Expression of airway mucus-associated proteins in rats with chronic obstructive pulmonary disease with a cold-dryness symptom pattern

Gao Zhen, Halmurat Upur, Wang Jing, Jing Jing, Li Zheng, Xu Dan, Li Fengsen

OBJECTIVE: To reveal the effects on expression of airway mucus-associated proteins in rats with chronic obstructive pulmonary disease (COPD) and a cold-dryness symptom pattern induced by elastase and smoking.

METHODS: The COPD model was established with an elastase dose into the trachea combined with exposure to smoking; the COPD model cold-dryness symptom pattern was further developed by exposure to a cold, dry environment. After 90 days, pathologic lung sections, inflammatory cytokine levels (measured by enzyme linked immunosorbent assay), mRNA and protein expression of mucus-associated proteins and aquaporins (measured by real-time polymerase chain reaction and western blots) were examined.

RESULTS: Cytokines interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) in the COPD and the cold-dryness symptom pattern COPD groups were all significantly higher than in controls (each \( P < 0.01 \)). IL-6 and IL-8 levels were higher in the cold-dryness symptom pattern COPD group than in the COPD group (each \( P < 0.05 \)). The AQP5 mRNA expression in the cold-dryness symptom pattern COPD and COPD groups was lower than in the control group (\( P < 0.01 \)), and that in the cold-dryness symptom pattern COPD group was lower than the COPD group (\( P < 0.05 \)). The expression of MUC5AC and MUC5B mRNAs in the cold-dryness symptom pattern COPD group and COPD group was higher than in the control group (each \( P < 0.01 \)), and that in the cold-dryness symptom pattern COPD group was higher than the COPD group (\( P < 0.05 \) and \( P < 0.01 \), respectively). The ratio of MUC5AC mRNA/MUC5B mRNA was COPD group < the cold-dryness symptom pattern COPD group < the control group. AQP4 and AQP5 protein expression in the cold-dryness symptom pattern COPD group was lower than that in the COPD group which was lower again than in the control group. MUC5AC and MUC5B expression in the cold-dryness symptom pattern COPD group was higher than in the COPD group and higher again than in the control group.

CONCLUSION: Cold-dryness affects the expression of mucus-associated protein mRNA and its corre-
sponding proteins, reducing the secretion of aquaporins and increasing the secretion of mucins. Imbalance in aquaporins and mucins can affect the function of mucus, increasing airway obstruction.

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Key words: Pulmonary disease, chronic obstructive; Cold-dryness syndrome; Aquaporins; Mucins

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a common disease of the respiratory system, characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response within the lungs to noxious gases or particles. The prevalence of COPD is 8.2% in people ≥ 40 years of age in China, with men having a higher prevalence (12.4%) than women (5.1%), and those living in rural areas have a higher prevalence (8.8%) than city dwellers (7.8%). The prevalence of COPD in people aged > 60 years in Hotan, a rural area of Xinjiang in China is 22.8%.

In the northwest of China, cold dryness is one of the common symptom patterns seen in COPD in Xinjiang, which is mostly mild to moderate disease. Lack of a biological basis for COPD has prevented treatment with traditional Chinese medicine (TCM). TCM may provide advantages, such as preventing disease exacerbation. Cold and dryness are closely related to body fluids, and the body fluid in airways is similar to mucus in TCM. Therefore, we propose that disturbance and imbalance in the secretion of mucus-associated proteins may play an important role in the progression of COPD. Cold and dryness may further aggravate the condition, developing it further into a more complicated condition-COPD with a cold-dryness symptom pattern. The cold-dryness symptom pattern was defined in terms of TCM. This study aimed to investigate the effect of COPD induced in tandem with cold and dryness, to investigate the effect of COPD with a cold-dryness symptom pattern on mucus-associated protein patterns.

MATERIALS AND METHODS

Instruments
FLI-2000H artificial climatic test chamber (EYELA Co., Tokyo, Japan); BUXCO MA1320 respiratory function test table (Buxco, Wilmington, North Carolina USA); BS-1105 electronic balance (Sai Duo Ke Shi Scales Co. Ltd., Beijing, China); slicer (Leica, Wetzlar, Germany); microscope (Leica, Wetzlar, Germany); 60 cm × 70 cm × 100 cm acrylic poisoning cabinet (self-made); high-speed centrifuge (Thermo Scientific Forma, Waltham, MA, USA); adjustable micro-pipetter (Eppendorf, Hamburg, Germany); low temperature freezer (Thermo Scientific Forma, Waltham, MA, USA); electronic balance (Sartorius, Gottingen, Germany); and micro-pipette (Gilson, Middleton, France).

Drugs and reagents
Cigarettes (tar content 12 mg, nicotine in smoke 1.0 mg, CO in smoke 13 mg, Xuelian brand, Xinjiang Cigarette Factory, Urumqi, China); elastase, 10% formalin solution, and 0.4% pentobarbital sodium (Shanghai Huayi Biologic Science and Technique Co., Ltd., Shanghai, China); IL-6 ELISA Kit, IL-8 ELISA Kit, TNF-α ELISA Kit (Bender Medsystems, Wien, Austria); The reverse-transcription polymerase chain reaction (RT-PCR) kits AB and Invitrogen, Carlsbad, CA, USA).

Animals
Ninety male Wistar rats (150 ± 20 g) were supplied by the Center of Experimental Animals, Xinjiang University of Medicine, Urumqi, China, License No: SCXK (Xin) 2003-0001. Rats were acclimatized for 2 days with the temperature (25 ± 3) °C and relative humidity 60.0%-80.0%, 5-7 rats in a cage. After which they were weighed, numbered according to body weight order and randomly divided into three groups (COPD model group (n = 35), cold-dryness COPD group (n = 35), and normal control group (n = 20) using a random number table.

Model design
A poisoning cabinet was used by the rats on study days 1-29 and 31-90. The cabinet had a volume of 420 L (length 60 cm, width 70 cm, and height 100 cm), with an exhaust diameter of 2 cm at the top and a central fan suspended from the ceiling to evenly distribute the smoke. There was also a 250 g gel dryer placed in the cabinet before each period of smoke. The poisoning cabinet was connected to a smoking apparatus, which supplied smoke at 15 sucks/min at all times to keep a relatively stable concentration of smoke. Eighteen rats smoked for 1 h each time, twice a day. The poisoning cabinet was cleared after each use, with the inlet and exhaust open, and residual smoke was dispersed by running the fan for 5 min. For the cold-dryness COPD model, rats were placed each night (10 h) in an artificial climate test chamber at (6 ± 1) °C and relative humidity 25.0%-32.8% . All other conditions were the same as for the COPD model group.

On Day 30, rats in the COPD and the cold-dryness COPD groups were given an endotracheal drip of elastase (20 U elastase in 0.8 mL saline for each 100 g body weight). Rats in the control group were given an endotracheal drip of the same volume of saline. Before the procedure, all instruments were disinfected with...
high pressure steam (121 °C, 120 kPa, 30 min). After anesthetization with 0.4% sodium pentobarbital (50 mg/kg body weight), rats were fixed on a plate with the head in a high position and the tail in a lower position. puncture trocar (0.7 mm × 19 mm, Becton Dickinson Medical Devices Co., Ltd., Suzhou, China) was slowly inserted along the tongue into the trachea and then the needle was withdrawn. A 1 mL needle tube containing the dose of elastase was angled towards the left at 45°, while half the volume of elastase solution was slowly injected; the other half of the elastase solution was injected with the needle angled right. Air (1 mL) was then injected, pushing the elastase solution completely into the lung, the back of the rat was patted, spreading the elastase solution evenly throughout the lung. Rats were kept warm, and after waking, were placed in a cage.

**Preparation of pathologic specimens**

Rats were anesthetized with an abdominal injection of 0.4% sodium pentobarbital (50 mg/kg body weight) on the 90th day, and killed by exsanguination from the inferior vena cava. Lungs were rapidly removed and the mid lobe of the right lung and part of the trachea and bronchus placed in 10% formal for fixation, routinely dehydrated, immersed in wax, embedded, and sectioned (3 continuous slices of 4 μm for each lung tissue). Sections were stained with hematoxylin and eosin (HE), and the morphological changes of the tissue were observed under a microscope and photographed.

**Detection of inflammatory cytokines in bronchoalveolar lavage fluid (BALF) and serum**

A transverse incision of the inferior trachea was made and the right main bronchus ligated. This was used to inject 3 mL of physiological saline solution into the left lung. Perfusate was retrieved immediately after each perfusion (60%-70%) and filtered using a Sterile bandage. Filtration was repeated three times to collect BALF for centrifugation. The supernatant was collected in a sterile bottle, which were then frozen at –70 °C. Enzyme-linked immunosorbent assay (ELISA) was used to detect IL-6, IL-8 and TNF-α, following the kit instructions exactly.

**Detection of airway mucus-associated proteins**

PCR was used to detect the presence of aquaporin (AQP)-5 mRNA, mucin (MUC)5AC mRNA and MUC5B mRNA. Western blots were used to detect AQ4P, AQ5P, MUC5AC and MUC5B.6

**Data analysis**

Continuous variables are expressed as mean ± standard deviation (x ± s). Analysis of variance and t-tests were performed for group comparisons. The Statistical Package for Social Sciences version 11.5 (SPSS Inc., Chicago, IL, USA) was used for data analyses. A P-value < 0.05 was considered significant.

**RESULTS**

**Comparison of pathologic morphologies**

On study day 90 the size of the alveoli was uniform and the thickness of alveolar wall was normal in the control group, with sparse or no inflammatory cell infiltration in the periphery (Figure 1A). In the COPD group, there was alveolar expansion and narrowing of the septum. The septum was also broken and fused into larger bursal lumens, with congestion and inflammatory cell infiltration (Figure 1B). In the cold-dryness COPD group, alveoli were expanded and the septum was narrowed. The septum was also broken and fused into larger bursal lumens, with congestion and inflammatory cell infiltration (Figure 1C).

**Levels of IL-8, IL-10 and TNF-α in BALF**

Table 1 shows that levels of IL-6, IL-8 and TNF-α in BALF were significantly lower in the control group than in the cold-dryness COPD group (P < 0.01 for all three cytokines) and the COPD group (P < 0.05 for all three cytokines). Levels of IL-6 and IL-8 in BALF were significantly lower in the COPD group compared with the cold-dryness COPD group (P < 0.05 for both cytokines).

**Expression of AQP5 mRNA, MUC5AC mRNA and MUC5B mRNA**

Results are presented in Table 2. AQP5mRNA expression in cold-dryness COPD and COPD groups was lower than in the control group, with the cold-dryness COPD group lower than the COPD group. MUC5ACmRNA expression in the cold-dryness COPD and the COPD groups was higher than in the control group, with the cold-dryness COPD group lower than the COPD group.

Figure 1 Comparison of pathologic morphologies among the three groups (hematoxylin eosin staining, × 100)

A: control group; B: COPD group; C: cold-dryness COPD group. The rats in the COPD and cold-dryness COPD groups were given an endotracheal dose of elastase, the control group were given an endotracheal dose of saline on study day 30. The double-arrow points to a merged alveolus. COPD: chronic obstructive pulmonary disease. There is no difference between B and C group.
Table 1 Levels of IL-6, IL-8 and TNF-α in BALF of rats after an endotracheal dose of either elastase and 90 days exposure to smoke (COPD and cold-dryness COPD groups) or control group (μg/mL, x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IL-6</th>
<th>IL-8</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>7±5</td>
<td>196±27</td>
<td>196±27</td>
</tr>
<tr>
<td>COPD</td>
<td>10</td>
<td>84±6</td>
<td>241±34</td>
<td>241±34</td>
</tr>
<tr>
<td>Cold-dryness COPD</td>
<td>10</td>
<td>105±22a</td>
<td>302±60b</td>
<td>302±60c</td>
</tr>
</tbody>
</table>

Notes: rats in the COPD and cold-dryness COPD groups were placed in a self-made poisoning cabinet with a volume of 420 L from the 1-29 th and 31-90 th days. The poisoning cabinet was connected to a smoking apparatus, smoked for 1 h each time, and were given an endotracheal dripping of saline on the 30th day. The poisoning cabinet was connected to a smoking apparatus, smoked for 90 h each time, and were given an endotracheal dripping of elastase, while in the normal control group were given an endotracheal dripping of saline on the 30th days. The poisoning cabinet was connected to a smoking apparatus, smoked for 90 h each time, and were given an endotracheal dripping of saline on the 30th day. COPD: chronic obstructive pulmonary disease; IL-6: interleukin-6; IL-8: interleukin-8; TNF-α: tumor necrosis factor; BALF: bronchoalveolar lavage fluid. Compared with the control group: a P < 0.01, b P < 0.05; compared with the COPD group, c P < 0.05.

DISCUSSION

Traditional Chinese Medicine is based on the belief that body fluid is the contact for internal organs functioning together; disorder in body fluid metabolism will further affect the function of internal organs. In addition to other substances, water is the main component of body fluid, which may be closely related to mucus under physiological conditions. Airway mucus, composed of a thin, serous layer and a relatively sticky mucous layer, is an effective barrier to prevent the attack of pathogenic microorganisms and toxins, and also an important component of the innate immune system, the mucociliary clearance system. The main component of mucus is mucin, a molecular glycoprotein, which affects the interaction of airway mucus and cilia, and determines the airway mucous membrane viscosity.

The correct ratio of water and mucin allows optimum clearance by cilia.1 Imbalance in water and mucin causes sticky mucus which can promote bacterial adhesion, cough with sputum, affect the mucociliary clearance function1 and prevent bacterial clearance10. Therefore, the water-mucin balance determines the functional status of mucus.

Mucus is a non-homogeneous, viscoelastic gel composed of water, electrolytes, lipids, proteins and glycoproteins. Mucin is a glycoprotein secreted by epithelial cells and is the main element maintaining the viscoelasticity of mucus. However, under conditions of chronic inflammation, abnormal secretion of mucin and dysfunction of the mucociliary clearance system leads to bacterial colonization of the respiratory tract, mucus plug formation, ventilatory dysfunction, pathophysiological changes and exacerbation of disease progression.11 Muc5AC is the most expressed mucin in the airway epithelium.12 Further research has concentrated on Muc5AC as one of the indicators to measure airway epithelial mucous secretions.13

Table 2 AQP5 mRNA, MUC5AC mRNA, MUC5B mRNA expression in rats after either an endotracheal dose of elastase and 90 days exposure to smoke (COPD and cold-dryness COPD groups) or control group (x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>AQP5 mRNA</th>
<th>MUC5AC mRNA</th>
<th>MUC5B mRNA</th>
<th>MUC5AC mRNA/MUC5B mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00±0.00</td>
<td>1.00±0.00</td>
<td>1.00±0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>COPD</td>
<td>0.57±0.07</td>
<td>1.79±0.24</td>
<td>2.10±0.27</td>
<td>0.85</td>
</tr>
<tr>
<td>Cold-dryness COPD</td>
<td>0.40±0.07a</td>
<td>2.53±0.31a</td>
<td>2.62±0.42a</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Notes: rats in the COPD and cold-dryness COPD groups were placed in a self-made poisoning cabinet with a volume of 420 L from the 1-29th and 31-90th days. The poisoning cabinet was connected to a smoking apparatus, smoked for 1 h each time, and were given an endotracheal dripping of elastase, while in the normal control group were given an endotracheal dripping of saline on the 30th day. COPD: chronic obstructive pulmonary disease; AQP: aquaporin; MUC: mucin. Compared with the control group, P < 0.01; compared with the COPD group, a P < 0.05, b P < 0.01.
Clinical trials have shown that patients with sputum production should receive mucolytic treatment; the mucus is then a good medium for bacterial growth and proliferation, which sets up a vicious cycle of increased inflammation and airflow obstruction. Airway water transport plays an important role in airway humidification of inhaled gases and maintains the volume and composition of airway surface liquids. Transport of water in the airway epithelia and endothelia is involved in airway humidification, alveolar fluid transport and secretion from mucus glands. AQPs are a family of protein-mediated transmembrane water transporters and are associated with water permeability. Studies have shown the expression of AQP5 and MUC5AC have a similar negative correlation in the lungs of asthmatic mice, and reduced expression of AQP5 by RNA interference results in a significant increase of MUC5AC expression. Downregulation of the bronchial mucosa AQP5 was correlated to mucus hypersecretion in non-smoking or patients with COPD who no longer smoke, indicating a direct link between AQP5s and mucin expression.

Table 3 Correlations between proinflammatory factors and airway mucus-associated proteins in bronchoalveolar lavage fluid (BALF) in rats after either an endotracheal dose of elastase and 90 days exposure to smoke (COPD and cold-dryness COPD groups) or control group

<table>
<thead>
<tr>
<th>Indicator</th>
<th>r value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8-AQP5</td>
<td>-0.80</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-8-MUC5AC</td>
<td>0.80</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-8-MUC5B</td>
<td>0.70</td>
<td>0.012</td>
</tr>
<tr>
<td>IL-6-AQP5</td>
<td>-0.95</td>
<td>0.000</td>
</tr>
<tr>
<td>IL-6-MUC5AC</td>
<td>0.84</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-6-MUC5B</td>
<td>0.85</td>
<td>0.001</td>
</tr>
<tr>
<td>TNF-α-MUC5AC</td>
<td>0.76</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Notes: IL: interleukin; COPD: chronic obstructive pulmonary disease; AQP: aquaporin; MUC: mucin.

Airway mucus hypersecretion is the result of inflammation; the mucus is then a good medium for bacterial growth and proliferation, which sets up a vicious cycle of increased inflammation and airflow obstruction. Airway mucus hypersecretion is a potential risk factor for lung function in patients with COPD, with the COPD clinical guidelines from the UK National Institute of Clinical Excellence (ICE), 2004 recommending that patients with long-term chronic cough and sputum production should receive mucolytic treatment. Clinical trials have shown that patients with moderate COPD who received mucolytics for eight months had significantly less acute exacerbations and lower hospitalization rates. Another study found that airway hyper-responsiveness and increases in mucus viscosity and mucus plug formation are closely related to the symptoms and mortality rate of asthma. Pathological examination revealed mucus accumulation in the bronchial system of the COPD patients resulting in bronchial obstruction. These results suggested MUC5AC not only led to distal bronchial stenosis and airflow obstruction by mechanical obstruction, but also induced terminal bronchiol wall collapse by changing the pulmonary surfactant - this constitutes the pathogenic basis of COPD. High expression of MUC5AC mRNA was an important reason for the increase in the secretion of phlegm and small airway obstruction in patients with acute exacerbation of COPD, suggesting MUC5AC is a more sensitive indicator in the acute phase of airway inflammation. Therefore, we can relieve acute exacerbation of COPD by blockade of mucus hypersecretion, or reducing the viscosity of mucus to prevent formation of mucus plug. In addition to an increase in the absolute amount of mucin, mucus retention during acute exacerbation or late stages of COPD was also related with imbalance of mucin/water and salt. Aquaporins (AQPs) play an important role in the body for liquid and water transport and metabolism. Airway water transport plays an important role in airway humidification of inhaled gases and maintains the volume and composition of airway surface liquids. Transport of water in the airway epithelia and endothelia is involved in airway humidification, alveolar fluid transport and secretion from mucus glands. AQPs are a family of protein-mediated transmembrane water transporters and are associated with water permeability. Studies showed AQPs are also involved in some important physiological and pathological functions, such as cell migration and lipid metabolism, suggesting AQPs are multifunctional proteins. Six of the thirteen identified AQPs were expressed in the lungs, where AQP5 is maximally expressed. AQP5 is localized on apical membranes of mucus glands and type I pulmonary epithelial cells and also plays an important role in mucus secretion. Viscous sputum is difficult to cough up in patients with COPD, and viscous mucus leads to bacterial colonization and recurrent infections. Downregulation of AQP5 in mucus glands was detected in COPD patients suggesting an increase in mucus viscosity may relate to decreases of AQP5. Studies have shown the expression of AQP5 and MUC5AC have a similar negative correlation in the lungs of asthmatic mice, and reduced expression of AQP5 by RNA interference results in a significant increase of MUC5AC expression. Downregulation of the bronchial mucosa AQP5 was correlated to mucus hypersecretion in non-smoking or patients with COPD who no longer smoke, indicating a direct link between AQP5s and mucin expression.
Differential expression of AQP1 and AQP5 is an important indicator of changes in the microenvironment of lung injury. Research on the subcellular distribution and function of water channels showed alveolar water transport, airway surface liquid regulation, airway humidification, and production of nasopharyngeal secretions are associated with the coordinated function of AQP5. AQP5 may be involved in the abnormal fluid transport of lung inflammation and knockout of AQP5 can diminish the bacterial clearance capacity of the lung. It has been reported that decrease in AQP5 mRNA expression, weak airway humidification and aggravation of airway inflammation in COPD rats can promote the development of COPD. Verkman et al. have found that AQP1 and AQP5 play a crucial role in the osmotic pressure-driven water transfer process in lungs.

In the present study, AQP5 mRNA expression in the cold-dryness symptom pattern COPD group and COPD group was lower than in the control group. By contrast, the MUC5AC and MUC5B mRNAs expression in the cold-dryness symptom pattern COPD group and the COPD group were higher than in the control group, and those in the cold-dryness symptom pattern COPD group was higher than in the COPD group. These results indicate that the cold-dryness can regulate the mucus function and increase the incidence of COPD by regulating the expression and secretion of AQP1s and mucins, as well as creating conditions for invasion of pathogenic factors. This may be one of the main mechanisms for TCM treatment of cold-dryness COPD by heating and moistening the lungs. Meanwhile, increase in mucin secretion, and decrease in AQP secretion in cold-dryness COPD result in a reduction of mucus, but thick sputum, which is difficult to cough up. This partly explains the mild sputum symptoms and main symptoms of shortness of breath, cough and asthma in COPD.

In summary, these results suggest the cold and dryness invading the lung first affects the expression of AQP5, MUC5AC and MUC5B mRNA and its corresponding proteins, which results in reducing the secretion of AQP5, increasing the secretion of mucins. The imbalance between AQP5s and mucins can affect the function of mucus and increase the degree of airway obstruction.

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