

Inhibitory Effect of Dexamethasone on Expression of Cysteine-rich 61 Protein in Airway Epithelial Cells of Allergic Mouse Models*

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Summary: In order to study whether cysteine-rich 61 protein (cyr61) is involved in the pathogenesis of asthma and its relation to airway inflammation, the effect of dexamethasone (Dxm) on the expression of cyr61 in the lung tissues of asthmatic mice was investigated. Forty BALB/c mice were divided into asthma group ($n=15$), control group ($n=10$) and Dxm group ($n=15$). The asthma group was sensitized and challenged by ovalbumin (OVA). The mice in Dxm group were intraperitoneally administered with Dxm after OVA challenge. The expression of cyr61 in the lung tissues was detected by using immunohistochemistry, and that of eotaxin protein in the bronchoalveolar lavage fluid (BALF) by using enzyme-linked immunosorbent assay (ELISA). The number of inflammatory cells in BALF was also analyzed. The results showed that the cyr61 expression was highest in asthma group ($P<0.05$), followed by Dxm group ($P<0.05$) and control group. The cyr61 had a positive correlation with the total nucleated cells ($r=0.867$, $P<0.05$), especially eosinophils ($r=0.856$, $P<0.05$), and eotaxin level ($r=0.983$, $P<0.05$) in the BALF. Our findings suggested that cyr61 is expressed in airway epithelial cells and has a positive correlation with eotaxin and number of airway infiltrating eosinophils.

Key words: asthma; airway inflammation; cysteine-rich 61; airway epithelial cells; eosinophils

Allergic asthma is a chronic inflammatory airway disease, in which many cells (mast cells, eosinophils, T lymphocytes, macrophages, neutrophils and epithelial cells) and cellular elements are involved in the inflammation. The accumulation and infiltration of airway eosinophils is one of the most important characteristics of asthma^[1]. Previous studies revealed that the infiltration of eosinophils can be induced by eotaxin secreted by airway epithelial cells^[2, 3]. However, the pathogenesis of airway inflammation hasn't been fully understood until now.

Cyr61/CTGF/NOV (CCN) family is a group of secreted proteins that are extremely cysteine-rich (10% by mass). It comprises cysteine-rich 61 (cyr61, CCN1), connective tissue growth factor (CTGF, CCN2), nephroblastoma overexpressed (NOV, CCN3), WNT 1-inducible signaling pathway proteins (WISP-1, CCN4), WISP-2 (CCN5), and WISP-3 (CCN6)^[4, 5]. They play important roles in many diseases by mediating cell adhesion, migration, proliferation, survival, and extracellular matrix synthesis. *In vitro*, CCN expression is finely regulated by diverse inflammatory mediators including cytokines (tumor necrosis factor- α , interleukin-1 β), small factors (prostaglandins, nitric oxide, histamine), and extracellular matrix enzymes^[6]. Moreover, cyr61 has been

identified to be involved in many lung diseases, such as ventilation-induced lung injury^[7], lung cancer^[8] and chronic obstructive lung disease^[9].

Studies^[6-11] have revealed that cyr61 plays an important role in various biological functions, such as angiogenesis, bone remodeling, cancer and endocrine pathways. Moreover, recent studies^[10, 11] demonstrated that cyr61 might contribute to inflammation by inducing migration of inflammatory cells and mediating adhesion of inflammatory cells. However, the role of cyr61 in asthma, which is also an inflammatory disease, hasn't been investigated to date. In order to identify whether cyr61 may be involved in the pathogenesis of asthma and its relation to airway inflammation, the effect of dexamethasone (Dxm) on the expression of cyr61 in the lung tissues of asthmatic mice was investigated in this study.

1 MATERIALS AND METHODS

1.1 Establishment of Allergic Mouse Models

All animal experiments were carried out according to the guidelines set by the Chinese Council on Animal Care and approved by the Tongji Medical College (China) Committee on Animal Experimentation. All animals were kept in SPF conditions at the animal facility of Tongji Medical College. Forty pathogen-free 6- to 8-week old BALB/c female mice were obtained from the Experimental Animal Center of Tongji Medical College. The animals were randomly divided into three groups: asthma group ($n=15$), control group ($n=10$) and Dxm

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group ($n=15$). The asthma group was treated by the method as described previously^[12]. Briefly, the mice in asthma group were sensitized via intraperitoneal injection of 40 μ g ovalbumin (OVA, Grade III, Sigma, USA) and 4.5 mg adjuvant [$\text{Al}(\text{OH})_3$] in 0.2 mL of saline three times at weekly intervals (day 0, 7, 14). The mice in control group received adjuvant alone. On the day 21–23, mice in asthma group received aerosolized challenges with 2% OVA for 30 min every day via an ultrasonic nebulizer. The Dxm group was administered with Dxm intraperitoneally (1.7 mg/kg, once a day) beginning on the day 21–23 prior to OVA challenge^[13]. All animals were killed on the day 24 and analyzed for the parameters described below. Meanwhile, lung tissue sections from asthma group were stained with hematoxylin and eosin (H&E). Then the H&E-stained tissue sections were examined by light microscopy (fig. 1).

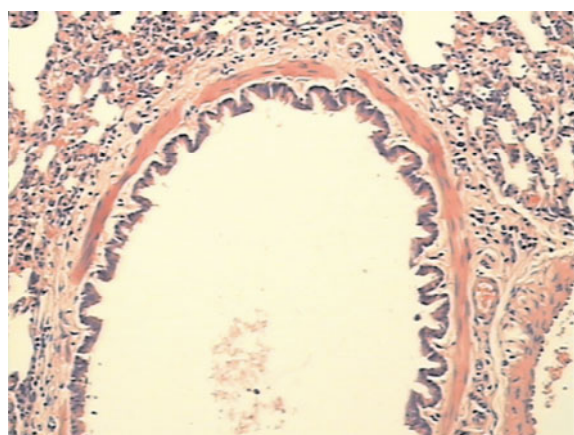


Fig. 1 Histopathologic changes of lung tissues in the allergic mouse (H&E staining, $\times 100$)

1.2 Bronchoalveolar Lavage Fluid (BALF) Collection

Immediately after the mice were killed, BALF was collected by flushing the lungs with 1 mL ice-cold sterile phosphate buffered saline (PBS) via the trachea three times. Then, BALF was centrifuged at 400 g for 5 min at 4°C to separate cells and supernatants. The total cell number was counted by using a hemocytometer. The supernatant from BALF was preserved at -70°C for the eotaxin detection.

1.3 Immunohistochemistry Staining

Lung sections were detected by polyclonal antibody

specific for mouse cyr61 (Santa Cruz, USA). As a negative control, the primary antibody was substituted with normal goat serum. Immunoreactivity was visualized with diaminobenzidine (DAB). The grey level of cyr61 positive granules was assayed by MIAS-300 image analyzer.

1.4 Expression of Eotaxin Detected by ELISA

The eotaxin level in BALF was measured by ELISA according to the manufacturer's instruction (Rapid-bio, USA). Briefly, samples of BAL fluid were aliquoted in duplicate into 96-well plates (50 μ L/well) pre-coated with antibody to mouse eotaxin and assayed according to the manufacturer's instructions. Absorbance (A) was measured at 450 nm. Cytokine concentration was determined by comparison with known standards.

1.5 Cells Count in BALF

The nucleated cells were prepared according to previous study^[14] and were stained by Wright-Giemsa staining. A differential count was performed by using the standard morphologic criterion of enumeration of at least 300 cells under an immersion objective microscope^[14].

1.6 Data Analysis

The data were expressed as $\bar{x} \pm s$. The results were analyzed by the Student's t test. Correlations analysis was performed by using Spearman nonparametric test. The difference was considered to be statistically significant at $P < 0.05$. All analyses were tested using SPSS statistical software (version 17.0).

2 RESULTS

2.1 Expression of Cyr61 in Lung Tissues

As shown in fig. 2 and 3, the expression of cyr61 was detected by using immunohistochemistry, and the grey level of cyr61 positive granules was assayed by MIAS-300 image analyzer. The cyr61 expression was detectable in the epithelial cells from small airway and nearby infiltrating inflammation cells mainly. The cyr61 expression was highest in asthma group ($P < 0.05$), followed by Dxm group ($P < 0.05$) and control group.

2.2 Nucleated Cells in BALF

As shown in fig. 4, the number of nucleated cells in asthma group was significantly greater than that in control group ($P < 0.05$), and that in Dxm group was lower than that in asthma group ($P < 0.05$), but higher than that in control group. Further analysis revealed the same trend to eosinophils in BALF.

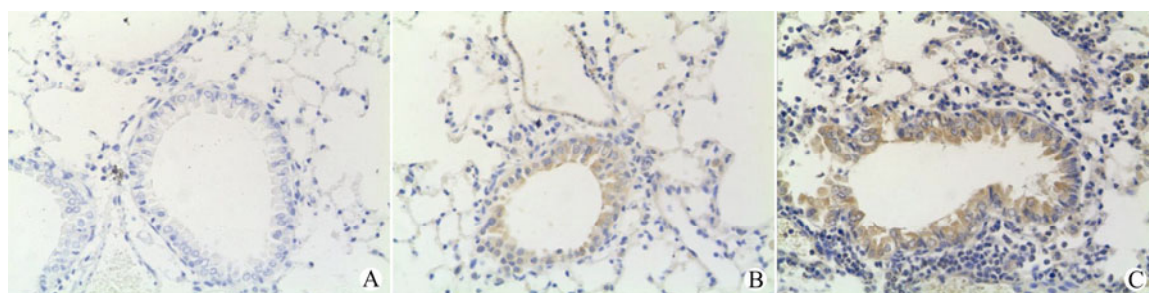


Fig. 2 Immunohistochemistry staining of cyr61 expression in the airway epithelial cells in the mice of different groups (DAB, $\times 200$)
A: control group; B: Dxm group; C: asthma group

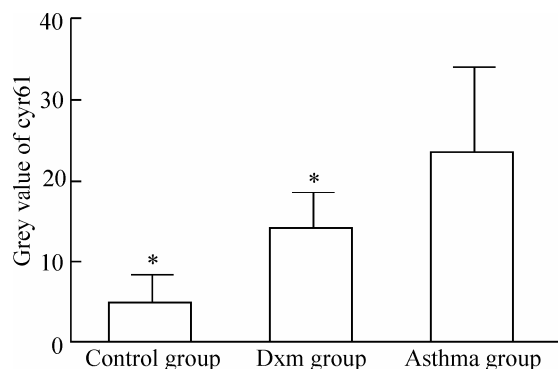


Fig. 3 The grey value of the cyr61 staining in different groups
Data were presented as $\bar{x} \pm s$. * $P < 0.05$ vs. asthma group

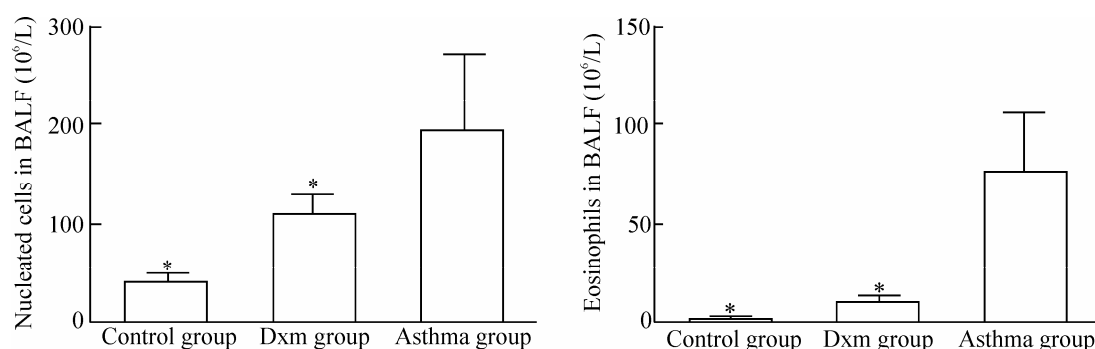


Fig. 4 The number of nucleated cells (left) and eosinophils (right) in BALF in the mice of different groups
Data were presented as $\bar{x} \pm s$. * $P < 0.05$ vs. asthma group

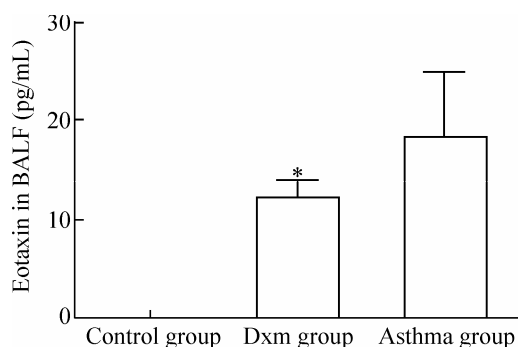


Fig. 5 The eotaxin level in BALF in the mice of different groups
Data were presented as $\bar{x} \pm s$. * $P < 0.05$ vs. asthma group

3 DISCUSSION

This study investigated the expression of cyr61 in mouse allergic asthma models induced by OVA. The expression of cyr61 was observed mainly in epithelial cells of the small airways and some surrounding inflammation cells in asthma group, significantly higher than that in control group. Moreover, the expression of cyr61 was lower in Dxm group than in asthma group. To our knowledge, asthma is characterized by airway inflammation, in which many cells, including airway epithelial cells, play important roles. Meanwhile, Dxm has great anti-inflammatory effects on asthma. Thus, cyr61 may have a possibility to play some roles in the pathogenesis

2.3 Correlation between Cyr61 and Inflammatory Cells

The positive correlation was found among cyr61, the nucleated cells ($r=0.867$, $P<0.05$), and eosinophils ($r=0.856$, $P<0.05$) in the BALF.

2.4 Determination of Eotaxin Levels

The level of eotaxin was below the limit of detection in the supernatant from BALF of control group. As shown in fig. 5, the level of eotaxin in Dxm group was lower than that in asthma group ($P<0.05$), but still higher than in control group ($P<0.05$). Moreover, the positive correlation was found between cyr61 and eotaxin ($r=0.983$, $P<0.05$).

of asthma.

The airway epithelial cells can act as airway protective barrier. Meanwhile, they are the first to be affected during immune responses through the expression of receptors and ligands, which directly interact with, guide, activate, and regulate other immune cell types. They release soluble factors such as chemokines and cytokines such as interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)- α , eotaxin, regulated on activation normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1, interferon gamma-induced protein (IP)-10 or lipid mediators (such as prostaglandins) to attract, activate and regulate immune cells in asthma^[15-17]. Lin *et al*^[10] found that cyr61 promoted Th17 development in rheumatoid arthritis by upregulating IL-6 production. Löbel *et al*^[11] revealed that cyr61 played an important role in regulating immune cell trafficking by attracting and locally immobilizing immune cells. All these suggested that cyr61 might play some roles in inflammation. On the basis of these, the over-expression of cyr61 in asthma epithelial cells might contribute to airway inflammation, and act as chemokines and cytokines described above. Thus, the correlation between the cyr61 and the infiltrating inflammatory cells was further analyzed. The results revealed that the positive correlation was found among cyr61, the nucleated cells, and eosinophils in the BALF, which suggested that the abnormal airway epithelial cells might contribute to airway inflammation through cyr61. However, it is still needed to identify the role.

In previous researches, *cyr61* was found to be involved in the pathogenesis of lung injury^[7] and fibrosis^[9]. *Cyr61* mediates its activities primarily through interaction with cell adhesion receptors, including integrin(s) and heparan sulfate proteoglycans (HSPGs). Some integrins ($\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$, $\alpha_M\beta_2$, and $\alpha_D\beta_2$) have since been identified as signaling receptors mediating *cyr61*. *Cyr61* induces fibroblast apoptosis through binding to $\alpha_6\beta_1$ and syndecan-4, leading to the p53-dependent activation of Bax and cytochrome c release^[18]. *Cyr61* can affect cells function through activation of extracellular signal-regulated kinase (ERK) and NF- κ B signal transduction pathway^[19-21]. However, as to the disease of asthma, whether *cyr61* plays the role through integrins or other signal pathways is unknown. Further investigation is needed in the future work.

We observed the positive correlation between the increased *cyr61* and eosinophils in asthma. Moreover, they were both affected by Dxm. Since eotaxin secreted by epithelial cell can induce the infiltration of eosinophils^[22], and that there was a positive correlation between *cyr61* and eotaxin in asthma in this study, *cyr61* may have a possibility to affect or be affected by eotaxin. Whether activation of *cyr61* might affect the secretion of eotaxin needs further study.

In summary, this study found that (1) *cyr61* protein was expressed highly in airway epithelial cells of asthma mouse models and decreased by Dxm; (2) *cyr61* had a positive correlation with the nucleated cells, especially eosinophils, in the BALF; and (3) *cyr61* had a positive correlation with eotaxin. In this study, some interesting phenomena about *cyr61* were observed in asthma mouse models. It suggested that *cyr61* might contribute to airway inflammation in asthma. More intervention trials are needed to identify the role of *cyr61* in the future.

Conflict of Interest Statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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