Effect of RNA interference therapy on the mice eosinophils CCR3 gene and granule protein in the murine model of allergic rhinitis

Xin-Hua Zhu¹, Bing Liao², Ke Liu¹, Yue-Hui Liu*¹

¹Department of Otorhinolaryngology Head and Neck Surgery, the Second Affiliated Hospital of Nanchang University, Nanchang 330006, China
²Department of Head and Neck Surgery, the Tumor Hospital of Jiangxi Province, Nanchang 330006, China

ARTICLE INFO

Article history:
Received 10 September 2013
Received in revised form 15 October 2013
Accepted 15 December 2013
Available online 20 March 2014

Keywords:
Allergic rhinitis
Eosinophil
CCR3
RNA interfering

ABSTRACT

Objective: To observe the clinical manifestations of allergic rhinitis mice and the expression changes of the eosinophils CCR3 and the granule protein mRNA in the bone marrow, peripheral blood and nasal lavage fluid. Methods: Twenty-four BALB/c mice were randomly divided into the control group, PBS therapy group, siRNA therapy group and the CCR3 siRNA therapy group (n=6). Allergic rhinitis model were sensitized and stimulated by ovalbunfin, and CCR3 siRNA therapy group were administered with CCR3 transnasally before stimulated. The levels of the eosinophils CCR3, MBP, ECP and EPO in bone marrow, peripheral blood and nasal lavage fluid were detected by RT–PCR. Results: Compared to the control group and CCR3 siRNA therapy group, the nasal mucosa of the PBS therapy group and siRNA therapy group developed epithelalysis, goblet cells hyperplasia, squamous epithelium metaplasia, epithelium necrosis, lamina propria and submucosa gland hyperplasia, vasodilatation, tissue edema, and the characterized eosinophil infiltration. RT–PCR indicated that the CCR3 mRNA, MBP , ECP and EPO expression in bone marrow, peripheral blood and nasal lavage fluid of the CCR3 siRNA therapy group was lower than the PBS therapy group and siRNA therapy group (P<0.05). Conclusions: The RNA interference therapy to CCR3 by local administration pernasal can suppress the process of the development, migration and invasion of the allergic rhinitis eosinophil, thus can reduce the effect of eosinophils and then reduce the inflammation effect of the allergic rhinitis. It may be a new treatment for respiratory tract allergic inflammation.

1. Introduction

Eosinophils (EOS) infiltration is an important pathological features of allergic diseases. In recent years, the study suggests that it is an important factor in the pathogenesis of allergy that EOS migration and activation to the local tissue. With the application of molecular biology techniques, the chemokine receptor 3 (CCR3) receptors were considered to be the EOS selective receptor which with a strong inducing chemotaxis effect. The traditional drug therapy has a poor specificity and the treatment is ineffective. RNA interference has the advantage of specific and targeting etc, is regulated by a large number of gene expression of a small amount of dsRNA. Currently the reports of the effect of inhibiting CCR3 expression on the allergic rhinitis is still rare. In this study, we established a mouse model of allergic rhinitis and used synthesized interfering RNA in vitro, and then observed the changes of mRNA of the EOS CCR3 and eosinophil granule proteins—major basic protein (MBP), cationic protein (ECP), peroxidase (EPO) expression in mice. This study is to explore the feasibility of the treatment of RNA interference (RNAi) for allergic rhinitis.

2. Materials and methods

2.1. Animals and reagents

Twenty four male BALB/c mice from 6 to 8 weeks, weight 20–30 g were purchased from animal and science of
2.2. Construction of CCR3siRNA expression plasmid vector

CCR3 full-length clone (NM_009914.4) was used as a template to amplify the coding frame sequence. Primer was designed and synthesized by BioWit-Technologies. shRNA sequences: shRNA1: GGTTGTTGTGATCCCTGATGAAAAGTACTGTTCAACCTGTTG; shRNA2: GGTTGTTGTGATCCCTGATGAAAAGTACTGTTCAACCTGTTG; shRNA3: GGGAGGCATGGGCTGATTTTGATG; shRNA4: GACCCACCCATGATGTGAA. RNAi target sequence was generated shRNA Lentiviral vector-pLVX-shRNA2-mCCR3-1 +2 +3 +4). Successfully constructed lentiviral vector plasmid (pLVX-ShRNA2-mCCR3-1 +2 +3 +4).

2.3. Animal grouping and sensitized model establishment.

In order to avoid the interference caused by external allergen, all the animals were kept in the laboratory of the department of animal science of medical college of Nanchang University, free diet, feeding by circadian rhythm. Twenty four clean grade BALB/c mice were randomly divided into 4 groups (n=6), one cage for each. Group one was the control group given normal saline. Group 2 was the PBS group given PBS. Group 3 was the siRNA group, given siRNA lentiviral vector. Group 4 was the CCR3 siRNA treatment group, given CCR3 siRNA. 8 μL treatment fluid were dropped into the nasal cavity on day 0 and day 14, respectively, twice a day. The ovalbumin (OVA) was injected into the intraperitoneal for sensitization on day 2 and day 16, twice a day. 300 mg OVA/mL was injected into the intraperitoneal for sensitization on day 2 and day 16. Each group was performed. The data which not normally distributed were analyzed by Kruskal-Wallis test. Then 1 mL syringe needle was put into the nasal cavity about 2 cm bilateral. 2 mL PBS buffer was used to lavage the nasal cavity. Pressing the eyeball at the mouse inner canthus, tear the retrobullar tissues and collect blood. Exposure the nasal and obtained the bilateral nasal mucosa. The mucosa was fixed in 4% paraformaldehyde solution, embedded in paraffin, made into 4 μm sections for HE staining. The bone marrow, peripheral blood, nasal lavage fluid were stored at –80 °C refrigerator, detected by CCR3, EPO, MBP and ECP. Performed real–time quantitative PCR for the CCR3, EPO, MBP, ECP, and housekeeping gene (β-actin). Reaction conditions: 95 °C denaturation 2 min, 40 cycles, 94 °C denaturation 20 s, 49.5 °C (CCR3), 55.2 °C (EPO), 52 °C (MBP), 50.9 °C (ECP), annealed 20 s, elongation at 72 °C for 30 s, plate–reading at 74 °C. PCR products were detected by agarose gel electrophoresis. Standardized cEOS, ICAM-1, Tenascin-C and PGF2a mRNA absorbance values with the internal reference β-actin optical density value, and obtained the relative content of CCR3, EPO, MBP and ECP mRNA expression.

2.5. Result determination

Grading: (1) 0: no sneezing, no scratching nose, no secretion in nasal cavity; (2) 1 score: times of sneeze <4, mild scratching nose, the secretions in anterior nostril; (3) 2 scores: times of sneeze: 4–10, frequent scratching nose, nasal secretions more than anterior nostril; (4) 3 scores: times of sneeze> 11, continuous scratching nose, rubbing, face covered with nasal secretions. Superimposed quantization method was used to record the score. Nasal symptoms of 1–4 groups were observed after 30 minutes of the stimulation, the behavior score was calculated according to this rating system (> 5 is an successful animal model).

2.6. Statistical analysis

The data were analyzed by SPSS 19. 0 statistics software, and the measurement data were expressed as mean± SD values. Normal test and homogeneity of variance in each group was performed. The data which not normally distributed were analyzed by Kruskal-Wallis test. The data were analyzed by SPSS 19. 0 statistics software, and the measurement data were expressed as mean± SD values. Normal test and homogeneity of variance in each group was performed. The data which not normally distributed were analyzed by Kruskal-Wallis test.
distributed were converted to normally distributed data by natural logarithm. $t$-test was applied in the comparison between two groups. $P<0.05$ was considered as statistical significance.

3. Results

3.1. Behavioral observation

The average score of the three groups was greater than 5 means successful model. The mice in the normal control group and CCR3 siRNA group had mild scratching nose and less sneezing, total score was $<5$. That means the allergic rhinitis animal model can be successfully established by this method, and the application of CCR3 siRNA treatment can effectively control the occurrence of allergic rhinitis.

3.2. Nasal histological changes

Compared group 1 with group 2 and group 3, the mucosa epithelium were shed and the goblet cell hyperplasia was obvious and coexisted with EOS and other inflammatory cell infiltration, also with mucosa layer thickening and the dilatation and congestion of lamina propria, and tissue edema, glandular hyperplasia, hypertrophy strong secretion. That is comply with the pathological features of allergic rhinitis (Figure 1).

3.3. RT-PCR test results

In this study, we detected bone marrow, peripheral blood and nasal lavage fluid of fresh specimens from 2–4 groups of the mice by RT–PCR, and found that after the treatment of pLVX–ShRNA2–mCCR3–1 +2 +3 +4 for topical nasal, the difference has statistically significant ($P<0.05$) (Figures 2, 3, Table 1).

The relative gray value of CCR3 mRNA of each sample in CCR3 siRNA treatment group were as follow: Bone marrow $(0.340 3\pm0.022 5)$, blood $(0.253 4\pm0.061 6)$, nasal lavage fluid $(0.00\pm0.00)$, which were significantly lower than the relative gray value of CCR3 mRNA of each sample in the PBS control group and the siRNA group ($P<0.05$).

The MBP mRNA relative gray value of each sample in the CCR3 siRNA group were as follow: Bone marrow $(0.324 0\pm0.024 5)$, blood $(0.015 3\pm0.012 8)$, nasal lavage fluid $(0.00\pm0.00)$ which were significantly lower than the MBP mRNA relative gray value of the PBS group and the siRNA group ($P<0.05$).

The ECP mRNA relative gray value of each sample in the CCR3 siRNA group were as follow: Bone marrow $(0.324 0\pm0.024 5)$, blood $(0.015 3\pm0.012 8)$, nasal lavage fluid $(0.00\pm0.00)$ which were significantly lower than the MBP mRNA relative gray value of the PBS group and the siRNA group ($P<0.05$).
The EPO mRNA relative gray value of each sample in the CCR3 siRNA treatment group were as follow: bone marrow (0.493±0.0115), blood (0.140±0.0260), nasal lavage fluid (0.00±0.00) which were significantly lower than the ECP mRNA relative gray value of the PBS–treated group and the siRNA group (P<0.05).

![Figure 5. EPO mRNA expression of bone marrow, blood, nasal lavage fluid of mice in each group.](image)

The EPO mRNA relative gray value of each sample in the CCR3 siRNA treatment group were as follow: bone marrow (0.159±0.0599), blood (0.084±0.1108), nasal lavage fluid (0.00±0.00) which were significantly lower than the EPO mRNA relative gray–value of the PBS–treated group and the siRNA group (P<0.05).

### Table 1

CCR3, MBP, ECP, EPO mRNA relative gray values of the bone marrow, the blood and the nasal lavage fluid in three groups (x±sd).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bone marrow</th>
<th>Blood</th>
<th>Nasal lavage fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control group</td>
<td>1.115±0.0480</td>
<td>0.670±0.0265</td>
<td>1.048±0.0132</td>
</tr>
<tr>
<td>CCR3</td>
<td>0.852±0.0650</td>
<td>0.783±0.0712</td>
<td>1.033±0.0469</td>
</tr>
<tr>
<td>MBP</td>
<td>0.977±0.0379</td>
<td>0.700±0.0416</td>
<td>0.990±0.0553</td>
</tr>
<tr>
<td>ECP</td>
<td>0.485±0.0546</td>
<td>0.697±0.1334</td>
<td>1.050±0.0410</td>
</tr>
<tr>
<td>EPO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>siRNA-treated</td>
<td>1.122±0.0461</td>
<td>0.657±0.0147</td>
<td>1.028±0.0260</td>
</tr>
<tr>
<td>control group</td>
<td>0.914±0.0362</td>
<td>0.697±0.0681</td>
<td>1.086±0.0531</td>
</tr>
<tr>
<td>CCR3</td>
<td>0.924±0.0467</td>
<td>0.745±0.2971</td>
<td>1.057±0.0647</td>
</tr>
<tr>
<td>MBP</td>
<td>0.561±0.0746</td>
<td>0.778±0.0171</td>
<td>0.976±0.0847</td>
</tr>
<tr>
<td>ECP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR3 siRNA</td>
<td>0.340±0.0225</td>
<td>0.253±0.0616</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>treatment group</td>
<td>0.324±0.0245</td>
<td>0.015±0.0128</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>CCR3</td>
<td>0.493±0.0115</td>
<td>0.140±0.0260</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>MBP</td>
<td>0.159±0.0599</td>
<td>0.084±0.1108</td>
<td>0.000±0.000</td>
</tr>
</tbody>
</table>

Note: Compared with the PBS–treated control group and the siRNA treatment group, P<0.05 (t test), the difference was not statistically significant. Compared PBS–treated control group with the siRNA–treated control group, P>0.05(t test), the difference was not statistically significant.

### 4. Discussion

Allergic rhinitis belongs to type 1 allergic reaction, which means after atopic individuals exposed to the allergens, the IgE–mediated media (mainly histamine) released and a variety of immune cells and cytokines involved in the nasal mucosa then caused this non–infectious inflammatory diseases. A large number of EOS infiltration in tissues is an important feature of allergic diseases, and EOS play a role of antigen–presenting cells in allergic airway disease[1,2]. EOS can release cytotoxic granule proteins, reactive oxygen species and lipid mediators, which can cause tissue damage, vascular leakage, mucus secretion and airway contraction[13]. It has been reported[3-5] that the pathogenesis of allergic diseases may be as follows: in the process of allergen challenge, the bone marrow can generate specific inflammatory cells (EOS progenitor cells), which can reach to the local tissue by peripheral blood circulation and then led to the local inflammation.

CCR3 is a G protein coupled receptor generated by transmembrane, primarily expressed in the EOS. Eosinophil activation chemokine (Eotaxin) belong to class CC chemokines, which combined with the CCR3 and induce eosinophil migrate to specific tissues, causing local tissue inflammation. Eotaxins can also play an important role in situ hematopoiesis of the EOS in sites of inflammation by CCR3 receptors[4-9]. RNAi is a mechanism which can make target gene silencing after specific transcription, dsRNA can cause the degradation of the homologous mRNA and lead to the undetectable expression of target gene. It can blocking the specific gene expression efficiently. Therefore, the use of RNAi technology for screening genome have been studied extensively. That is, design corresponding dsRNA for the different part of the genome, construct dsRNA libraries, introduced into different individual cells, the protein expression can be changed to filter the gene and determine gene function[11-15]. Therefore, our experiments adopted the external synthesized interference RNA, specifically inhibit the eosinophil CCR3 expression, observed the EOS CR3 and eosinophil granule proteins–MBP, ECP, EPO mRNA changes in mice bone marrow, peripheral blood and nasal lavage fluid EOS.

In our previous work, we first establish the animals model, calculated by behavioral scoring system≥5 is a successful animal model, we found that the average score of group 2 and group 3 were all≥5. The normal group and CCR3 siRNA group only with mild scratching nose and less sneezing, score≤5. HE staining showed that compared with group 1, group 2 and group 3 were with significantly mucosal epithelium, goblet cell hyperplasia and EOS and other inflammatory cells, mucous layer thickening, dilatation and congestion of the lamina propria, tissue edema, gland hyperplasia and great hypertrophy secretion. That means
the allergic rhinitis animal model can be successfully established by this method, and the application of CCR3 siRNA treatment can effectively control the occurrence of allergic rhinitis.

Our previous studies has applied lentiviral vectors to constructed simultaneously for four fragments (mCCR3-1, mCCR3-2, mCCR3-3 and mCCR3-4) CCR3 siRNA expression shuttle plasmid (pLVX–ShRNA2–mCCR3–1 +2 +3 +4) successfully, and have a higher silencing effect which identified by QPCR on EOS CCR3 gene[16]. Detected by RT–PCR it was found that EOS CCR3 mRNA granule protein mRNA expression were high in the bone marrow, peripheral blood and nasal lavage fluid of the allergic rhinitis mice, which indicating that the EOS involved in the localization and activation of the tissue during the disease pathogenesis. In the experimental group, after treatmen of topical nasal pLVX–ShRNA2–mCCR3–1 +2 +3 +4, the test results show the corresponding EOS CCR3 mRNA and granule protein mRNA (MBP, ECP, EPO) expression was decreased, and reducing the changes of nasal cavity mucosa pathological, which indicate that the application–specific CCR3 siRNA can inhibit CCR3 expression and also decreased the eosinophil granule protein mRNA expression. This shows that the CCR3 may be involved in the whole process of the development, migration and invasion of EOS, which can not only block the CCR3 receptor expression, but also block its maturation, activation and chemotaxis, and blocking the recruitment of hematopoietic progenitor cells, mature eosinophil to sites of inflammation, improve the inflammatory response of allergic rhinitis[6,17,18].

In this study, we investigated the use of RNAi technology to treat allergic rhinitis of mice model, we found that inhibit the expression of CCR3 mRNA can reduce the eosinophil granule protein mRNA expression in the the bone marrow, peripheral blood and nasal lavage fluid, improved pathological changes in the nasal mucosa of mice, which indicated that EOS is suppressed in the development, migration and invasion, so as to relieve nasal allergy of the allergic rhinitis. Thus, based on the efficient and specific sequence interference for the target gene CCR3, we can provide some ideas for future exploration of the targeting siRNA gene therapy for allergic rhinitis.

Conflict of interest statement
We declare that we have no conflict of interest.

References


