

Basic Investigation

Functional mechanism of Pingchuanning Decoction on adjustment of clara cell secretory protein in airway remodeling of asthmatic rats

FANG Xiang-ming 方向明, LI Jun 李俊, LI Ze-geng 李泽庚, DONG Xiao-bo 董小波

FANG Xiang-ming, LI Jun, Anhui Medical University, Hefei 230032, China

FANG Xiang-ming, LI Ze-geng, DONG Xiao-bo, Anhui University of Traditional Chinese Medicine, Hefei 230038, China
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Correspondence to: LI Jun, Anhui Medical University, Hefei 230032, China; Fang Xiang-ming, Anhui Medical University, Hefei 230032, China. Anhui University of Traditional Chinese Medicine, Hefei 230038, China. fxm.bsh@163.com

Telephone: +86-551-5169049

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Abstract

OBJECTIVE: To study the functional mechanism of Pingchuanning Decoction in treatment of airway remodeling in asthmatic rats.

METHODS: Eighty healthy Wistar male rats were randomized into eight groups ($n=10$ rats each): Normal group, Asthma model group, Dexamethasone group, Guilong Kechuanning group, Xiaoqinglong Decoction group, and Pingchuanning Decoction low-, middle-, and high-dose groups. The rats of all but the Normal group were made into asthma models through intraperitoneal injection and aerosol inhalation of ovalbumin. All treatments were administered at the first stimulation of asthma onset (third week of modeling), and the rats were killed after stimulating asthma attacks for 4 weeks. The general conditions of rats and pathomorphological changes of the lung tissues were observed. The expression of nerve growth factor (NGF) of the lung tissues was measured with immunohistochemical

methods, and the content of Clara cell secretory protein (CCSP) mRNA was determined with RT-PCR.

RESULTS: Compared with the Normal group, the contents of NGF and CCSP mRNA in the lung tissues of the Model group were significantly changed ($P<0.01$). Compared with the Model group, the indices of Pingchuanning Decoction and other treatment groups were improved to some extent ($P<0.05$ or $P<0.01$).

CONCLUSIONS: Pathological changes of airway inflammation and remodeling were present in these rat asthma models. Pingchuanning Decoction had an intervention effect on these experimental models. Its functional mechanism may be related to multiple factors, including alleviation of airway inflammation, relief of bronchial smooth muscle spasm, and inhibition of airway remodeling.

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Key words: Pingchuanning decoction; Asthma; Nerve growth factor; Clara cell secretory protein; Functional mechanism

INTRODUCTION

Bronchial asthma (hereafter referred to as asthma) is a chronic airway inflammatory disease involving many kinds of cells and cellular components. Its basic pathological changes are airway inflammation, airway hyper-responsiveness, and airway remodeling. The concept of airway remodeling was first proposed by Huber and Koessler in 1922. The morphological manifestations of asthma are thickening of the airway walls, increasing of

the cross-sectional area of airway walls, and narrowing of the inner diameter of airways. Nerve growth factor (NGF) plays an important role in the inflammatory response under conditions of airway inflammation, and it participates the airway remodeling through its auto-secretion. Clara cells and Clara cell secretory protein (CCSP) may offer protection in the process of airway inflammatory injury, repair, and remodeling in asthma^[1]. This study was performed to evaluate the effect of Pingchuanning Decoction on asthma by observing NGF and CCSP levels in lung tissues of rats with asthma based on previous studies.

METHODS

Experimental animals

Eighty healthy male Wistar rats weighing 200 ± 20 g were selected for this experiment. The rats and forage were provided by the Experimental Animal Center of Anhui Medical University.

Medicines and reagents

Materials and equipment for this study were as follows: ovalbumin (OVA) (production batch number: A8040; Sigma, US), dexamethasone (production batch number: 100402; Shanghai Xinyi Pharmaceutical Co., Ltd.), Guilong Kechuanning capsules (production batch number: 100605; Shanxi Guilong Medicine Co., Ltd.), Pingchuanning Decoction (Anhui Weimin Pharmacy), Xiaoqinglong Decoction (Anhui Weimin Pharmacy), ultrasonic nebulizer (Jiangsu Yuyue Medical Equipment Co., Ltd.), and HH-2 digital constant temperature water bath cabinet (Jiangsu Jintan Ronghua Instrument Manufacturing Co., Ltd.).

The main reagents and instruments for the pathological study were an embedding machine (Changzhou Zhongwei Electron Instrument Co.), RM2126 paraffin microtome (Leica, Germany), and photomicroscope (Olympus, Japan).

The main reagents for the immunohistochemical study were an anti-NGF and DAB reagent box (Beijing Biosynthesis Biotechnology Co., Ltd.) and an SP immunohistochemistry kit (Beijing Biosynthesis Biotechnology Co., Ltd.).

The main reagents and instruments for the RT-PCR study were DEPC (Gibco, US), TRIZOL Reagent (Invitrogen, US), RT-PCR kit (Shanghai Biological Engineering Co., Ltd.), primer synthesis (Shanghai Biological Engineering Co., Ltd.), PCR marker (100, 250, 500, 750, 1000, and 2000 bP; Takara, Japan), low-temperature high-speed centrifuge (Shanghai Minqiao Precision Instrument Co., Ltd.), UV-visible spectrophotometer (Biometra, Germany), PCR instrument (Eppendorf, Germany), electrophoresis apparatus (Beijing Liuyi Instrument Factory), and gel density scanners and Labworks 3.0 analysis software (UVP, US).

Grouping

Eighty male Wistar rats were randomized into eight groups ($n=10$ rats each): A, Normal group; B, Asthma model group; C, Dexamethasone group; D, Guilong Kechuanning group; E, Xiaoqinglong Decoction group; F, Pingchuanning Decoction low-dose group; G, Pingchuanning Decoction middle-dose group; and H, Pingchuanning Decoction high-dose group. Animals were kept and fed in a room temperature (22 ± 2)°C environment for 7 days for adaptive feeding before modeling.

Modeling

After adaptive feeding for 7 days, the asthma modeling was begun^[2]. Groups B, C, D, E, F, G, and H received 1 mL of 10% OVA suspension by intraperitoneal injection on days 1 and 8 of modeling. Group A received 1 mL of normal saline instead of OVA suspension. Two weeks later, Groups B, C, D, E, F, G, and H were placed in sealed glass containers and nebulized with 1% OVA solution for 20 min once a day for 4 weeks. Successful asthma attack stimulation was manifested as an increased respiratory rate, lip cyanosis, abdominal cramps, breathing with nod, and unstable standing. Group A was nebulized with normal saline.

Preparation of medicines

Composition of Pingchuanning Decoction: Mahuang (Herba Ephedrae) 9 g, Xi Xin (Radix et Rhizoma Asari) 3 g, Su Zi (Fructus Perillae) 6 g, Xing Ren (Semen Armeniacae Amarum) 9 g, Chen Pi (Pericarpium Citri Reticulatae) 6 g, Ban Xia (Rhizoma Pinelliae) 9 g, Huang Qi (Radix Astragali) 9 g, Tai Zi Shen (Radix Pseudostellariae) 9 g, Fang Feng (Radix Saposhnikovia) 6 g, Di Long (Pheretima) 6 g, and Zhe Bei Mu (Bulbus Fritillariae Thunbergii) 9 g.

Preparation: The standard dose of crude medicine required based on each animal's body weight was converted from the standard dose for human body weight with the formula ($Db = Da \cdot Rab$)^[3], which equated to 9.80 g crude medicine per kilogram of rat body weight. The Pingchuanning Decoction high dose was 19.60 g/kg (Group F), middle-dose was 9.80 g/kg (Group G), and low dose was 4.90 g/kg (Group H). The middle dose was equal to the dose for clinical use in adults, twice the low dose, and half the high dose. The medicines were soaked in distilled water for 1 h, removed and placed into an earthenware casserole, and decocted three times with a slow fire for 30 min after boiling. The decoctions were mixed and concentrated to 0.98 g crude medicine/mL. A portion of this mixture was then concentrated to 1.96 g crude medicine/mL, and another portion was diluted to 0.49 g crude medicine/mL. The final product was stored in the refrigerator until ready for use.

Dexamethasone: The tablets were crushed and dissolved with normal saline to a concentration of 0.04

mg/mL. The final product was stored in the refrigerator until ready for use.

Guilong Kechuanning capsules: The powder was poured out and dissolved with normal saline to a concentration of 1 g/mL. The final product was stored in the refrigerator until ready for use.

Composition of Xiaoqinglong Decoction: Ma Huang (Herba Ephedrae), Xi Xin (Radix et Rhizoma Asari), Shao Yao (Radix Paeoniae), Fa Ban Xia (Rhizoma Pinelliae Praeparatum), Gui Zhi (Ramulus Cinnamomi), Wu Wei Zi (Fructus Schisandrae Chinensis), Gan Jiang (Rhizoma Zingiberis), and Zhi Gan Cao (Radix et Rhizoma Glycyrrhizae Praeparatum cum Melle).

Preparation: According to the original party proportion and decocting requirements of the original prescription, the medicines were decocted and the decoction was paired with distilled water to a concentration of 1 g crude medicine/mL.

The rats were administered treatments daily 30 min before stimulating asthma onset. Groups A and B were given equal amounts of distilled water per administration every day. Group C was administered dexamethasone 0.4 mg/kg daily. Group D was administered Guilong Kechuanning 10 g/kg daily. Group E was administered Xiaoqinglong Decoction 10 g/kg daily. Group F was administered Pingchuanning Decoction 4.9 g/kg daily. Group G was administered Pingchuanning Decoction 9.8 g/kg daily. Group H was administered Pingchuanning Decoction 19.6 g/kg daily. The doses for Groups F, G, and H were 0.5, 1, and 2 times the adult daily dose. The intragastric volume was identical to that at 25 mL/kg body weight. After the experiment was finished, the administrations were stopped and fasting was adopted for 1 day before obtaining the ex-

perimental materials.

Detection of NGF in lung tissues

The dewaxed paraffin sections were stained with immunohistochemical reagent box (SABC method). The concentration of anti-NGF was 1%. The negative control was phosphate-buffered saline. The procedure was performed following the instructions of the immunohistochemical detection kit. The gray value of the NGF expression area was measured by the MIAS-2000 Medical Image Analysis System. NGF was expressed as brown granular material and was present mainly in the cytoplasm of airway epithelial cells, smooth muscle cells, and lung interstitial cells. Five horizons were taken from every immunohistochemical staining section under high magnification. The positive-area gray values of each horizon were detected, and the average gray values (A) were calculated by the same observer.

Detection of total RNA in lung tissues

After euthanasia, the left lower lung was removed and stored in liquid nitrogen. The total RNA in 100 mg of lung tissues was extracted with a one-step method. The RNA content was measured by a UV spectrophotometer. All materials were soaked in DEPC water overnight, sterilized by high pressure, and dried. All operations were carried out on ice.

RT-PCR

Reagents and total RNA were added one by one according to the kit instructions. The reaction volume was 25 μ L. The primer design of expressed genes according to RT-PCR were as follows.

Gene name	Primers	PCR product length
GAPDH	forward: 5'-ACCACAGTCCATGCCATCAC-3'	452 bp
	reverse: 5'-TCCACCACCCTGTTGCTGTA-3'	
CCSP	forward: 5'-CATCAGCCCACATCTACAGAC-3'	373 bp
	reverse: 5'-GGGCTTTAGCGTAGAATATCT-3'	

Operation

1) Template RNA (1 μ L), oligo (dT) 18 (1 μ L), and nuclease-free water (10 μ L) were added to 0.2-mL EP tubes. They underwent centrifugal mixing for a few seconds, hatching at 70°C for 5 min, and rapid quenching on ice for 10 min. 2) Next, 5 $\times$ buffer (4 μ L), dNTP mixture (2 μ L), RNase inhibitor (40 U/ μ L) (1 μ L), reverse transcriptase (1 μ L), and nuclease-free water were added to a total volume of 25 μ L. 3) Centrifugal mixing was performed at 42°C for 60 min and 72°C for 10 min. Then, cDNA was obtained for PCR amplification. 4) Nuclease-free water (8.5 μ L), cDNA (2 μ L), forward primer (10 μ mol/l) (1 μ L), reverse prim-

er (10 μ mol/L) (1 μ L), and PCR Master Mix (12.5 μ L) were added. Reaction conditions were as follows: GAPDH 94°C, 5 min; 94°C, 45 s; 60°C, 60 s; 72°C, 60 s, 35 cycles; 72°C, 7 min; CCSP 94°C, 5 min; 94°C, 45 s; 60°C, 60 s; 72°C, 60 s, 35 cycles; 72°C, 10 min. 5) PCR products were stored at 4°C until ready for electrophoresis.

Detection of CCSP mRNA expression with Sq RT-PCR

The PCR products of CCSP and GAPDH were respectively added (5 μ L each) along with the loading buffer (1 μ L) into 1% agarose gel and electrophoresed for 40

min at 100 V. The average optical density of each band was analyzed with the GDS gel imaging system. The relative expression of CCSP was calculated with GAPDH as an internal reference.

Determination of RT-PCR products

After purification, the RT-PCR products were measured by an ABI PRISM 377 automatic sequencer and compared with the CCSP sequence published in the literature.

Statistical analyses

SPSS17.0 software was used for statistical analyses. Data were presented as mean ± standard error ($\bar{x} \pm s$). ANOVA for among-group comparison, SNK test for two-two comparison between groups, and correlation analysis for analysis of the correlation between factors were adopted. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Immunohistochemical changes of NGF in lung tissues

Table 1 Comparison of immunohistochemical result of NGF in lung tissues ($\bar{x} \pm s$)

Group	Rats(<i>n</i>)	Dose g·kg ⁻¹ ·d ⁻¹)	Average gray value (A)
A	10	—	81.326 ± 3.136
B	8	—	115.389 ± 2.839 [”]
C	9	0.4	95.648 ± 3.032 ^{▲▲}
D	9	10	96.562 ± 2.974 [▲]
E	8	7.8	96.895 ± 2.373 [▲]
F	8	4.9	99.276 ± 3.317 [▲]
G	9	9.8	96.020 ± 2.648 ^{▲▲}
H	9	19.6	96.630 ± 2.825 ^{▲▲}

Note: Compared with the normal group: ^{*}*P*<0.05, [”]*P*<0.01; compared with the model group: [▲]*P*<0.05, ^{▲▲}*P*<0.01. The data of NGF expression in the lung tissues of each group were analyzed by analysis of variance (*F*=123.1, *P*<0.01) and further by the SNK test: 1) Compared with the Normal group, the NGF expression in the lung tissues of the Model group was significantly different (*P*<0.01). 2) Compared with the Model group, the NGF expressions in the lung tissues of all treatment groups were different (*P*<0.05, *P*<0.01). 3) In comparison of the Pingchuanning Decoction middle- and high-dose groups with the Dexamethasone group, there was no significant difference (*P*>0.05). In comparison of the Guilong Kechuaning group with the

Xiaoqinglong Decoction group, there was no significant difference (*P*>0.05). In comparison of the Dexamethasone group with the Guilong Kechuaning group, there was a significant difference (*P*<0.05). In comparison among the Pingchuanning Decoction groups, the high- and middle-dose groups were better than the low-dose group (*P*<0.05) (Figures 1-8). There was a significant positive correlation between NGF expression in the lung tissues and bronchial wall thickness as shown in the correlation analysis (*r*=0.924492, *P*<0.05).

Changes in CCSP mRNA in lung tissues

Table 2 Comparison of the relative contents of CCSP mRNA in lung tissues of rats ($\bar{x} \pm s$)

Group	Rats(<i>n</i>)	Dose(·kg ⁻¹ ·d ⁻¹)	CCSP mRNA
A	10	—	0.67 ± 0.045
B	8	—	0.49 ± 0.038 [”]
C	9	0.4	0.61 ± 0.072 ^{▲▲}
D	9	10	0.57 ± 0.023 ^{▲▲}
E	8	7.8	0.56 ± 0.019 [▲]
F	8	4.9	0.55 ± 0.094 [▲]
G	9	9.8	0.60 ± 0.091 ^{▲▲}
H	9	19.6	0.59 ± 0.057 ^{▲▲}

Note: Compared with the normal group: ^{*}*P*< 0.05, [”]*P*< 0.01; compared with the model group: [▲]*P*< 0.05, ^{▲▲}*P*< 0.01. The data of CCSP mRNA expression in the lung tissues of each group were analyzed by analysis of variance *F*=7.497, *P*<0.01) and further by the SNK test: 1) Compared with the Normal group, the CCSP mRNA expression in the lung tissues of the Model group was significantly different (*P*<0.01). 2) Compared with the Model group, the CCSP mRNA expressions in the lung tissues of all treatment groups were different (*P*< 0.05, *P*<0.01). 3) In comparison of the Pingchuanning Decoction middle- and high-dose groups with the Dexamethasone group, there was no significant difference (*P*>0.05). In comparison of the Guilong Kechuaning group with the Xiaoqinglong Decoction group, there was no significant difference (*P*>0.05). In comparison of the Dexamethasone group with the Guilong Kechuaning group, there was a significant difference (*P*<0.05). In comparison, among the Pingchuanning Decoction groups, the high- and middle-dose groups were better than the low-dose group (*P*<0.05) (Figures 9, 10). There was a significant negative correlation between the CCSP mRNA expression in the lung tissues and bronchial wall thickness as shown in the correlation analysis (*r*=0.986541, *P*<0.05).

Immunohistochemical Staining of NGF

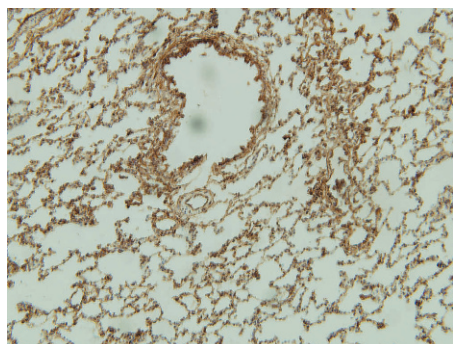


Figure 1 Normal group (×400).

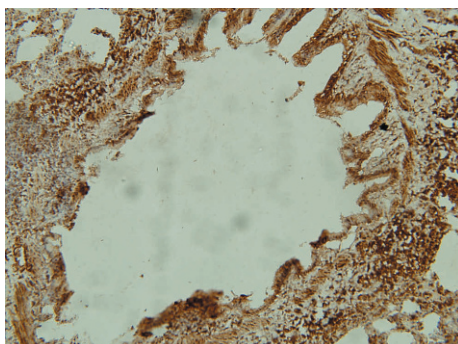


Figure 2 Model group (×400).

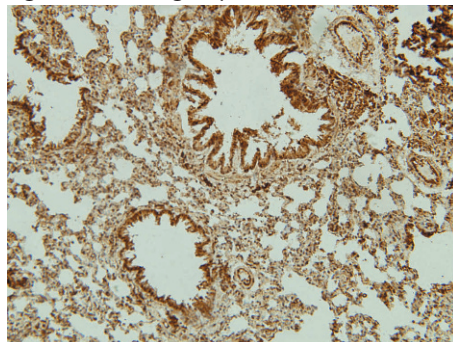


Figure 3 Dexamethasone group (×400).

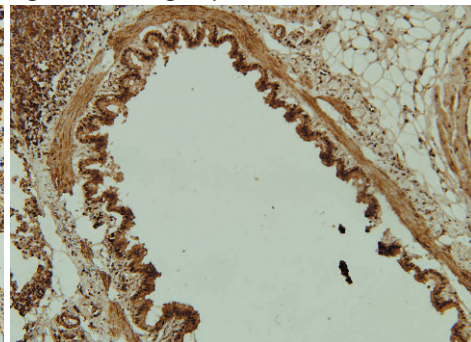


Figure 4 Guilong Kechuanning group (×400).

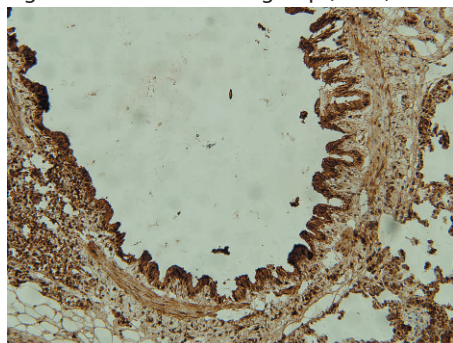


Figure 5 Xiaoqinglong Decoction group (×400).

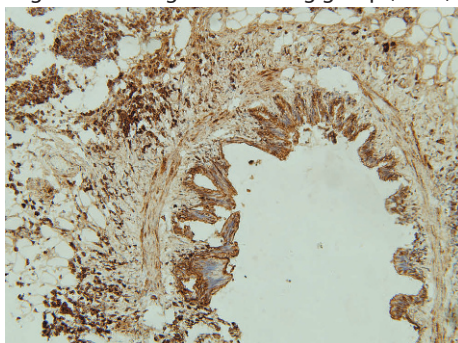


Figure 6 Pingchuanning low-dose group (×400).

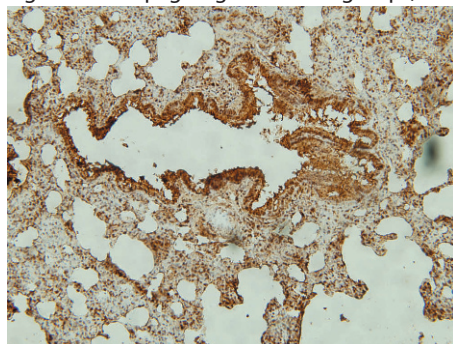


Figure 7 Pingchuanning middle-dose group (×400).

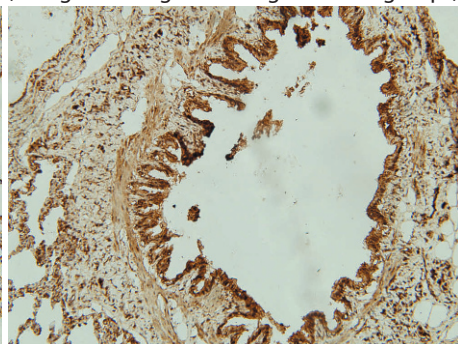


Figure 8 Pingchuanning high-dose group (×400).

Electrophoresis of the PCR Product of CCSP mRNA

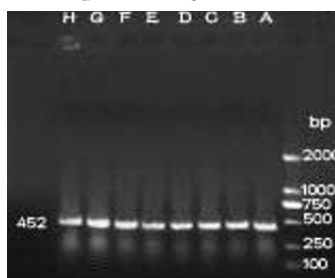


Figure 9 GAPDH electrophoresis. Figure 10 CCSP mRNA electrophoresis.

DISCUSSION

Bronchial asthma is a chronic airway inflammatory disease involving a variety of cells and cellular components. It is a polygenic hereditary disease affected by environmental factors. The main pathological changes are immune inflammatory infiltration, airway hyperresponsiveness, and airway remodeling. It belongs to the Xiao-asthma syndrome in TCM. According to TCM, the main pathogenesis of this disease is invasion of the body surface by the exogenous pathogen, and phlegm-fluid affects the lung, which fails to disperse and descend. The phlegm-fluid causes *Qi* stagnation, leading to an obstruction of *qi* of the lung. The repeated asthma attacks, followed by spleen and kidney deficiency and liver *Qi* stagnation, result in the failure of the lung to disperse and descend. In emergency cases, the acute symptoms are treated first; when these are relieved, the fundamental cause is treated. For acute attacks of asthma cold syndrome, the main treatment principle is to dispel wind cold and dissolve phlegm to relieve the asthmatic breathing with regulation of *Qi* and activation of blood. Reinforcement of *Qi* and strengthening of the spleen is the accessory treatment. Pingchuanning Decoction is modified from Shegan Mahuang Tang (Belamcanda and Ephedra Decoction) and Sanzi Yangqin Tang (Three-Seed Filial Devotion Decoction). It is an effective prescription for asthma cold syndrome. It comprises 11 medicines that function well in warming the lung, dissolving phlegm, stopping cough and asthma, expelling wind, relieving spasm, regulating *Qi*, and activating blood.

NGF is a member of the neurotrophic factor family. It is expressed in a variety of cells of different structures and inflammatory cells in the lung tissues, playing an important role in airway inflammation, hyperresponsiveness, and remodeling^[4]. After allergen inhalation, an immediate or delayed allergic reaction occurs, inducing acute attack of asthma and accumulation and activation of inflammatory cells in the airway. These cells further release inflammatory mediators, postponing the anti-inflammatory response. Therefore, airway remodeling is the result. NGF can stimulate sensory neurons to secrete calcitonin gene-related peptide or tachykinin, causing neurogenic inflammation by changing airway neurons and their afferent nerve fibrous functions and aggravating the airway inflammation that already exists^[5]. After antigen stimulation, NGF integrates with the surface receptors of mast cells, promotes mast cell release into inflammatory reaction medium, adds immediate hypersensitivity, and causes persistent high reactivity of the airway through the remodeling network of airway neurons^[6]. NGF induces smooth muscle remodeling and subepithelial fibrosis, excessive blood vessel formation, excessive nerve regeneration, and smooth muscle hyperplasia^[7], possibly through autocrine regulation of these cells in airway re-

modeling. NGF can increase the release of vascular endothelial growth factor to promote angiogenesis in airway smooth muscle proliferation and airway remodeling in asthma^[8,9]. Matrix metalloproteinase-2 (MMP-2) can promote the proliferation of myofibroblasts. Induction of MMP-2 expression by NGF may be one of the mechanisms of inflammation and airway remodeling in asthma^[10].

In the present study, the NGF expression in the lung of the Model group was significantly higher than that of the Normal group, and was positively correlated with the degree of airway remodeling (bronchial wall thickness). It can thus be predicted that NGF may become the key link in evaluation of airway remodeling severity, diagnosis, and treatment in asthma in the future. Among the treatment groups, the NGF expression in the Pingchuanning Decoction groups was significantly lower than that in the Model group, but still higher than that in the Normal group. This indicates that Pingchuanning Decoction can inhibit NGF expression and reduce airway inflammation and smooth muscle remodeling. The effect of a middle dose is as effective as that of dexamethasone.

A decrease of the level of CCSP has been found in bronchoalveolar lavage fluid (BALF)^[11] and serum^[12] of asthma patients. Immunohistochemical studies showed decreased numbers of Clara cells in the small airways. This decrease in Clara cells and CCSP in asthma patients would result in more epithelial injury and generation of inflammatory mediators in the airways, causing or aggravating the airway inflammation. Thus, pulmonary CCSP reduction or absence has close ties with bronchial asthma^[13].

After antigen inhalation, phospholipase A₂ and arachidonic acid contents are significantly increased in the airways patients with asthma. CCSP is a strong inhibitor of phospholipase A₂ in reducing the release of inflammatory mediators such as prostaglandin, thromboxane A₂, leukotrienes, and platelet activators, which are derived from arachidonic acid. In addition, CCSP can inhibit the production of interferon- γ , interleukin-1, interleukin-6, and tumor necrosis factor- α and the biological activity of IFN- γ , and decrease the chemotactic activity of macrophages and neutrophils.

In a pathological study involving Clara cell secretory protein gene-deficient rats (CCSP^{-/-}) and wild rats (CCSP^{+/+}) sensitized by OVA, Wang *et al.*^[14] observed that the number of neutrophils in BALF of CCSP^{+/+} rats increased significantly, myeloperoxidase activity and macrophage inflammatory protein- α levels increased, and lung inflammation was enhanced. This suggests that CCSP plays an important role in the inhibition of allergic inflammation in asthma.

Airway remodeling is a pathological change involving airway wall thickening, subepithelial fibrosis, smooth muscle hypertrophy, myofibroblast proliferation, salivary gland metaplasia, increased extracellular matrix de-

position, etc. It is a complex, progressive process. Fehrenbach *et al.*^[15] found that keratinocyte growth factor could promote the proliferation of bronchial epithelial cells, which are CCSP-positive cells only; consequently, Clara cells were found to be important in repairing bronchial epithelial injury.

Lesur *et al.*^[16] found that CCSP was significantly lower in the BALF and plasma of patients with asthma, the CCSP level in plasma depended on the genotype of CCSP, the changes in the CCSP gene on the 38th bit might be related to asthma, the plasma CCSP level could reflect the decreased secretion of CCSP caused by small airway remodeling, and the level of CCSP was unrelated to the steady state or onset of asthma but was related to the course of asthma.

Luo *et al.*^[17] found that Clara cells decreased in number and bronchial walls thickened in asthmatic rats. They conjectured that Clara cells secreted less CCSP in the process of repairing epithelial injury in airway inflammation. The reduction in Clara cells led to delayed repair of epithelial injury, and fibroblast migration to the subepithelial level played the role of alternative repair, thus promoting remodeling.

In the present study, the CCSP mRNA content, namely the CCSP expression, in the lung tissues of the Model group was significantly lower than that in the Normal group and was negatively correlated with the severity of airway remodeling (bronchial wall thickness). This is why CCSP may become an evaluation factor in determination of airway remodeling severity and treatment of airway remodeling in the future. Among the treatment groups, the CCSP expression in the Pingchuanning Decoction groups was significantly higher than that in the Model group but still lower than that in the Normal group. This indicates that Pingchuanning Decoction can promote CCSP expression, reduce airway inflammation, and delay the repair of epithelial injury and that a middle dose is as effective as dexamethasone.

The results of this study show that Pingchuanning Decoction is effective in lowering the NGF level and increasing CCSP expression. In this way, it intervenes in airway remodeling in patients with asthma. Detailed studies on the pathogenesis of airway remodeling contribute to the control of asthma. Today, when prevention and control of asthma is becoming increasingly urgent, the use of TCM is one of the most effective treatment methods.

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