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Effect of tranilast on bleomycin induced pulmonary fibrosis in a rat model

Liantao Tang, Tao Jiang*, Xiaoli Han and Dongling Chen

Department of Respiratory, the First Affiliated Hospital, Chongqing Medical University, Chongqing, China.

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This study aimed to investigate the effect and mechanism of tranilast (TR) on bleomycin (BLM) induced pulmonary fibrosis in a rat model, pulmonary fibrosis was induced in rats by using intratracheal bleomycin and the serum and lungs were collected 28 days later. The serum levels of IL-4 and IFN-γ were determined by ELISA and IL-4/IFN-γ was calculated. Reverse transcription polymerase chain reaction (RT-PCR) was employed to measure the mRNA expression of TGF-β1. Immunohistochemistry was employed to detect the protein expression of TGF-β1 followed by evaluation of alveolitis and fibrosis. When compared with BLM group and glucocorticoid (GC) group, the INF-γ level was markedly increased and IL-4 level dramatically decreased in the TR group with a significant decrease of IL-4/INF-γ ratio. The mean optical density and mRNA expression of TGF-β1 were markedly decreased accompanied by low scores of alveolitis and fibrosis. Significant differences were observed between TR groups (medium and high dose) and BLM group as well as control group (P < 0.05) in the aforementioned parameters. Moreover, marked difference was also observed among TR groups (P < 0.05) in the aforementioned parameters. Both TR and GC can affect the Th1/Th2 lymphocyte balance and inhibit the TGF-β1 expression improving the BLM induced pulmonary fibrosis.

Key words: Th1/Th2, tranilast, pulmonary fibrosis, interferon-γ, transforming growth factor-β1.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease, with a median survival of 3 to 5 years (Barqaqli et al., 2009). The precise pathologic mechanisms of pulmonary fibrosis are not fully understood. Fibrosis is thought to be the result of an abnormal wound-healing response to successive lung injury. Oxidative stress with reactive oxygen species (ROS) plays an important role in the process of pulmonary fibrosis (Barqaqli et al., 2009). Excessive levels of reactive oxygen species (ROS) may damage cellular macromolecules. Antioxidant enzymes, such as extracellular superoxide dismutase (ECSOD), may directly inactivate ROS and prevent ROS-initiated reactions (Rahman et al., 2006). Furthermore, chemokines recruit leukocytes, fibroblast precursors and other key effector cells to sites of tissue injury, and therefore, represent a potential of antifibrotic therapy. Macrophages are integrated into all stages of the fibrotic process, perhaps because they serve as key regulators of fibroblast recruitment, proliferation and activation. They promote fibrosis by secreting chemokines and specific matrix metalloproteinases that degrade extracellular matrix (ECM) components, thus facilitating the recruitment of inflammatory cells to sites of tissue injury (Wynn, 2011).

The imbalance of Th1/Th2 lymphocytes has been found in patients with pulmonary fibrosis, and increasing evidence reveals the correlation between Th1/Th2 lymphocyte imbalance and pulmonary fibrosis. In the airways of silica-exposed mice, over expression of Th2 cytokines (IL-4, IL-5, IL-6) and signal molecules (Stat3 and Socs3) were observed while Th1 (IL-1β and TNF-α) cytokines are under expressed (Tripathi et al., 2010). This change in responsiveness to fibrosis could be reversed by the transcription factor Nrf2. Transforming growth factor-β1 (TGF-β1) is secreted by macrophages, epithelial cells and fibroblasts, and possessed the profibrotic effect. Tranilast has been used in allergic diseases, because of its inhibitory effect on mast cells; but it also has an anti-fibrotic effect. Tranilast can inhibit
the secretion of IL-13 by Th2 cells and then, regulates the Th1/Th2 lymphocyte balance (Tao et al., 2011). Tranilast also inhibits the activation of macrophages, decreases the activity of fibroblasts and then inhibits the expression of TGF-β1 (Platten et al., 2011). The present study aimed to evaluate the effects of tranilast on bleomycin (BLM) induced pulmonary fibrosis.

MATERIALS AND METHODS

Animals

Female (female rats were more responsive to BLM than males) Sprague Dawley (SD) rats weighing 200 ± 10 g (specific pathogen free; n = 60) were purchased from the Animal Center of Chongqing Medical University, the study was approved by the ethical committee in research. In the study, we choose the female rats, because the weight and size in female rats were smaller than male rats of the same age, and female rats were easy to operate in anesthesia and intrastrastic administration. In addition, adult female rats were more sensitive to the bleomycin than males. Pulmonary fibrosis, morbidity and mortality were higher in female rats and the difference in the results could be more obvious.

Drugs

Tranilast was purchased from the Out-Patient Pharmacy of affiliated the First Hospital of Chongqing Medical University and diluted in distilled water for use. Bleomycin hydrochloride injection (Nippon Kayaku Co. Ltd).

Primers (4)

The primers used in the present study were synthesized in Shanghai Sangon.

Animal processing and sampling

These animals were randomly assigned into following groups (n = 10 per group): control group; pulmonary fibrosis group (BLM); high dose tranilast (TR) group; medium dose tranilast group; low dose tranilast group; prednizone acetate group (GC group). All rats were given ad libitum access to water and food. Animals in the control group did not received treatment and those in the remaining groups were intratracheally treated with bleomycin to induce pulmonary fibrosis. In brief, rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g body weight) and tracheotomy was performed. Bleomycin was intratracheally instilled (Luo et al., 2010). One day later (24 h), rats in low, medium and high dose tranilast groups were intragastrically given at 50, 100 and 200 mg/kg per day, respectively, and those in prednizone group were intragastrically treated with prednizone at 10 mg/kg per day. Rats in the control group and pulmonary fibrosis group were given ad libitum access to water and food. Four weeks after establishment of pulmonary fibrosis model, rats were anesthetized and blood was collected from the carotid vein followed by isolation of serum. The serum levels of IL-4 and IFN-γ were measured using ELISA kit according to the manufacturer’s instructions followed by calculation of IL-4/INF-γ ratio. The hilum of right lung was ligated and the lungs were homogenated was mixed; (2) the tubes were centrifuged for 3 min at room temperature; (3) the supernatant (about 0.6 ml) was transferred into another tubes; (4) the tubes that was added 0.6 ml isopropanol was oscillated for 30 s and centrifuged for 25 min; (5) the supernatant was discarded; (6) 1ml 75% alcohol was added into the tubes; (7) the tubes were centrifuged for 1 min at 13000 to 15000, and the supernatant was discarded; (8) 50 ul diethyl pyrocarbonate treated water was added into tubes. RNA was stored at -80°C.

Detection of mRNA expression of TGF-β1 in the lung

About 50 to 100 mg of lung tissues were homogenated and total RNA was extracted:(1) 0.2 ml chloroform was added into homogenated lung tissues, the centrifuge tubes were oscillated and the homogenated was mixed; (2) the tubes were centrifuged for 3 min at room temperature; (3) the supernatant (about 0.6 ml) was transferred into another tubes; (4) the tubes that was added 0.6 ml isopropanol was oscillated for 30 s and centrifuged for 25 min; (5) the supernatant was discarded; (6) 1ml 75% alcohol was added into the tubes; (7) the tubes were centrifuged for 1 min at 13000 to 15000, and the supernatant was discarded; (8) 50 ul diethyl pyrocarbonate treated water was added into tubes. RNA was stored at -80°C.

The mRNAs of TGF-β1 and β-actin were amplified using corresponding primers and the products stored at 4°C. These products were then subjected to 2% agarose gel electrophoresis for the analysis of products. The gel was collected and the bands were observed under an ultraviolet lamp and photographed. The optical density of these bands were scanned into a computer and analyzed using a gene analysis system. The optical density of target gene was normalized by that of β-actin as the relative expression of target gene.

Immunohistochemistry

Surfactant protein (SP) methods were employed to detect the TGF-β1 expression by immunohistochemistry. In brief, the paraffin-embedded sections were heated at 68°C for 60 min, and then deparaffinized in xylene and dehydrated in alcohol. These sections were treated with 3% H2O2 at 37°C for 10 min to inactivate the endogenous peroxidase followed by washing in phosphate buffered saline (PBS) thrice (5 min for each) and then antigen retrieval. These sections were blocked in normal goat serum at 37°C for 20 min, and then treated with primary antibody at 4°C overnight (in the negative control, primary antibody was replaced with PBS). After washing in PBS thrice (5 min for each), sections were incubated with biotinylated secondary antibody at 37°C for 30 min. After washing in PBS thrice (5 min for each), these sections were treated with horseradish peroxidase conjugated streptomycin avidin solution at 37°C for 30 min followed by PBS washing thrice (5 min for each). Visualization was performed with diaminobenzidine (DAB) followed by washing with water. After counterstaining, dehydration and transparentization, sections were dried and mounted.

Statistical analysis

All data were expressed as means ± standard error (SE) and analyzed by SPSS13.0. Differences between mean values from the various treatments and control groups were assessed for statistical significance by analysis of variance and if significant were followed by Tukey’s test. A P value < 0.05 was considered statistically significant.

RESULTS

Serum levels of IL-4 and IFN-γ

The IL-4 level in medium and high dose tranilast groups was lower than that in the bleomycin group and glucocorticoid group, but no significant difference was found between low dose tranilast group and the later two
mRNA expression of TGF-β1 from RT-PCR

When compared with bleomycin group, the mRNA expression of TGF-β1 in the control group and three tranilast groups was significantly decreased (P < 0.05). When compared with glucocorticoid group, the mRNA expression of TGF-β1 in the medium and high dose tranilast groups was markedly reduced (P < 0.05). In addition, the mRNA expression of TGF-β1 in the medium dose tranilast group was lower than that in the low dose tranilast group (P < 0.05) (Figure 1).

TGF-β1 protein expression from immunohistochemistry

The relative optical density was expressed as the protein expression of TGF-β1. The TGF-β1 expression is shown in the Table 1.

The optical density in three tranilast groups and the bleomycin group were significantly higher than that in the control group (P < 0.01). No marked difference was found in the optical density between the bleomycin and the glucocorticoid group (P = 0.411). However, the optical density in the high and medium dose tranilast groups was lower than that in the bleomycin group and glucocorticoid group. There was no marked difference between low dose tranilast group and glucocorticoid group (P = 0.184). Significant difference in the optical density was found between low dose tranilast group and bleomycin group (P < 0.01). The optical density in the medium and high dose tranilast groups was different from that in the earlier two groups (P < 0.01). The optical density in the low dose tranilast group was dramatically higher than that in the medium and high dose tranilast group (P < 0.05), and that in medium dose tranilast group was higher than that in the high dose tranilast group (P < 0.01).
Table 1. The mean optical density of TGF-β1 expression in different groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean optical density</th>
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<tbody>
<tr>
<td>Glucocorticoid group</td>
<td>0.51±0.06 #</td>
</tr>
<tr>
<td>Low dose tranilast group</td>
<td>0.41±0.06 *#</td>
</tr>
<tr>
<td>Medium dose tranilast group</td>
<td>0.30±0.05 *#</td>
</tr>
<tr>
<td>High dose tranilast group</td>
<td>0.18±0.03 *#</td>
</tr>
<tr>
<td>Bleomycin group</td>
<td>0.60±0.06 #</td>
</tr>
<tr>
<td>Control group</td>
<td>0.10±0.03 *#</td>
</tr>
</tbody>
</table>

*P <0.01 versus bleomycin group; *P <0.01 versus high dose tranilast group; *P<0.05 versus medium dose tranilast group; *P<0.05 versus low dose tranilast group; *P <0.01 versus glucocorticoid group; *P<0.01 versus control group.

Pathological examination of the lung

**Gross features**

**BLM group:** The lung volume was markedly decreased and had irregular size. Lungs in BLM group became hard and pale, and pleural fibrosis was evident. Bullae were found in the lung and yellow-white plaques were noted on the surface of lungs.

**GC group:** The lung volume was reduced but larger than that in the BLM group. The lungs were relatively soft as compared to BLM group and become red. Pleural fibrosis and bullae were not found. Yellow-white plaques were also absent.

**TR groups:** The lung volume was reduced but larger than that in the aforementioned two groups. The lungs were relatively soft and red. The lungs in the low dose TR group were similar to those in GC group in the morphology. When compared with low dose TR group, the reduction of the lungs in the medium and high dose TR groups was small and the lungs were soft. The lungs in the high dose TR group were similar to those in the control group.

**Control group:** The lungs were red, smooth and soft. Hemorrhage and ecchymosis were not found.

**Microscopic findings**

**BLM group:** The structure of a lot of alveoli collapsed and was damaged. The alveolar septum was thickened. The amount of collagens in the alveolar spaces was less than that in the BLM group. The pathological features were similar to low dose TR group.

**TR groups:** Few inflammatory cells were found in the alveolar spaces and the structure of a minority of alveoli collapsed. Small amount of collagens were found in the alveolar septum. The amount of collapsed alveoli and collagens in the medium and high dose TR groups were smaller than that in the low dose TR group. The pathological features in the high dose TR group were similar to control group.

**Control group:** No features of alveolitis or fibrosis were found.

**Scoring for alveolitis and pulmonary fibrosis**

The alveolitis was scored according to the criteria developed (Szapiel et al., 1979). The alveolitis was scored into 4 grades: Grade 0: absence of alveolitis (-), score 0; Grade 1: mild alveolitis (+), widened alveolar septum due to cell infiltration and the lesioned tissues accounted for < 20% of total lung, score 1; Grade 2: moderate alveolitis (++), and the lesioned tissues 20 to 50% of total lung, score 2; Grade 3: severe alveolitis (+++), diffuse distribution and lesioned tissues > 50% of total lung, score 3. Pulmonary fibrosis was scored into 4 grades: Grade 0: absence of fibrosis (-), score 0; Grade 1: mild fibrosis (+), and the affected tissues accounted for < 20% of total lung, score 1; Grade 2: moderate fibrosis (++), the affected tissues 20 to 50% of total lung, and the structure of alveoli was irregular, score 2; Grade 3: severe fibrosis (+++), affected tissues > 50% of total lung, alveolar fusion and irregular structure of lung parenchyma, score 3. Significant difference was found between TR groups (medium and high dose) and GC group as well as BLM group in the scores of alveolitis and pulmonary fibrosis (P < 0.05) (Table 2).

DISCUSSION

TR is also known as N-[3,4-dimethoxycinnamonyl]-anthranilic acid and was developed by Kissei Pharmaceuticals. It was used as an antiallergic drug and can inhibit the degranulation of mast cells and basophils, which then suppresses the release of mediators involving in the anaphylaxis including histamine and 5-HT. Studies have demonstrated that TR is effective in the treatment of rat cutaneous anaphylaxis and experimental asthma (Kim et al., 2009). Clinically, TR is applied in the treatment and
prevention of bronchial asthma and allergic rhinitis.

Pulmonary fibrosis involves several pathological processes, such as damage to epithelial cells, inflammation and fibrosis. TGF-β1 has been found to be closely related to the pulmonary fibrosis. TGF-β1 can promote the differentiation and proliferation of fibroblasts, stimulate the synthesis of type I, II and IV collagens, increase the synthesis of protease inhibitors and facilitates the production of extracellular matrix. In the study of Tarantal et al. (2010), exogenous and transient TGF-β1 over-expression in fetal monkey lung was achieved by transabdominal ultrasound-guided fetal intrapulmonary injection of adenoviral vector expressing TGF-β1 at the second or third trimester of pregnancy. Their results showed severe pulmonary and pleural fibrosis, increased proliferation of myofibroblasts in the fibrotic foci and massive deposition of collagen fibers on the inner and outer sides of the pleural membrane. These findings suggest that TGF-β1 over-expression may cause pulmonary and pleural fibrosis. In addition, TGF-β inhibitor has been shown effective in the treatment of BLM induced pulmonary fibrosis in mice (Wang et al., 2010), in which the mRNA expressions of IFN-γ, type I collagen and fibronectin were markedly decreased in the lungs (Arribillaga et al., 2011). In a lot of animal studies, the TGF-β1 level has been regarded as an indicator to determine the therapeutic efficacy.

In the present study, when compared with BLM group, the protein and mRNA expressions of TGF-β1 were dramatically reduced following TR treatment, which implies TR may decrease the transcription and expression of TGF-β1 in the lung TGF-β1. TGF-β1 can stimulate the mesenchymal cells to produce collagens and fibronectin, and promote the transformation of mesenchymal cells into epithelial cells resulting in damage to tissues (Sureshbabu et al., 2011). In addition, MASSON staining also revealed that TR treatment significantly reduce the collagen content as compared to BLM group. Therefore, TR may suppress the generation of fibrocytes and reduce the accumulation of collagens and fibronectin through inhibiting TGF-β1 production. Moreover, when compared with GC group, the down-regulation of TGF-β1 by TR was more obvious.

Some researchers speculate that the imbalance of Th1/Th2 lymphocytes is closely related to the pulmonary fibrosis and inflammation. In the Th2 response, the number of eosinophils and mast cells significantly increases, resulting in increased production of Th2 cytokines, including IL-4, IL-12 and IL-13. In rats, the response to injury is characterized by Th2 response following which these rats are susceptible to pulmonary fibrosis (Kunkel et al., 1996). Adults with activation of IL-4 gene are susceptible to pulmonary fibrosis (Vasakova et al., 2006).

In the present study, the IL-4 level in the GC group, TR groups and BLM group was increased and increased accompanied by decrease of IL-4/IFN-γ, which suggests that rats with pulmonary fibrosis were characterized by Th2 response in immune function. When compared with BLM group, the IL-4 level reduced, IFN-γ level increased and IL-4/IFN-γ ratio increased in the TR groups, which indicates TR can exert anti-fibrotic effect via regulating Th1/Th2 balance (Kikuchi et al., 2010). Moreover, the ability of TR to regulate the Th1/Th2 balance is superior to that of GC. The higher the TR dose, the lower the TGF-β1 level, and the collagen accumulation is also reduced. Thus, the regulation of Th1/Th2 lymphocyte balance by TR is more evident. These findings suggest the anti-fibrotic effect of TR depends on the dose of TR, which is seldom reported in previous studies. These in vivo findings suggest a mechanism as to how tranilast may represent a protective factor for development of lung fibrosis. Further studies may concern morbidity, mortality, survival rates and respiratory function analysis in pulmonary fibrosis rats.

In the rat pulmonary fibrosis model, the female sex hormone is a likely factor to enhance the severity of disease and decreased survival rates and maybe we could expect that male rats may have a higher survival rate. Female lung tissue showed greater degrees of lung inflammation and fibrosis. Fibroblasts from BLM-treated rats exhibited an altered phenotype by increased responsiveness to estradiol treatment, causing increase in procollagen1, IL-4 and TGF-β1 mRNA expression (Ghararee-Kermani et al., 2005). Furthermore, the male rats performed more offensive and these were the reasons for choosing the female rats. Although, tranilast

<table>
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<th>Group</th>
<th>Alveolitis</th>
<th>Pulmonary fibrosis</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(-) (+) (++) (+++)</td>
<td>(-) (+) (++) (+++)</td>
</tr>
<tr>
<td>GC group</td>
<td>0 8 2 0</td>
<td>1 5 4 0</td>
</tr>
<tr>
<td>Low dose TR group</td>
<td>3 7 0 0</td>
<td>4 6 0 0</td>
</tr>
<tr>
<td>Medium dose TR group</td>
<td>6 4 0 0</td>
<td>7 3 0 0</td>
</tr>
<tr>
<td>High dose TR group</td>
<td>9 1 0 0</td>
<td>9 1 0 0</td>
</tr>
<tr>
<td>BLM group</td>
<td>0 6 4 0</td>
<td>0 0 4 6</td>
</tr>
<tr>
<td>Control</td>
<td>10 0 0 0</td>
<td>10 0 0 0</td>
</tr>
</tbody>
</table>

Table 2: Scores of alveolitis and pulmonary fibrosis in different groups.
cannot currently be used for the treatment of pulmonary fibrosis, we believe that the present results may lead to new therapeutic options.

REFERENCES


