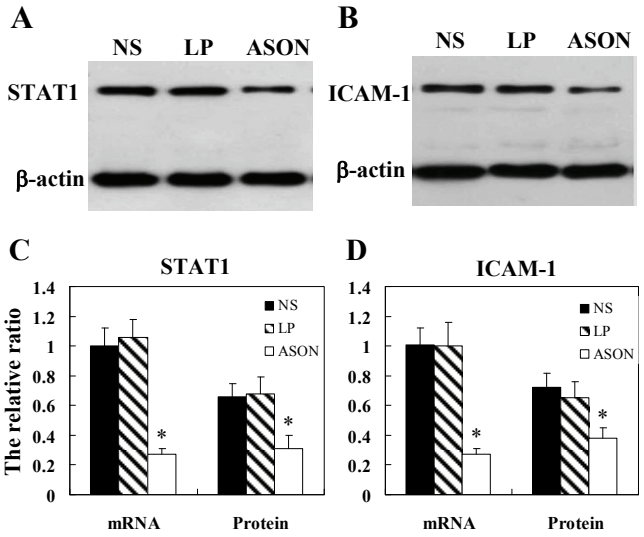

Figure 2. The mRNA and protein expressions of STAT1 and ICAM-1 in AMs. The protein expressions of STAT1 (A) and ICAM-1 (B) in AMs were detected by Western blotting. The mRNA levels of STAT1 and ICAM-1 were measured by FQ-PCR. The mRNA and the protein expressions of STAT1 (C) and ICAM-1 (D) in AMs in ASON, NS and LP group were detected. *$p < 0.05$, compared with NS group and LP group.

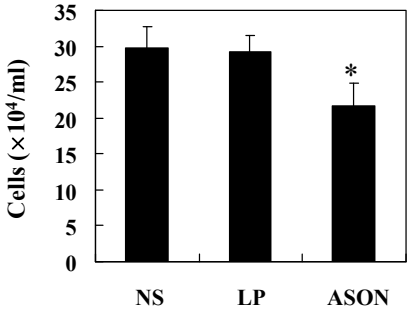

Figure 3. The total number of cells in BALF. Rats were aerosolized with NS, liposome and phosphorothioated STAT1 ASON respectively, and then sacrificed. The cells from the bronchoalveolar spaces were harvested by BAL. The total number of cells in BALF was counted in Neubauer haemocytometer chamber. *$p < 0.05$, compared with NS group and LP group.

were frozen, and then sectioned into 8 μm thick slices. The fluorescence was seen in cells (mainly in AMs) and the lung tissues, which confirmed STAT1 ASON could enter into lung and cells by aerosoliaztion (Figure 1).

Protein expression of STAT1 and ICAM-1 in AMs from rat pulmonary fibrosis

In addition, we performed Western blotting analysis of total proteins derived from AMs to assess local STAT1 and ICAM-1 expression in AMs. As shown in Figures 2A and 2B, the downregulation of STAT1 and ICAM-1 protein expression was found in STAT1 ASON treated AMs but not in AMs treated with liposome or NS. Both STAT1 and ICAM-1 protein expressions of AMs in ASON group were significantly lower than those in NS group and LP group ($p < 0.05$). There was no statistic difference between NS group and LP group ($p > 0.05$) (Figures 2C and 2D).

Effect of aerosolized STAT1 ASON on STAT1 and ICAM-1 mRNA expression in AMs of rat pulmonary fibrosis

STAT1, ICAM-1 and β-actin mRNA expressions by AMs were analysed by FQ-PCR after aerosolized with liposome, NS or STAT1 ASON/liposome complex respectively. Compared with LP group and NS group, STAT1 and ICAM-1 mRNA in AMs in STAT1 ASON group decreased ($p < 0.05$). The mRNA expressions of STAT1 and ICAM-1 in ASON group were 0.2708 and 0.2665 of that in NS group, respectively. However, liposome treatment had no effect on STAT1 and ICAM-1 mRNA expression in AMs, and there was no statistic difference between LP group and NS group (Figures 2C and 2D).

Effect of STAT1 ASON inhalation on liver and kidney functional parameters in serum of rats with pulmonary fibrosis

The concentrations of ALT, AST, BUN and Scr in serum had no statistic difference among NS group, LP group and ASON group ($p > 0.05$). These results suggested that STAT1 ASON inhalation has no toxic side effect of liver and kidney in BLM-induced rat pulmonary fibrosis (Table 1).

Effect of STAT1 ASON treatment in vivo on the total number of cells in BALF

Using the established model, we studied the effect of STAT1 ASON treatment *in vivo* on the total number of cells in BALF. We observed that the number of cells in the STAT1 ASON/liposome complex treatment group was significantly lower than that in the NS group and LP group ($p < 0.05$). However, there was no statistic difference in the total number of cells in BALF after treatment with NS or liposome alone ($p > 0.05$) (Figure 3).

Table 1. The effect of aerosolized STAT1 ASON on liver and kidney function in bleomycin-induced rat pulmonary fibrosis

Group	n	ALT (U/L)	AST (U/L)	BUN (mmol/L)	Scr (μmol/L)
NS	5	48.36 ± 8.21	175.88 ± 33.27	8.20 ± 1.20	42.08 ± 11.61
LP	5	52.78 ± 8.43	189.68 ± 43.08	6.82 ± 2.04	39.60 ± 5.44
ASON	5	51.38 ± 6.14	164.60 ± 12.50	8.48 ± 0.78	38.80 ± 5.99

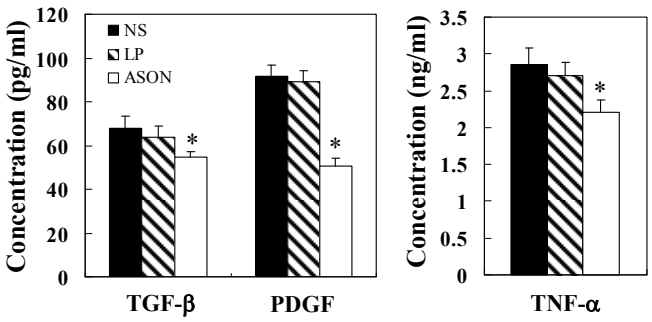

Figure 4. The concentrations of TGF-β, PDGF and TNF-α in BALF. The concentrations of TGF-β, PDGF and TNF-α in BALF were measured by ELISA. *$p < 0.05$, compared with NS group and LP group.

The effect of STAT1 ASON on TGF-β, PDGF and TNF-α production in BALF

The concentrations of TGF-β, PDGF and TNF-α in BALF were measured by ELISA. As shown in Figure 4, compared with NS group and LP group, the concentrations of TGF-β, PDGF and TNF-α in BALF in ASON group significantly reduced ($p < 0.05$).

Correlation assay

The concentrations of TGF-β, PDGF and TNF-α in BALF were significantly positively correlated with the total number of cells in BALF ($r = 0.887$, 0.759 and 0.866, respectively, $p < 0.01$).

Discussion

Pulmonary fibrosis is a common consequence, and often a central feature, of many lung diseases. It is also a highly lethal disorder. Current therapies for pulmonary fibrosis utilizing corticosteroids and/or immunosuppressants have shown little benefit (13). To date, there are no effective therapies for treating pulmonary fibrosis (14). Tran et al. reported positive effects of adenovirus-mediated transfer of the bacterial BLM resistance gene in mouse model of BLM-induced pulmonary fibrosis. However, this approach is limited to BLM-induced pneumopathy, and is not applicable to other types of interstitial pulmonary fibrosis. Recently, a novel variation in gene delivery was introduced wherein oligonucleotide phosphorothioates, being shown to be stable in most tissues. These analogues of oligonucleotides were more resistant to nuclease than their unmodified counterparts, and were therefore expected to exhibit longer activities. This approach presents a more promising method of therapy. Current therapeutic applications of ASON in lung diseases include asthma (15-17) and irradiation lung damage (12).

It has been demonstrated that AMs play a key role in the pathogenesis of IPF by virtue of their ability to release a variety of cytokines and inflammatory mediators (3). In previous *in vitro* study, we found that STAT1 ASON could attenuate the release of proinflammatory cytokines in AMs of BLM-induced rat pulmonary fibrosis and inhibit lung fibroblast proliferation and hydroxyproline secretion (10, 11). In the present study, we examined whether aerosolization of STAT1 ASON could depress STAT1 and ICAM-1 expression and inflammatory mediator release in BLM-induced rat pulmonary fibrosis.

ASON is generally short, single-stranded oligonucleotides that hybridize to their target mRNAs with high specificity and avidity through Watson-Crick base pairing. In this study, we choose aerosolization model to deliver STAT1 ASON to lung. As we know, there are many methods of delivering ASON to its target tissue. For example, intravenous or intraperitoneal administration, but by the two methods, ASON is easy to be decomposed by endonucleases in blood or abdomen where endonucleases are abundant. Moreover, the used dosage by aerosolization is usually lower than that by either intravenous or intraperitoneal administration. Consequently, it may cause to less toxic side effect. Another method of delivery of ASON to lung tissue is intratracheal injection. But, for practical clinical pulmonary fibrosis, the requirement for this method with exposure of trachea and associated trauma for injection would be contraindicated. In clinical trials, inhalation delivery of antibiotic, antifungal, and immunostimulatory agents has gained satisfying curative effect. The lung has a very large absorption surface area, at the same time, it is lined with surfactant, a cationic glycolipid at physiologic pH, which serves as a carrier to facilitate ASON uptake into lung cells. Ali et al. reported that aerosolized ASON was mainly distributed in lung tissue other than in other organs including heart, liver and kidney, and had no toxic effects (18). But, by intravenous or intraperitoneal administration, ASON mainly localized in liver and kidney. In the present study, the concentrations of ALT, AST, BUN and Scr in serum of BLM-induced rat pulmonary fibrosis showed no difference among NS group, LP group and ASON group, suggesting that the STAT1 ASON inhalation has no toxic side effect of liver and kidney.

In this study, oligonucleotides were synthesized with a phosphorothioate backbone to improve resistance to endonucleases. On the other hand, treatment of target cells with ASON requires an appropriate delivery system because ASON are anionic molecules that cross cell membranes poorly. AMs can rapidly internalize liposome and hence are apt to be exposed to STAT1 ASON after aerosolization. Liposome-mediated gene transfer has a higher efficacy than adenovirus-mediated gene transfer due to the lack of immunogenicity and toxicity and holds greater potential for long-term transgene therapy. For these reasons, liposome was chosen in the study.

Recently, Bringardner et al. propose that inflammation plays a critical role in the pathogenesis of IPF (19). BAL is named as "fluid lung biopsy" and the total number of cells in BALF can reflect the extent of lung inflammation. Our data showed that STAT1 ASON administration reduced the inflammatory cell infiltration as shown by a significant decrease in total number of cells in BALF in BLM-induced rat pulmonary fibrosis. The possible mechanism was that

aerosolized STAT1 ASON could lead to downregulation of ICAM-1 expression.

TGF-β is a critical mediator of fibrogenesis by exerting immunological actions, having direct effects on structural cells involved in the synthesis of ECM and affecting fibroblast proliferation and the differentiation of fibroblasts into myofibroblasts (20). Several experimental animal studies have demonstrated that it is possible to decrease bleomycin-induced lung fibrosis by inhibiting TGF-β type I receptor (21) or using TGF-β neutralization antibodies or inhibiting TGF-β signaling (22). TNF-α also plays a key role in the development of interstitial inflammation and fibrosis. Agents with anti-TNF-α properties such as pirfenidone and etanercept have shown promise in treatment of patients with IPF (23). PDGF, another important inflammatory and fibrogenic mediator, induces fibroblast chemotaxis, fibroblast proliferation and promotes fibroblast-mediated tissue matrix contraction (24).

In this study, we also observed the effect of aerosolized STAT1 ASON on TGF-β, PDGF and TNF-α. The results demonstrated that STAT1 ASON by aerosol delivery *in vivo* reduced the concentrations of TGF-β, PDGF and TNF-α in BALF in BLM-induced rat pulmonary fibrosis. In addition, the concentrations of TGF-β, PDGF and TNF-α were positively correlated to the total number of cells in BALF.

In conclusion, aerosolization of STAT1 ASON could ameliorate alveolitis in BLM-induced rat pulmonary fibrosis. However, we have no evidence to prove that aerosolized STAT1 ASON has direct effect on pulmonary fibrosis, which needs further study.

Acknowledgement

This work was supported by a grant from the National Natural Science Foundation of China (No. 30570814 to Xianming Fan).

References

1. Gharaee-kermani M, Hu B, Thannickal VJ, Phan SH, Gyetko MR. Current and emerging drugs for idiopathic pulmonary fibrosis. Expert Opin Emerg Drugs. 2007;12:627-646.
2. Scotton CJ, Chambers RC. Molecular targets in pulmonary fibrosis. The myofibroblast in focus. Chest. 2007;132:1311-1321.
3. Scheule RK, Perkins RC, Hamilton R, Holian A. Bleomycin stimulation of cytokine secretion by the human alveolar macrophages. Am J Physiol. 1992;262:L386-L391.
4. Prasse A, Pechkovsky DV, Toews GB, et al. A vicious circle of alveolar macrophages and fibroblasts perpetuates pulmonary fibrosis *via* CCL18. Am J Respir Crit Care Med. 2006;173:781-792.
5. Baker SJ, Rane SG, Reddy EP. Hematopoietic cytokine receptor signalling. Oncogene. 2007;26:6724-6737.
6. Mertens C, Darnell Jr JE. Snapshot: JAK/STAT signaling. Cell. 2007;131:612.
7. Ramana CV, Chatterjee-Kishore M, Nguyen H, Stark GR. Complex roles of stat1 in regulating gene expression. Oncogene. 2000;19:2619-2627.
8. Gharavi NM, Alva JA, Mouillesseaux KP, et al. Role of the JAK/STAT pathway in the regulation of interleukin-8 transcription by oxidized phospholipids *in vitro* and in atherosclerosis *in vivo*. J Biol Chem. 2007;282:31460-31468.
9. Fan XM, Wang ZL, Li ZH. STAT1 activation and STAT1-dependent immune-response gene ICAM-1 expression in alveolar macrophages of rats suffered from interstitial pulmonary fibrosis. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2003;19:3-6.
10. Fan XM, Wang ZL. STAT1 antisense oligonucleotides attenuate the proinflammatory cytokines release by alveolar macrophages in bleomycin-induced fibrosis. Cell Mol Immunol. 2005;2:211-217.
11. Fan XM, Liu CT, Zhan XQ, et al. The effect of STAT1 antisense oligonucleotides on lung fibroblast proliferation and hydroxyproline secretion. Zhonghua Jie He He Hu Xi Za Zhi. 2005;28:709-713.
12. Carpenter M, Epperly MW, Agarwal A, et al. Inhalation delivery of manganese superoxide dismutase-plasmid/liposomes protects the murine lung from irradiation damage. Gene Ther. 2005;12:685-693.
13. Balamugesh T, Behera D. Idiopathic pulmonary fibrosis. J Assoc Physicians India. 2007;55:363-370.
14. Tabata C, Tabata R, Kadokawa Y, et al. Thalidomide prevents bleomycin-induced pulmonary fibrosis in mice. J Immunol. 2007;179:708-714.
15. Stenton GR, Ulanova M, Dery RE, et al. Inhibition of allergic inflammation in the airways using aerosolized antisense to Syk kinase. J Immunol. 2002;169:1028-1036.
16. Duan W, Chan JH, Mckay K, et al. Inhaled p38α mitogen-activated protein kinase antisense oligonucleotide attenuates asthma in mice. Am J Respir Crit Care Med. 2005;171:571-578.
17. Isogai S, Athiviraham A, Fraser RS, Taha R, Hamid Q, Martin JG. Interferon-γ-dependent inhibition of late allergic airway rsponses and eosinophilia by $CD8^+$ γδT cells. Immunology. 2007;122:230-238.
18. Ali S, Leonard SA, Kukoly CA, et al. Absorption, distribution, metabolism, and excretion of a respirable antisense oligonucleotide for asthma. Am J Respir Crit Care Med. 2001;163:989-993.
19. Bringardner BD, Baran CP, Eubank TD, Marsh CB. The role of inflammation in the pathogenesis of idiopathic pulmonary fibrosis. Antioxid Redox Signal. 2008;10:287-301.
20. Coker RK, Laurent GJ, Shahzeidi S, et al. Transforming growth factors-β1, -β2, and -β3 stimulate fibroblast procollagen production *in vitro* but are differentially expressed during bleomycin-induced lung fibrosis. Am J Pathol. 1997;150:981-991.
21. Nagano J, Iyonaga K, Kawamura K, et al. Use of tacrolimus, a potent antifibrotic agent, in bleomycin-induced lung fibrosis. Eur Respir J. 2006;27:460-469.
22. Li YJ, Azuma A, Usuki J, et al. EM703 improves bleomycin-induced pulmonary fibrosis in mice by the inhibition of TGF-β signaling in lung fibroblasts. Respir Res. 2006;7:16.
23. Selman M, Thannickal VJ, Pardo A, Zisman DA, Martinez FJ, Lynch JP. Idiopathic pulmonary fibrosis: pathogenesis and therapeutic approaches. Drugs. 2004;64:405-430.
24. Bonner JC. Regulation of PDGF and its receptors in fibrotic diseases. Cytokine Growth Factor Rev. 2004;15:255-273.