Expression and Regulation of Interleukin-37 in Pathogenesis of Nasal Polyps

Jie Cheng · Hong Ouyang · Jingdong Du

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Abstract Interleukin-37 (IL-37) belongs to IL-1 family and is recently identified as a natural suppressor of innate inflammatory and immune responses. Its role in digestive system was well characterized, however, little is known about its function in respiratory diseases. This study is aimed to investigate the expression and regulation of IL-37 in patients with nasal polyps (NPs). Twenty-five patients with NPs and sixteen normal controls were included, and IL-37 production was determined by immunohistochemistry and enzyme-linked immuno sorbent assay, respectively. The relationship between IL-37 expression and Th1/Th2 cytokines was also evaluated. Besides, the effect of IL-37 on dispersed nasal polyp cells (DNPCs) was investigated. We observed significantly decreased IL-37 mRNA and protein levels expression in NPs compared with normal control. IL-37 was found negatively with Th2 cytokines and had no relation with Th1 cytokines. Furthermore, we provided the first evidence that IL-37 down-regulates Th2 cytokine expressed by DNPCs. Our results demonstrate that enhanced Th2 cytokine levels was related to decreased IL-37 expression in NPs, and provide a possible explanation for IL-37's regulatory role in the pathogenesis of NPs.

Keywords Nasal polyps · IL-37 · Th2 cytokine levels

Introduction

Chronic rhinosinusitis (CRS) is a widespread disease, but its pathogenesis is not fully understood. The most severe form of CRS is nasal polyps (NPs). Histomorphological of NPs include mucosal epithelial hyperplasia, infiltration of inflammatory cells, neo-vascularization, and remarkable edema [1–3]. Activation of Th2 cells and released cytokines play important roles in accumulation of inflammatory cells, such as B cells, plasma cells, eosinophils, macrophages, and neutrophils, which contribute to the development of NP [4–6].

IL-37 is a newly described anti-inflammatory cytokine and can be induced by Toll-like receptor (TLR) agonists in monocytes and binds to IL-18-receptor (R) and IL-18-binding protein (BP) [7, 8]. Endogenous IL-37 exerts anti-inflammatory effects by suppressing innate immune responses through decreasing the production of inflammatory cytokines induced by TLR agonists, as well as that induced by IL-1 and tumor necrosis factor (TNF) [9, 10]. IL-37 has been found in many human inflammatory and autoimmune disease states. For example, the IL-37 protein was detected in synovial cells of patients with rheumatoid arthritis, in alveolar macrophages from patients with *Mycobacterium avium* infections, in the foam-like cells of atherosclerotic coronary and carotid artery plaques, in psoriatic plaques, and in the lamina propria macrophages of patients with Crohn's disease [9, 11]. However, the role of IL-37 in respiratory disease is not characterized.

Thus, the present study was aimed to investigate the expression of IL-37 in patients with NP and its regulatory role in Th2 inflammation.

J. Cheng · H. Ouyang (✉) · J. Du
Department of Otolaryngology, RenHe Hospital, China Three Gorges University, No. 410, Yiling Road, Yichang 443001, Hubei, China
e-mail: ouyang@163.com

J. Cheng
e-mail: lw120103@sohu.com

Methods

Patients

Twenty-five patients with NPs were enrolled in the study. The diagnosis was established on history, clinical examination, nasal endoscopy, and computed tomography (CT) scan of the sinuses, which accorded with the standard criterion by E3POS guideline [12]. Sixteen patients undergoing septoplasty due to anatomical variations were considered as controls and the tissues from the inferior turbinate during septal surgery were sampled. None of the subjects used oral or nasal corticosteroids 4 weeks before surgery. All subjects were free from upper respiratory tract infection for 4 weeks preceding the study. Details of all subjects are summarized in Table 1. This study was approved by the local Ethical Committee and informed consent was obtained from each subjects.

Each specimen was cut into two portions. One portion was immediately frozen in liquid nitrogen and stored at -80°C for mRNA and protein analysis. The other portion was fixed with 4 % paraformaldehyde and embedded in paraffin for immunohistochemistry (IHC) staining. In addition, fresh polyp tissues were further used for isolation and culture of dispersed nasal polyp cells (DNPCs).

Immunohistochemical Staining

For IHC, the slides were placed in 0.3 % H_2O_2 for 20 min at room temperature to reduce nonspecific background staining caused by endogenous peroxidase. After washing with PBS again, the slides were boiled in 10 mM citrate buffer for 15 min followed by cooling at room temperature. Mouse antihuman polyclonal antibody for IL-37 (LifeSpan Biosciences, Wash, USA) and control antibody (Dako, Denmark) were incubated overnight at 4°C for immunohistochemical staining, respectively. The next day, the slides were washed with PBS and incubated with secondary antibody (Gene Tech, Shanghai, China) at room temperature for 1 h.

After washing, DAB (Gene Tech, Shanghai, China) staining was performed under microscope. After rinsing with distilled water, the sections were counterstained with

Mayer's hematoxylin (Zhongshan Golden bridge, Beijing, China) for a further 25 s, dehydrated with series ethanol (90, 100, 100 %), cleared with xylene (3 times), and mounted with neutral balsam (Zhongshan Golden bridge, Beijing, China). Control for nonspecific staining was routinely performed with PBS instead of primary antibodies and all proved negative.

The sections were blindly examined and coded with no awareness of the clinical data. They were visualized with an Olympus CX40Microscope (Olympus Europa GmbH, Germany). In high-power fields ($400\times$), the count of IL-37 stained sections was performed. Ten fields were counted in each specimen and the median was calculated for each antibody.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-37 Protein and Th1/Th2 Cytokine Expression

100 mg of freshly obtained tissue specimen was weighed, and 1 mL protease inhibitor cocktail (Keygentec, Nanjing, China) was added per every 100 mg tissue. The tissue was then homogenized with a homogenizer (Kinematica, Switzerland) for 1 min on ice. After homogenization, the suspension was centrifuged at 4,000 rpm for 20 min at 4°C , and the supernatants were stored at -80°C until analyzed.

Enzyme-linked immunosorbent assay (ELISA) kits were used for measuring tissue IL-37, IL-12, IFN- γ , IL-4, IL-13, IL-5, IL-10 levels (R&D systems, USA) according to the manufacturer's protocols. Total serum IgE level was measured using a Roche COBAS-CORE II (Roche Diagnostics, Basal, Switzerland). The detection limits of the assays were as follows: IL-37, 125 pg/mL, IL-12, 1.56 pg/mL, IFN- γ , 8 pg/mL, IL-4, 1.56 pg/mL, IL-13, 93.8 pg/mL, IL-5, 7.8 pg/mL, IL-10, 3.9 pg/mL.

Real-Time PCR Analysis

Real-time PCR was performed as we described previously [15]. Total RNA was extracted from SIP or mucosa tissues using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription (RT) was performed, and cDNA was synthesized from 2 μg of total RNA using an oligo (dT) 18 primer and M-MLV reverse transcriptase (TAKARA, Syuzou, Shiga, Japan). The mRNA expression was determined using an ABI PRISM7300 Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix TaqTM (TAKARA). The sequences of the primers were as follows: IL-37, sense 5'-CTCCTGGGGGTCTCTA AAGG-3', anti-sense 5'-TACAATTGCAGGAGGTGCAG-3'; β -actin forward: 5'-AAGATG ACC CAG ATC ATG TTT GAG ACC-3'; β -actin reverse: 5'-AGC CAG GTC

Table 1 Demographic characteristic of NPs patients and normal controls

Groups	NPs	Controls
Number	25	16
Sex (male:female)	13:12	8:8
Age (years)	32.6	33.4
Skin prick test-positive	6	0

CAG ACG CAG GAT-3'. PRISM samples contained $1 \times$ SYBR Green Master Mix, $1.5 \mu\text{L}$ of $5 \mu\text{M}$ primers, and 25 ng of synthesized cDNA in a $25\text{-}\mu\text{L}$ volume. Reactions were heated to 95°C for 10 min , followed by 40 cycles of denaturation at 95°C for 10 s , and annealing extension at 60°C for 60 s . All PCR reactions were performed in duplicate. Melting curve analysis was used to control for amplification specificity. The mean value of the replicates for each sample was calculated and expressed as a cycle threshold (Ct) value. The relative expression of each target gene was determined as the difference (ΔCt) between the Ct value of the target gene and the Ct value of $\beta\text{-actin}$. Fold changes in the target gene mRNA were determined as $2^{-\Delta\Delta\text{Ct}}$.

DNPCs Preparation

dispersed nasal polyp cells were prepared from fresh NPs by means of enzymatic digestion, as previously described [16]. Briefly, the minced NPs were incubated for 2 h at 37°C in RPMI 1,640 containing 2.0 mg/mL protease, 1.5 mg/mL collagenase, 0.75 mg/mL hyaluronidase, and 0.05 mg/mL DNase. The cell suspension was then filtered through a 70-mm cell strainer (BD Falcon, Bedford, Mass) to remove any undigested tissue and washed 2 times with washing medium (RPMI 1,640 supplemented with 2% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin). The cell pellet was resuspended in erythrocyte lysis buffer and washed with washing medium. After washing, DNPCs were suspended in culture medium (RPMI 1,640 supplemented with 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin). Viability was assessed through the exclusion of trypan blue stain. Different concentrations of recombinant IL-37 (R&D systems, $1\text{--}200 \text{ ng/mL}$) were incubated with DNPCs ($500 \mu\text{L}$, $10^5/\text{mL}$) treated with or without Poly I:C (500 ng/mL , Sigma, USA) in 48-well plate. PBS was used as negative control. At different time points of incubation, the superants were collected and analyzed.

Statistical Analysis

All data were expressed as mean $\pm$ SD except additional note. Statistical significance between different groups was determined using nonparametric Mann–Whitney U test. The Spearman rank correlation test was used to analyze the correlation among the expression of biomarkers and clinical stage. $P < 0.05$ was considered as significant difference.

Results

Decreased IL-37 Expression in NP by IHC

In normal tissues, most epithelial cells, interstitial cells and glandular cells were positive for IL-37 staining, while IL-37 immunoactivity in NPs decreased significantly (Fig. 1a, b). The number of IL-37 positive cells per high power field (HPF, $200\times$) in NPs was significantly lower than that of normal controls (Fig. 1c).

Decreased IL-37 mRNA and Protein Levels as well as Th2 Cytokines Protein in NP

The IL-37 mRNA and protein expression in NPs were significantly lower than those in the normal controls, especially in those patients with asthma ($P = 0.001$, Fig. 2a, b).

IL-4, IL-5, IL-13 protein level also increased in NPs compared with normal controls and their protein levels were negatively correlated with IL-37, respectively ($P < 0.001$, Fig. 3a–f). IL-12, IFN- γ and IL-10 decreased in NPs compared with normal controls (Fig. 3g, h) and their protein levels were not correlated with IL-37 (data not shown).

IL-37 Regulated Th2 Cytokines Expression in DNPCs

Our results showed that rhIL-37 did not affect Th2 cytokine levels in DNPCs. However, after stimulated with Poly I:C, IL-37 can down-regulate Th2 cytokine level in DNPCs

Fig. 1 IHC showed IL-37 expression in NPs and control

Fig. 2 Decreased IL-37 mRNA and Protein Levels as well as Th2 Cytokines Protein in NP

Fig. 3 IL-4, IL-5, IL-13 protein level were negatively correlated with IL-37, respectively ($P < 0.001$, a–f)

Fig. 4 IL-37 can down-regulate Th2 cytokine level in DNPCs and did not regulate IL-10 expression

(Fig. 4a–c). Furthermore, our results showed that IL-37 did not regulate IL-10 expression, which is also an anti-inflammatory cytokine (Fig. 4d).

Discussion

Chronic rhinosinusitis with NPs is a common disease in clinical which characterized by persistent inflammation of the nasal and paranasal mucosa, typically showing a Th2 skewed eosinophilic inflammation with high levels of IL-5 and IgE [12, 13]. Most studies about its pathogenesis focused on inflammatory cytokines rather than anti-inflammatory cytokines. In NPs, only anti-inflammatory cytokine IL-10 was investigated and its protective role was clarified.

IL-37 (IL-1F7) was firstly described in 2000 and is one of the most recently characterized members of IL-1 family [14]. IL-37 expression was found in the cytoplasm of plasma cells in epithelial crypts and germinal centers of tonsils, lamina propria of normal colon, stroma of colon carcinomas, ductal mammary carcinoma, normal thalamus,

and placenta. It was also found in blood monocytes, fully differentiated keratinocytes in stratum granulosum of skin, PBMC and dendritic cell [15–18]. In general, IL-37 has been shown to have potent anti-inflammatory properties and many studies have elucidated its precise role in chronic intestinal inflammation. However, its effect in upper airway disease was not reported so far.

Our present study provides the first evidence that IL-37 expression was closely related to Th2 inflammation in NPs. Decreased IL-37 mRNA and protein levels were found in NPs tissues compared with controls, suggesting its protective role in occurrence of NPs. In normal tissues, we found IL-37 was expressed by most epithelial cells and some interstitial cells and glandular cells. Besides, IL-4, IL-5, IL-13 protein level also increased in NPs compared with normal controls and their protein levels were positively correlated with IL-37. On the contrary, our data showed that Th1 cytokines such as IL-12 and IFN- γ were not correlated with IL-37. Thus, we postulated a possible negative regulation of IL-37 on Th2 cytokines expression. Next, we established DNPCs model to simulate the environment of NP. Interestingly, our results showed that IL-37

alone did not affect Th2 cytokine levels in DNPCs. After stimulated with Poly I:C, a simulator of viral infection, IL-37 can down-regulate Th2 cytokine level in DNPCs. We also found that IL-37 did not affect IL-10 expression. These results suggested that IL-37 regulation of Th2 cytokines needs pathologic stimulator and its process was IL-10 independent, which was consistent with McNamee's study [11]. Inconsistent with our results, studies of IL-37 in autoimmune disease such as SLE or inflammatory bowel disease showed high expression of IL-37, which may be attributed to that IL-37 play different role in different disease backgrounds.

In summary, our data demonstrated that IL-37 can suppress Th2 cytokine production by DNPCs when combined with poly I:C. Besides, IL-37 is an independent regulator in NPs. Our study suggested that IL-37 can be used as a potential treatment method for NPs in future.

Conflict of interest The authors declare that they have no conflict of interest.

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