Original Article

Clearing heat and promoting diuresis method on TGF-β1/Smad signal pathway in airway remodeling of asthmatic rats

Peng Zhang¹*, Bo Nie²*, Yan Li³, Junhong Wang³, Huiping Yu³, Mian Sang⁴

Departments of ¹Orthopedics, ³Paediatrics, Dongzhimen Hospital of Beijing University of Chinese Medicine, Beijing 100700, China; ²Key Laboratory of Chinese Internal Medicine of Ministry of Education, Dongzhimen Hospital of Beijing University of Chinese Medicine, Beijing 100700, China. *Equal contributors.

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Abstract: Objective: The present study was designed to observe the effects of clearing heat and promoting diuresis method on transforming growth factor-β1 (TGF-β1)/Smad signal pathway in airway remodeling of asthmatic rats. Methods: 40 male SD rats were randomly classified into group A (normal group), group B (model group), group C (Chinese medicine group) and group D (Western medicine group), with 10 rats in each group. Ovalbumin (OVA) was used to perform sensitization and to stimulate the establishment of rat models with asthmatic airway remodeling. There was no modeling in group A. Enzyme-linked immunosorbent assay (ELISA) was applied to test the concentration of TGF-β1 in serum. Collagen-I, Collagen-III, Smad2, Smad3 and Smad7 protein expressions in lung tissues were tested by immunohistochemistry, while TGF-β1, Smad2, Smad3 and Smad7 mRNA expressions were examined by fluorogenic quantitative PCR. Results: Compared with group B, TGF-β1, Smad2 and Smad3 protein and mRNA expressions in lung tissues of group C and D decreased while Smad7 protein and mRNA expressions increased (P<0.01). Conclusions: The clearing heat and promoting diuresis method could improve airway remodeling of asthmatic rats and its mechanism was likely to have correlation with the regulation of TGF-β1/Smad signal pathway.

Keywords: Clearing heat and promoting diuresis method/traditional Chinese medical therapy, asthma, airway remodeling, signal pathway

Introduction

Bronchial asthma (asthma for short) is a kind of airway allergic inflammation disease involving eosinophil (EOS), macrophage, lymphocyte, mastocyte, epithelial cell, smooth muscle cell and various cytokines, inflammatory mediator. Airway remodeling, which is one of the three major pathological features of asthma (airway allergic inflammation AAI, airway hyper-reactivity AHR, airway remodeling), belongs to irreversible pathological change of airway concerning multiple cytokines. The forming process is complex, which is exactly the major reason of asthma being hard to cure. The previous studies indicate that asthma occurs after it develops to a certain stage, but it is able to exist being independent of AHR and AAI in the early stage of disease [1, 2]. Therefore, the currently simple control of inflammation, remission of symptoms and immunotherapy still cannot prevent the occurrence and development of airway remodeling, which cannot treat asthma fundamentally. Early and precise diagnosis, treatment of asthma and prevention of the occurrence and development of airway remodeling are the important entry points of reversing the difficult rule. At present, treatments of asthma primarily include anti-inflammatory therapy, symptomatic treatment and immunotherapy [3]. Nevertheless, reliable detection indexes and effective therapeutic drugs for the developing step of airway remodeling are lacking. The present experiment aimed at investigating the influences of clearing heat and promoting method of diuresis on airway remodeling of asthmatic rats...
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and TGF-β1/Smad signal pathway with the aid of asthmatic airway remodeling model.

Materials and methods

Materials

Cleaning-grade male SD rats aged 4 weeks and weighed 48-50 g were supplied by Beijing Weitonglihua Laboratory Animal Technology Co., LTD. The cleaning heat and promoting diuresis recipes were Chinese medicine artemisia capillaris thunb relative prescription recipes (containing 9 g artemisia capillaris thunb, 9 g gardenia jasminoides, 9 g radix sophorae flavescentis, 9 g large-leaved gentian, 9 g acorus gramineus soland, 9 g curcuma aromatics, 9 g herba pyrrosiae, 9 g plantain, 9 g fritillaria thunbergii, 6 g trichosanthes kiriowii maxim and 3 g liquorice root), which were provided by Beijing kagrentang pharmaceutical co., LTD. The recipes were confected into solution with the concentration of 0.5 g/ml; OVA and dexamethasone (American Sigma Company); rabbit anti-Collagen-I antibody, rabbit anti-Collagen-III antibody, rabbit anti-Smad2 antibody, rabbit anti-Smad3 antibody, rabbit anti-Smad7 antibody (Becton, Dickinson and Company); immunohistochemical staining kit, DAB kit (Invitrogen, USA); hyperpure RNA extraction kit, HiFi-MMLV cDNA first synthetic kit, SYBR Green PCR Mixture, Dnase 1 (kangwei century biology).

Methods

Animal grouping and the establishment of asthmatic models: The OVA was applied to conduct sensitization and the method of activation was chronically taken in for improvement. Asthmatic rat models were prepared and were divided into sensitization stage and activation stage. Sensitization stage: 1 ml 1% freshly prepared OVA physiological saline solution was injected into abdominal cavity and 200 mg 10% aluminium hydroxide gel was injected into right leg muscle on the first day; sensitization was performed on the eighth day and the above processes were repeated then. Activation stage: the rats were placed into transparent sealed containers from the fifteenth day and were activated for 30 min by aerosol atomized from 1% freshly prepared OVA by ultrasonic atomization device. The atomization flow was 3 ml·min⁻¹ and went once every other day. Afterwards, 1% OVA aerosol concentration was added once every 8 days and atomization was conducted once every other day. The atomization would be terminated until the thirty-ninth day. SD rats in blank control group were fed normally without any drug. The performances of successful activation were as follows: rats appeared to breathe faster or with mouth open and to be dysphoric with cyanosis of mouth, lips, claws and nails and twitching of abdominal muscle, etc.

40 male SD rats were randomly classified into four groups-normal group (group A), model group (group B), Chinese medicine group (group C) and Western medicine (dexamethasone) group (group D), with 10 rats in each group. Except normal group, other groups were prepared asthmatic models.

Administration methods: Groups except normal and model groups were given corresponding liquid medicine for intragastric administration from the seventeenth day, which was regulated once every week according to rats' weight and lasted for 4 weeks. All rats were allowed to drink freely without being used other anti-asthmatic drugs during the experiment. The dosage of administration of each group was based on clinical adult drug dosage and conversion coefficient converting into dosage of rats. Western medicine group was given 1 ml/kg dexamethasone and Chinese medicine group was given 5 ml/kg granular solution of artemisia capillaris thunb relative prescription recipe one time a day. The intragastric administration was respectively performed with equal amounts of normal saline in blank control group and model group. 10% chloral hydrate (400 mg/kg) of all rats was injected anesthesia in the enterocoelia at the end of the fourth week. Collecting blood of aorta abdominals and serum was collected by conventional centrifuge. Blood samples were respectively loaded into EP tubes and stored at -80°C for back-up. Right upper lobe lung tissue was placed in 4% paraformaldehyde for immobilization about 12 h. Gradient alcohol was used to accomplish the dehydration and the conventional paraffin was used to embed section.

Immunohistochemistry: The immunohistochemistry was adopted to examine the distributions and expressions of Collagen-I, Collagen-
Table 1. Comparison of serum TGF-β1 content in rats (X±s)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>TGF-β1 content (ng/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal group (A)</td>
<td>10</td>
<td>103.13±11.68*</td>
</tr>
<tr>
<td>Model group (B)</td>
<td>10</td>
<td>208.07±18.42</td>
</tr>
<tr>
<td>Chinese medicine group (C)</td>
<td>10</td>
<td>127.75±18.53*</td>
</tr>
<tr>
<td>Western medicine group (D)</td>
<td>10</td>
<td>131.17±13.46*</td>
</tr>
</tbody>
</table>

*: P<0.01 compared with group B; #: P<0.01 compared with group A.

III, Smad2, Smad3 and Smad7 in lung tissues. They were taken out to recover to room temperature and were douched by PBS with 3 min x 3 times. 3% H₂O₂ deionized water (colorless liquid) was applied to carry out the incubation for 10 min in order to remove the endogenous peroxidase activity, and using PBS to douch it as the method above. We could drop reagent A and incubate for 20 min at room temperature, then we dropped reagent B, it was incubated for 10-15 min at room temperature or 37°C. PBS was used to douch the wet box at 4°C for overnight. The dilute primary antibody was taken out to recover to room temperature, using PBS to douch it like the method above. Dropping reagent C, it was incubated at room temperature or 37°C for 15 min then it was douched by PBS like the method above. DAB chromogenic agent appeared coloration. Tap water was used to douching completely for about 5 min. The hematoxylin was adopted to redye it and turned out to be dehydrating and transparent. The neutral resin was applied to seal sheet.

Serum levels of TGF-β1 in rats tested by ELISA (in accordance with kit instructions): 100 μl corresponding serum was added into every hole in coated well in groups. The coated well was placed at the condition of 37°C to react for 90 min, then throwing off the liquid in coated well and sopping up with the absorbent paper with douching. TGF-β1 antibody working solution of antibiotic rats was added into coated well with 100 μl in each hole for reaction at 37°C for 60 min. Starting to wash for three times with 0.01 mol/l PBS every time for 1 min. 100 μl ABC working solution was added into each hole to react at 37°C for 30 min, then 0.01 mol/l PBS was used to wash for 1 min x 5 times. Meanwhile, TMB substrate was placed at 37°C for balance. Adding 100 μl TMB substrate into each hole of coated well to react in dark at 37°C for 20-25 min, then adding 100 μl stop buffer and blue turned to yellow immediately. OD value was determined at 450 nm of the coated well and the corresponding sample concentration was calculated on the basis of standard curve.

Extraction of mRNA and TGF-β1, Smad2, Smad3 and Smad7 mRNA expressions in lung tissues tested by qRT-PCR: Hyperpure RNA extraction kit was adopted to extract the total RNA in lung tissue samples based on product specification. HiFi-MMLV cDNA first synthetic kit (CWbio.Co.Ltd, Cat#CW0744) was employed to conduct reverse transcription according to product specification. RT-PCR reaction was performed with 7500 fluorescent quantitative PCR device and SYBR PCR Mixture was used to perform amplification in accordance with product specification. The reaction system was totally 20 μl and the amplification conditions were as follows: 95°C predegeneration for 10 min and 45 cycles of 95°C degeneration for 10 s, 59°C annealing for 60 s, and 72°C extension for 15 s. β-actin amplification acted as reference. qRT-PCR was employed to calculate the relative expression levels of mRNA in every group and the results of PCR expression was performed with relative quantitative analysis of data with the method of 2⁻ΔΔct.

Statistical methods: SPSS 16.0 was employed to accomplish the statistical analysis and the data were expressed by X±s. Furthermore, mean comparisons among groups adopted single factor variance to be analyzed, which P<0.05 was considered to have statistically significant differences. Semi quantitative data were conducted imaging analysis through HPIAS-2000 high-definition color image-text pathological analysis system. Moreover, statistical treatment was carried out with SPSS 16.0, which P<0.05 was regarded as the difference with statistical significance.

Results

Determination of serum TGF-β1 content

Serum TGF-β1 content in group B was evidently higher than that in group A, illustrating obvious
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difference (P<0.01). Serum TGF-β1 content in group C and D was apparently lower than that in group B after treatment, which demonstrated obvious differences compared with group B (P<0.01). Table 1 displayed that serum TGF-β1 expressions of group C and D were higher than that of blank control group and were obviously different from control group (P<0.05).

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum TGF-β1</th>
</tr>
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<tbody>
<tr>
<td>Blank control</td>
<td>Lower</td>
</tr>
<tr>
<td>A: Normal</td>
<td>Higher</td>
</tr>
<tr>
<td>B: Model</td>
<td>Higher</td>
</tr>
<tr>
<td>C: Chinese</td>
<td>Higher</td>
</tr>
<tr>
<td>D: Western</td>
<td>Higher</td>
</tr>
</tbody>
</table>

Collagen-I, Collagen-III, Smad2, Smad3 and Smad7 protein expressions in airway

Immunohistochemical staining revealed that brown yellow immune positive reaction occurred in airway sections of rats in all groups and the positive mainly expressed in epithelial cell, submucosa and smooth muscle layer of the airway.

The gray values demonstrated that airway Smad2 (Figure 1) and Smad3 (Figure 2) protein expressions of rats in group B increased and were evidently different from group A (P<0.05). However, airway Smad2 and Smad3 protein expressions of rats slightly increased after the intervention of Chinese medicines. In addition, airway Smad7 (Figure 3) protein expressions of
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rats in group B decreased, which had obvious differences compared with group A ($P<0.05$). After the intervention of Chinese medicines, the airway Smad7 protein expressions of rats still reduced slightly compared with group A. **Figure 3** informed that there was no difference between group C and D.

The airway Collagen-I (**Figure 4**), Collagen-III (**Figure 5**) protein expressions of rats in group B increased, stating apparent differences compared with group A ($P<0.05$). Yet the airway Collagen-I, Collagen-III protein expressions of rats slightly reduced after the intervention of Chinese medicines and group C had no difference with group D.

**TGF-β1, Smad2, Smad3 and Smad7 mRNA expressions in lung tissues of rats**

The experimental results suggested that TGF-β1, Smad2 and Smad3 mRNA expressions in lung tissues of rats in group B were higher than that in blank control group, while Smad7 protein expression evidently decreased. Additionally, TGF-β1, Smad2 and Smad3 mRNA...
expressions in lung tissues of rats in Chinese and Western medicine groups were lower than that in model group while Smad7 protein expression increased (Figure 6).

Discussion

Airway remodeling, one of the characteristic pathophysiological changes of asthma, is explicitly put forward in 1992. It can be defined that the cell and extracellular matrix forming airway wall change in composition, organizational structure and quantity up to now, of which the major features are as follows: holistic loss of epithelial cell, thickness of basement membrane of airway tissue, epithelial fibrosis, enlargement of goblet cell and submucosal...
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In recent years, plenty of studies have confirmed that TGF-β (the cytokine promoting fibrosis), which comes from various kinds of cells (such as macrophage, epithelial cell, fibroblast and eosinophil granulocyte), is the one of the main factors of structure remodeling in lung tissue of asthmatic patients [6]. TGF-β1, which has the functions of regulating the growth and differentiation of a variety of cells, can produce the most intense stimulative affects on extracellular matrix accumulation in all cytokines. Additionally, smooth muscle cell, fibroblast, alveolar macrophage, airway epithelial cell and eosinophil have expressions of related cytokines as well [7, 8]. After extensive researches, Holgate et al. propound the epithelial-mesenchymal trophic unit theory and think that the primary pathogenesis of refractory asthma is airway remodeling, which is caused by the secretion imbalances of TGF-β receptor and epidermal growth factor receptor on account of the normal repair mechanism damage of airway epithelial cell [9]. Chen G et al. have discovered that TGF-β1 can stimulate DNA synthesis of airway smooth muscle cell and is capable of promoting cell division and proliferation, resulting in permanent phenotypic changes of airway smooth muscle cell, which prompts that TGF-β1 involves in the airway remodeling of asthma directly or indirectly and the mutations of thickness of airway wall, airway stenosis and limitation of ventilation appear [10]. The recent studies also have proved that the occurrence of airway remodeling exists in childhood. Furthermore, it is reported that TGF-β1 expression of asthmatic children is apparently higher than that of normal children and the increase degree is related to the severity of asthma, suggesting its involvement with the onset of asthma [11-13]. TGF-β1 is able to stimulate the division and proliferation of airway smooth muscle cell and has a promoting effect on the accumulation of Collagen-I, Collagen-III in the airway wall, which brings about proliferation and migration of fibroblast and induces bronchial epithelial cell to compound Collagen-IV involving and accelerating the formation of airway remodeling [14-16]. The methods of controlling the production of TGF-β1 or other biological functions have significant influences on the prevention and cure of the airway remodeling and further development of asthma [17, 18].

Smad protein, which conducts molecule for downstream critical signal of TGF-β1 signal pathway, can directly transmit the signal from cell membrane to nucleus and mediate the conduction of TGF-β1 intracellular signal. Rosendahl et al. adopt egg albumin to make asthmatic mice models for comparing with normal mice, finding that Smad3 level obviously rises in lung tissue of the former and Smad2 expression can also rapidly enhance in bronchial epithelial cells, fibroblasts and vascular endothelial cells of induced dense mice [19]. Smad6 and 7, which the functions are contrary to regulatory proteins, belong to inhibitory proteins and play inhibitory effects in TGF-β1/Smad signal pathway. The combination of Smad7 and activated TGF-β1 receptor is applied to prevent Smad2 from combining with type I receptor and the phosphorylation of them in order to interdict signal transmission of TGF-β mediated, and the inhibitory effect of Smad7 is much more apparent than Smad6 [20]. The regulation of any step and mediation in TGF-β1/Smads signal pathway is able to effectively prevent the occurrence and development of airway remodeling. Consequently, it follows that the control of TGF-β1/Smads signal pathway is the key to the prevention and cure of asthma.

The present study, starting from TGF-β1/Smads signal pathway with the methods of immunohistochemistry and qRT-PCR, selected TGF-β1 and Smad2, Smad3, Smad7 proteins with opposite effects in the downstream of signal transduction as test indexes to illuminate molecular mechanism of artemisia capillaris thunb rela-
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tive prescription (clearing heat and promoting diuresis method representative prescription) to regulate TGF-β1/Smads signal pathway and to control bronchial asthma. The results turned out that serum TGF-β1 expression in model group was evidently higher than normal control group in the assaying of serum TGF-β1, revealing the statistically significant differences between them ($P<0.01$), which illustrated that TGF-β1 involved in the generating process of airway remodeling.

The immunohistochemical results displayed that airway Smad2, Smad3 protein expressions of rats in model group were up-regulated while Smad7 protein expression was down-regulated compared with blank control group, revealing the up-regulations of Smad2, Smad3 and down-regulation of Smad7 in the pathogenetic process of asthma. Thus it suggested that the downstream Smads proteins in TGF-β1/Smads signal pathway might play a certain effect on the airway remodeling of asthma. The airway Smad2, Smad3 expressions of rats were lower while Smad7 expression was higher in Chinese and Western medicine groups compared with model group, which indicated obvious differences ($P<0.05$) and stated that the intervention of clearing heat and promoting diuresis method could regulate the expressions of Smads proteins, control the mediation of TGF-β1/Smads signal pathway, relieve the occurrence of airway remodeling and further play a role in the treatment of asthma. Therefore, the control of artemisia capillaris thunb relative prescription (clearing heat and promoting diuresis method representative prescription) on the development of asthma through participating in the regulation of airway TGF-β1/Smads signal transduction pathway could be one of the action mechanisms of treating asthma by clearing heat and promoting diuresis method.

The results of TGF-β1, Smad2, Smad3 and Smad7 mRNA expressions in lung tissues of rats were as follows: lung tissue TGF-β1, Smad2 and Smad3 mRNA expressions of rats were higher and Smad7 protein expression was lower in model group than that of blank control group. In addition, lung tissue TGF-β1, Smad2 and Smad3 mRNA expressions were lower and Smad7 protein expression was higher in Chinese and Western medicine groups compared with model group. The above conclusions further clarified from gene level the molecular mechanism of artemisia capillaris thunb relative prescription (clearing heat and promoting diuresis method representative prescription) regulating TGF-β1/Smads signal pathway and controlling bronchial asthma.

From what was said above, we concluded that artemisia capillaris thunb relative prescription (clearing heat and promoting diuresis method representative prescription) could improve airway remodeling of asthmatic rats, and its mechanism seemed to be in correlation with the regulation of TGF-β1/Smad signal pathway, which could provide scientific evidences for the clinical application of traditional Chinese medicine pediatrics with the purpose of enriching the clinical medication theory.

Disclosure of conflict of interest

None.

Address correspondence to: Mian Sang, Department of Paediatrics, Dongzhimen Hospital of Beijing University of Chinese Medicine, No. 5 Haiyuncang Street, Dongcheng District, Beijing 100700, China. E-mail: xndbsgg43@yeah.net

References


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