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Effect of ligustrazine on the levels of collagen and transforming growth factor-β1 in rats with asthma

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Abstract: Objective The formation of subepithelial fibrosis and increased deposition of types I and III collagen are important features of airway remodelling in asthma, while transforming growth factor-β (TGF-β1) is the major regulative factor of airway remodelling. The study aims at exploring the effect of ligustrazine on the collagen deposition and the expression of TGF-β1 in the airway wall of asthmatic rats. **Methods** Forty SD rats were randomly assigned into 5 groups (n=8 each): a normal group (A), an asthma model group (B), a dexamethasone group (C), a low-dose ligustrazine group (D) and a high-dose ligustrazine group (E). The asthma model was established by repeated inhalation of oval bulium. Groups C, D and E were treated by injection of 0.5 mg dexamethason and 40 mg or 80 mg ligustraizine respectively before stimulation, while Group A was treated with normal saline instead. The levels of collagen and TGF-β1 expression in airway wall were semi-quantitatively measured by the immunohisto-chemistry technique. **Results** The levels of collagen type III and TGF-β1 expression were significantly higher in Group B than those in Group A ($P<0.01$). In the Group C, Group D and Group E, the levels of collagen type III and TGF-β1 expression in airway wall were lower than those in Group B ($P<0.01$), and there was no difference among Groups E, C and A ($P>0.05$). The contents of collagen type I had no difference among all five groups. A close correlation between TGF-β1 expression and collagen type III content in various groups was found ($r_s=0.7063$, $P<0.01$). **Conclusions** Ligustrazine may reduce the deposition of collagen type Ⅲ and inhibit the expression of TGF-β1.

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Key words: Asthma; Ligustrazine; Collagen; Tranforming growth factor-β1; Rat

川芎嗪对哮喘大鼠气道壁胶原沉积和TGF-β1表达的影响

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[摘 要] 目的 上皮下胶原沉积及纤维化是哮喘气道重建的典型特征,转化生长因子β1(TGF-β1)在此过程中发挥了重要作用。该研究旨在观察川芎嗪对大鼠哮喘模型气道壁胶原及TGF-β1含量的影响。**方法** 40只SD大鼠随机分成对照组(A)、哮喘组(B)、激素干预组(C)、小剂量(D)和大剂量(E)川芎嗪干预组,以卵蛋白致敏并长期吸入激发制备哮喘模型,C,D,E组分别在每次激发前腹腔注射地塞米松0.5 mg/只,川芎嗪40 mg/kg及川芎嗪80 mg/kg。4周后处死大鼠,收集肺组织标本观察病理改变,并采用免疫组化半定量测定气道壁Ⅰ,Ⅲ型胶原和TGF-β1的含量。**结果** B组气道壁Ⅲ型胶原和TGF-β1含量均显著高于A组,C,D,E组均较B组降低,差异有显著性(均$P<0.01$),E组与A组、C组含量接近(均$P>0.05$),D组Ⅲ型胶原和TGF-β1含量高于E组,差异有显著性($P<0.01$);气道壁TGF-β1表达与Ⅲ型胶原含量呈显著正相关($r_s=0.7063$, $P<0.01$)。各组间Ⅰ型胶原含量差异无显著性(均$P>0.05$)。**结论** 川芎嗪减少哮喘大鼠气道壁Ⅲ型胶原沉积和TGF-β1的表达,对Ⅲ型胶原的抑制可能部分通过抑制TGF-β1的表达而实现。

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Airway remodelling is a typical pathological feature of chronic asthma, mainly presenting hyperplasia and hypertrophy of airway smooth muscle, incrassation of basement membrane, extracellular matrix deposition and subepithelial fibrosis. Airway remodelling leads to continuous airway hypersensitivity and inflexible air block, making asthma chronic and stubborn, finally decreasing the lung function. According to the result of immunohistochemistry the deposition of types I and III collagen increases in the airway wall, while transforming growth factor β1 (TGF-β1) is the major regulative factor of airway remodelling in asthma, directly affecting the collagen deposition in airway wall and promoting fibrosis. Ligustrazine is extracted from *chuanxiong*, a traditional Chinese medicine with an effect of activating the blood and loosening the stagnation, which plays a role in inhibiting collagen synthesis by fibroblast. Ligustrazine is effective for the therapy of rat lung fibrosis model and clinical lung fibrosis patients[1,2]. Thus in this study the difference of collagen deposition in the airway wall and TGF-β1 expression were observed before and after ligustrazine administration using rat lung fibrosis model so as to understand its influence on airway remodelling and the possible mechanism.

Material and methods

Reagents

The reagents used in this experiment were as follows: oval albumin (OVA, Sigma), vaccine of B. pertussis (Beijing Bioproducts Institute), rabbit polyclonal antibody against collagen types I and III from rat, TGF-β1 kit, SABC kit, DAB kit (Boster Biological Technology Co. Ltd, Wuhan), ligustrazine injection (The 1st Biochemical Pharmaceutical Company, Shanghai. Lot No. 011201, MW 208.69), and dexamethasone sodium phosphate injection (The 2nd Pharmaceutical Factory, Nanjing. Lot No. 021205).

Methods

Laboratory animals and grouping

Forty healthy male SD rats were provided by the Animal Center of School of Medicine of Southeast University, with an average body weight of 200 ± 20 g. They were randomly assigned into five groups (8 in each group): control (A), asthma (B), hormone interference (C), low dose (D) and high dose (E) ligustrazine interference. The asthma model was established according to reference[3], i. e. the rats were hyper-sensitized by i. p. injection of 1ml 10% OVA containing 100 mg dry powder of aluminum hydroxide and 5×10^9 inactivated vaccine of B. pertussis and asthma was induced by inhaling 1% OVA atomized liquid 2 weeks later. A successful asthma rat model should manifest hyperpnea, lip cynosis, abdominal muscle spasm, nodding respiratory and staggering, etc. Then rats from Groups B were placed in transparent airtight container and were stimulated by inhaling of 1% OVA atomized liquid (moderate) for about 20 minutes once daily for the first 3 days and then every other day for 4 weeks in all. Normal saline was injected i. p. before stimulation. Rats from Groups C, D and E were treated in the same way except that normal saline was substituted by 0.5 mg dexamethasone injection in Group C, and by 40 mg/kg or 80 mg/kg ligustrazine injection in Groups D and E. Normal saline was given via both i. p. injection and atomized inhaltion to rats in Group A instead of OVA. All animals were sacrificed within 24 hours after the last stimulation, and the lung tissues were fixed by intubation gavage of 4% poly-formaldehyde PBS via the lung artery.

Pathological observation of the airway wall

The fixed lung tissues were dehydrolyzed by gradient ethanol to prepare paraffin slice measuring 3 μm thick. The slices were routinely stained by Hematoxylin-Eosin (HE) to observe pathological changes.

Detection of types I and III collagen and TGF-β1

The paraffin slices were routinely de-waxed and the antigens were rehabilitated by enzyme digestion or microwave. Intrinsic peroxidases were inactivated by H_2O_2-methanol solution. The slices were blocked at room temperature for 30 minutes by normal goat serum. First antibody (rabbit against collagen types I and III from rat and TGF-β1) in 1:200 dilution was added and left overnight at 4℃. The slices were then

washed with PBS and after biotinized goat anti-rabbit IgG had been added they were left at 37℃ for 20 minutes. They were then washed with PBS and avidin-peroxidase conjugates were added and they were then left at 37℃ for 20 minutes. They were then washed with PBS 4 times (5 minutes each time) and DAB solution was added for staining at room temperature. The reaction time (10 minutes or so) was controlled under the microscope. Slices were then routinely washed and gently restained with Hematoxylin and sealed by neutral gum. The contents of collagen type I and III and TGF-β1 in various groups were semi-quantitatively measured through IHE (via OD value) by computer image process software (The Medical morphology recognition and statistics system was provided by Nanjing Medical University Pathology Laboratory).

Statistical process

All quantitative data were expressed as $\bar{x} \pm s$ and processed by Prism 3.0 software. The differences between groups were analyzed by ANOVA and the correlation of groups was analyzed by Spearman.

Results

Clinical manifestations and pathological changes in various groups

Obvious dyspnea appeared in Group B after hyper-sensibilization and stimulation and different degrees of dyspnea appeared in Groups C, D and E, while there was no reaction in Group A. According to pathological observation, the airway wall grew thick and the cavity grew narrow, while the plica in the mucous membrane of the airway increased, the epithelial cell fell off, the airway smooth muscle and reticular basement membrane incrassated, eosinophil and lymphocyte invaded under the mucous membrane and in the wall in Group B. The pathological changes in Group D was the same with Group B but in a minor degree. No epithelial cell fell off in Groups C and E, while the plica in the mucous membrane of the airway increased and the airway smooth muscle and reticular basement membrane incrassated in a similar degree with Group A. Only a little eosinophil and lymphocyte invasion appeared in the wall.

Collagen deposition in the airway wall in various groups

The collagen of the airway wall mainly distributed in the subepithelial layer. In Group B the whole subepithelial layer was dyed deeply, even part of dark brown. While in Group A it was weakly or not stained at all. The coloration degree in Groups C, D and E was between Groups A and B. The content of collagen type III in Group B was higher than that in Group A by semi-quantitative measurement, and also higher than that in Groups C, D and E ($P<0.01$). The content of type III collagen in Group D was higher than that in Group E ($P<0.01$). The distribution of collagen type I was similar with collagen type III, and there were no differences in the collagen type I content between different groups ($P>0.05$). All these data are shown in Table 1.

Table 1 Contents of types Ⅲ and Ⅰ in the 5 groups ($\bar{x} \pm s$)

Group	n	Collagen type Ⅲ	Collagen type Ⅰ
A	8	19.1 ± 7.5^{a}	33.7 ± 12.5
B	8	55.4 ± 6.8	44.4 ± 7.9
C	8	20.9 ± 6.7^{a}	37.1 ± 12.2
D	8	$31.9 \pm 7.7^{a,b,c}$	35.4 ± 8.0
E	8	$20.5 \pm 5.1^{a,d}$	38.4 ± 8.4

Note: a vs Group B $P<0.01$; b vs Group A $P<0.01$; c vs Group C $P<0.01$; d vs Group D $P<0.01$

Comparison of TGF-β1 expression in airway wall in various groups

TGF-β1 was mainly expressed in the epithelial layer of airway wall, and also to a high degree in smooth muscle and eosinophil. In Group B the epithelial layer of airway wall was stained dark brown or yellow, and in Group A there was just a thin yellow coloration or none at all, while in Groups C, D and E the coloration was from thin yellow to dark yellow. The content of TGF-β1 in the airway wall of Group B was obviously higher than that of Groups A, C, D and E ($P<0.01$), while that of Group D was lower than that of Group B ($P<0.01$) and higher than that of Groups A, C and E ($P<0.05$). No difference was found among Groups A, C and E ($P>0.05$). (Table 2)

Table 2 Levels of TGF-β1 expression in the 5 groups ($\bar{x} \pm s$)

Group	n	TGF-β1
A	8	19.3±4.6
B	8	68.5±14.3[a]
C	8	25.2±9.3[b,c]
D	8	38.1±10.1[a,b]
E	8	25.8±5.2[b,c]

Note: a vs Group A $P<0.01$; b vs Group B $P<0.01$; c vs Group D $P<0.05$

The relationship between the content of type III collagen in airway and the content of TGF-β1

TGF-β1 expression is positively correlated with the content of type III collagen in airway wall in various groups ($r_s=0.7063$, $P<0.01$).

Discussion

Airway remodelling, also referred as airway remold or reconstruction, is a repair reaction of the body to injury stimulation. The epithelial cell and macrophage, etc in the airway wall will secrete a lot of cytokines and growth factors (TGF-β1, PDGF and endothelin, etc) under the stimulation of long time chronic inflammation. This can promote the smooth muscle and fibroblast to proliferate, differentiate and produce extracellular matrix, resulting in incrassation of connective tissue rendering fibrosis.

TGF-β1 is the major regulative factor of airway remodelling, which can be secreted by macrophage, epithelial cells, the smooth muscle cells and eosinocyte in the airway wall under the condition of asthma[4]. Under the influence of TGF-β1, the smooth muscle cells proliferate and incrassate and the fibroblasts differentiate into fibrocytes, which can increase production of fibronectin (FN) and collagen. TGF-β1 also improves the deposition of collagen in the extracellular matrix and inhibits the production of collagenase and proteinase resulting in the decrease of collagen disaggregation.

Theoretically inhibiting the production of TGF-β1 in the lung tissue can restrain or lessen the airway remodelling in asthma. Glucocorticoid can inhibit the production of TGF-β1, as reported by Kong et al[5] that dexamethasone can restrain the expression of TGF-β1 in the airway wall of guinea pig asthma model and that the thickness of the airway wall is positively related with the level of TGF-β1. In this report dexamethasone effectively interfered with the expression of TGF-β1 in the airway wall of rat asthma mode, which was consistent with the former result.

Collagen deposition in the matrix of basement membrane is a pathological feature of chronic asthma. The major compositions in the incrassated reticular layer are type III, V and a little I collagen and FN[6] as reflected from immunohistochemistry. The ratio between types I and III collagen in normal lung tissue is about 2∶1, while the ratio can reach 3～4∶1 in lung tissues with fibrosis. At the early stage of fibrosis it is type III collagen that takes up most of the collagen increase, while at the late stage it is type I collagen. Adrenocortical hormone can inhibit the deposition of collagen in asthma. Trigg et al[7] reported that inhaling adrenocortical hormone can decrease the deposition of beclometasone Dipropionate type III collagen in the basement membrane. This study showed that the content of type III collagen noticeably decreased, even was close to that in the normal control group, in the airway wall of asthma rats treated with adrenocortical hormone.

The major ingredient of ligustrazine, an extract from *chuanxiong*, is tetramethyl-pyrazinamide. Wang et al[8] reported that ligustrazine can restrain the formation and development of fibrosis in the matrix of injured kidneys. Hou et al[1,2] proved that Salvia miltiorrhiza Bge. and ligustrazine is effective for the therapy of the rat lung fibrosis model and clinical lung fibrosis patients. In this report it was found that two doses of ligustrazine could both obviously restrain the expression of TGF-β1 and deposition of collagen in the airway wall of asthma rats. The effect of high-dose ligustrazine is close to that of dexamethasone, and that of the low-dose is relatively small. The expression of TGF-β1 in the airway wall is markedly positively correlated with the content of collagen, i.e., the inhibition effect of ligustrazine on the deposition of collagen may be partially attained by restraining the expression of TGF-β1. Another result is that both adrenocortical hormone and ligustrazine can inhibit the expression of type III collagen in reticular

layer, while having no influence on type I collagen. Maybe since the fibrosis on the airway remodelling of asthma is in the early stage, the drugs affect more on the actively synthesizing type III collagen and less on the dormancy type I collagen.

It is an effective way for restraining asthma, especially chronic and stubborn asthma, by preventing and reversing airway remodelling. Ligustrazine has a repressive effect on the expression of TGF-β1 and collagen synthesis of airway wall, partially similar with dexamethasone. Thus it is worthy to study, in detail, the treatment of asthma with ligustrazine alone or in combination with glucocorticoid.

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·消息·

中国当代儿科杂志被美国《化学文摘》收录

从2004年第6卷第1期起，中国当代儿科杂志被国际著名检索系统美国《化学文摘》(CA)收录。这标志着中国当代儿科杂志学术水平不断地提高，在与国际接轨的进程中又迈出了重要的一步。现将收录函公布如下：

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