

Expressions of nuclear factor-kappa B p50 and p65 and their significance in the up-regulation of intercellular cell adhesion molecule-1 mRNA in the nasal mucosa of allergic rhinitis patients

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Abstract The purpose of the study was to investigate expressions of nuclear factor-kappa B (NF- κ B) and intercellular cell adhesion molecule-1 mRNA (ICAM-1 mRNA) in the nasal mucosa of allergic rhinitis (AR) patients. Expressions of NF- κ B and ICAM-1 mRNA were studied using immunohistochemistry and reverse transcription-PCR (RT-PCR) in AR tissues and corresponding normal nasal mucosa. The correlation between NF- κ B and ICAM-1 mRNA was studied using linear correlation analysis. The results of immunohistochemistry showed that expression of NF- κ B was significantly up-regulated in the nasal mucosa of AR compared with that in normal tissue ($P < 0.01$), over-expression of NF- κ B p50 was found in the cytoplasm and nucleus ($P < 0.01$), and NF- κ B p65 was mainly expressed in the cytoplasm ($P < 0.01$). ICAM-1 mRNA was strongly expressed in the nasal mucosa of AR compared with that in normal tissue as shown by RT-PCR ($P < 0.01$). Up-regulation of ICAM-1 mRNA was significantly correlated with

over-expressions of NF- κ B p50 and NF- κ B p65 ($r = 0.8995$, $P < 0.01$; $r = 0.7601$, $P < 0.01$). In conclusion, NF- κ B plays a key role in AR. Excessively activated NF- κ B promotes the transcription of ICAM-1 mRNA. ICAM-1 is related to the pathogenesis and development of AR.

Keywords Allergic rhinitis · Nuclear factor-kappa B · Intercellular adhesion molecule-1

Introduction

The incidence of AR which has had an upward trend in recent years, seriously impacts the health and quality of life of patients. Genetic research on AR has primarily focused on a number of susceptibility genes that could contribute to the allergic process, multiple genes and their associated transcription factors involved in the process [1]. NF- κ B is a multi-cellular nuclear transcription factor, and it plays a central role by regulating the amplified cascade effect of immune and inflammatory cytokines and inflammatory mediators [2]. ICAM-1 plays an important role in local inflammation, and gene expression is regulated by NF- κ B [3]. This study attempted to detect expression and activation of NF- κ B in AR nasal mucosa from the perspective of molecular biology, and it promoted expression of ICAM-1 mRNA, in order to further investigate the pathogenesis of allergic rhinitis.

Methods

Source of specimens

All tissues were obtained from patients who saw a doctor in the Second Hospital of Shandong University from April

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2008 to November 2010. All patients were consistent with the diagnostic criteria of allergic rhinitis while deflection of nasal septum patients was used as the control group. Written informed consents were obtained from the patients regarding the surgical operation and the use of resected specimens for research. The specimens, whose size was 0.3×0.3 cm, were collected from inside the middle turbinate mucosa. A specimen was taken from each side of the nasal cavity. One was fixed for 24 h in 4 % paraformaldehyde solution, placed in a gradient alcohol dehydration, soaked in wax, and paraffin blocks were made. The other was placed in an Eppendorf tube and stored at -80°C in an ultra-low temperature refrigerator.

Nasal mucosa NF- κ B immunohistochemical analysis

Formalin-fixed and paraffin-embedded nasal mucosa specimens were cut into 4- μm slices. Experiments were performed following the manufacturer's instructions. The four-micron slices were de-waxed in xylene, cultured in 3 % hydrogen peroxide to eliminate intrinsic peroxidase and repaired antigen with low-medium heat in a microwave oven for about 15 min, and then incubated overnight at 4°C with rabbit anti-human p50 polyclonal antibody/mouse anti-human p65 monoclonal antibody (Santa Cruz Biotechnology, Inc.) against purified recombinant NF- κ B. Then, sheep anti-rabbit/mouse immunoglobulin and streptavidin conjugated to horseradish peroxides were added. Finally, 3,3-diaminobenzidine was used for color development and hematoxylin was used for counter staining. Each slice was randomly taken 5 photos at 400 X magnification under the microscope, the images were inputted into Imagepro Plus 5.0.2 professional image analysis software, the average optical density (OD) value of positive cells in the nasal tissue was measured, and the data were expressed as $X \pm S$.

Extraction of nasal mucosa RNA and detection of reverse transcription-PCR

Total RNA was extracted from the stored frozen tissues of the nasal mucosa using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quantity and purity of total RNA were measured using a Nanodrop reader. 1 μg of total RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Invitrogen) and RNasin (Takara, Otsu, Shiga, Japan). The cDNA sequence was then amplified using gene-specific primers and Go taq Polymerase (Promega, Madison, WI, USA). The PCR primer sequences were as follows: ICAM-1, sense 5'-CACAAGCCACGCCCTCCCTGAACCT-3' and reverse 5'-TGTGGGCCTTTGTGTTTTGATGCTA-3'; β -actin, sense 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse 5'-CTCCTTAATGTCACGCACGATTTC-3'. PCR

conditions were 94°C for 1 min, annealing temperature was 58°C for 1 min, and extension temperature was 72°C for 1 min. After 35 cycles, the eventual extension temperature was 72°C for 7 min. PCR products were verified by electrophoresis of the PCR products on a 2 % agarose gel with ethidium bromide staining. The band intensity of ethidium bromide fluorescence was measured using NIH/1D Image Analysis Software Version 1.61 (National Institutes of Health, Bethesda, MD, USA). The relative intensity (RI) of each band was determined with the use of the ratio to β -actin.

Statistical analysis

NF- κ B and ICAM-1 mRNA levels between AR and deflection of nasal septum groups were compared by independent-samples t test using statistic analysis software SPSS (version 14). The correlations between the OD values of NF- κ Bp50/p65 and expression intensity of ICAM-1 mRNA were analyzed by linear correlation analysis. Statistical significance was considered at the value of $P < 0.05$.

Results

Pathological changes of AR in nasal mucosa

In HE sections, the mucosa had edema, the ciliated columnar epithelial goblet cells had hyperplasia, and a large number of eosinophilic granulocytes were infiltrated and exuded in the mucosa and mucosal surfaces. The interstitial space had edema, and a certain number of lymphocytes, and plasma cells and mast cells were infiltrated. Mucus secretion was increased in glands, and basal layers were thickened (Fig. 1).

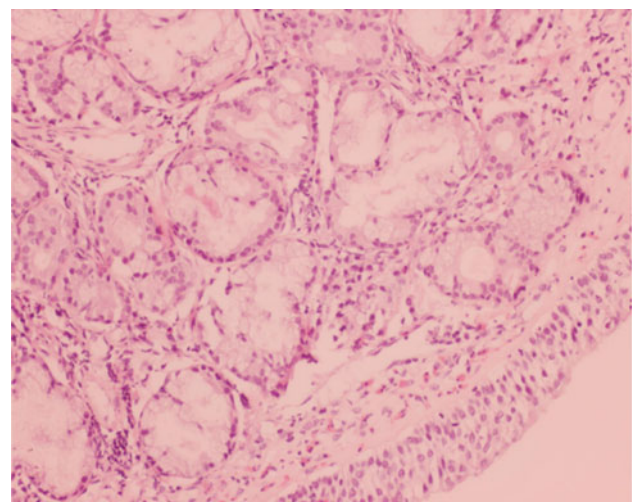


Fig. 1 AR middle turbinate mucosa with HE staining, $\times 400$

NF- κ B expression in nasal mucosa

NF- κ B immunohistochemistry showed strong immunoreactivity in the cytoplasm and nucleus of pseudostratified ciliated columnar epithelium and glandular epithelium in the AR nasal mucosa groups (Figs. 2, 3). Among the NF- κ B, there were a large proportion of p50 positive nuclei, and a relatively small proportion of p65 positive nuclei. There was positive staining in the nuclei of stromal vascular endothelial cells and infiltrative inflammatory cells. However in the deflection of the nasal septum groups, the majority of p50 and p65 were expressed in the

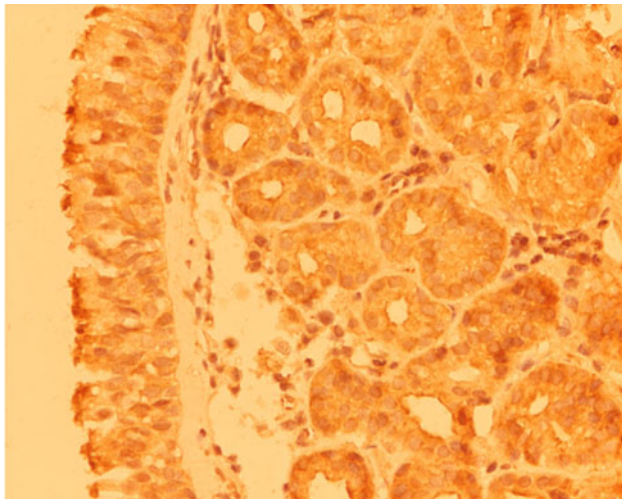


Fig. 2 Expression of p50 in AR nasal mucosa ciliated pseudostratified epithelium, serous cells and interstitial vascular endothelial cell, inflammatory cell nuclei by immunohistochemical staining, $\times 400$

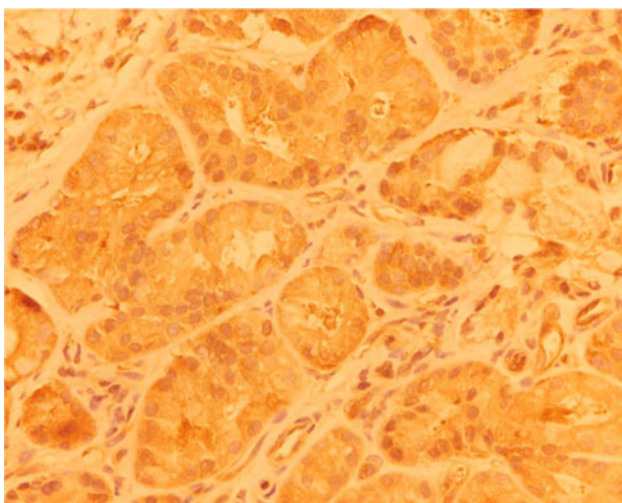


Fig. 3 Expression of p50 in AR nasal mucosa ciliated pseudostratified epithelium, serous cells and interstitial vascular endothelial cell, inflammatory cell nuclei by immunohistochemical staining, $\times 400$

cytoplasm of the middle turbinate epithelium mucosal cells, and occasionally in the nucleus (Fig. 4). There was no obvious staining in the negative control groups. Image analysis results are shown in Table 1.

Expression of ICAM-1 mRNA in nasal mucosa

Expression levels of ICAM-1 mRNA in AR nasal mucosa groups and deflection of nasal septum groups were examined by quantitative reverse transcriptase-PCR. There was a specific ICAM-1 gene band at 458 bp in AR groups, while brightness and sharpness of the band were significantly higher than the control groups (Fig. 5). The internal standard β -actin gene band was at 539 bp, and gene expression in AR groups and control groups had no significant difference (Fig. 6). Optical density scanning semi-quantitative analysis was used for expression of the relative strength coefficients (Table 2). ICAM-1 mRNA expression level in AR nasal mucosa groups was significantly higher than that of control groups ($P < 0.01$).

The correlation between NF- κ B p50, NF- κ B p65 and ICAM-1 mRNA in AR nasal mucosa

ICAM-1 mRNA up-regulation was correlated with over-expression of NF- κ B p50 and NF- κ B p65. Among them, NF- κ B p50 and the corresponding ICAM-1 mRNA expression had a significant positive correlation ($r = 0.8995$, $P < 0.01$), and NF- κ B p65 and the corresponding ICAM-1 mRNA expression had a significant positive correlation ($r = 0.7601$, $P < 0.01$) (Figs. 7, 8).

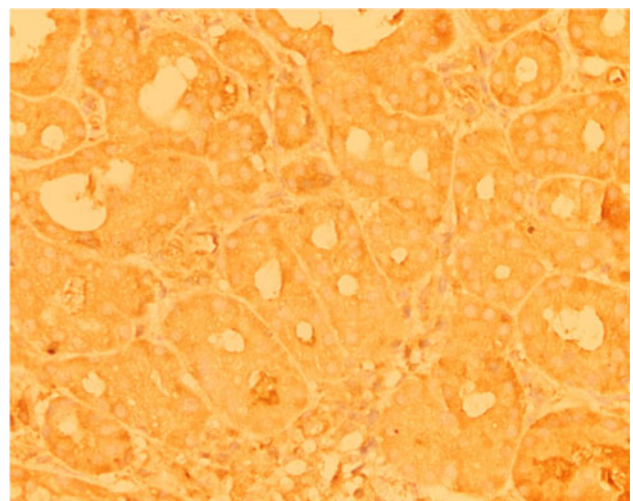
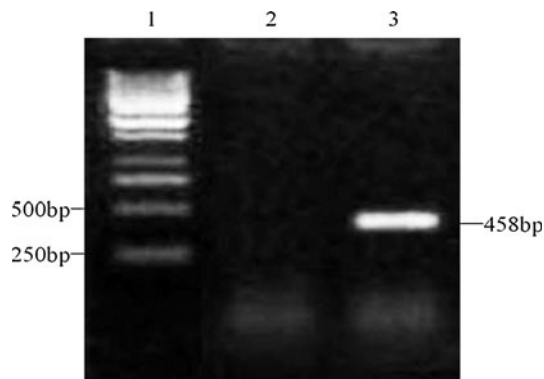
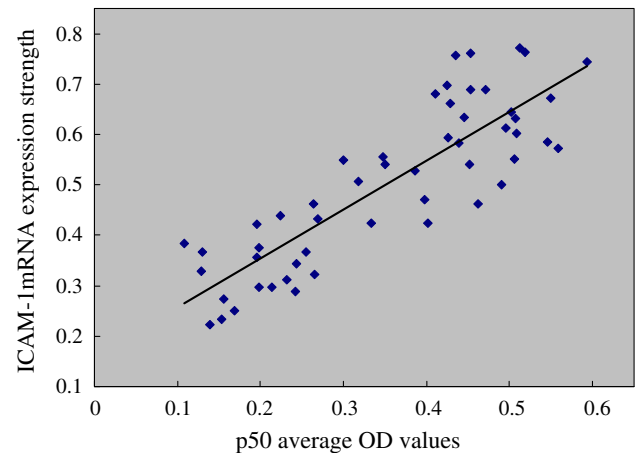
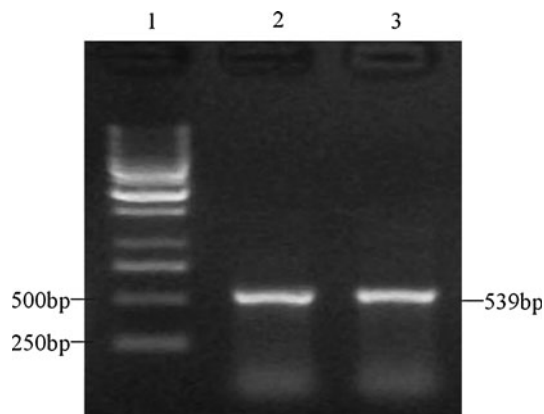
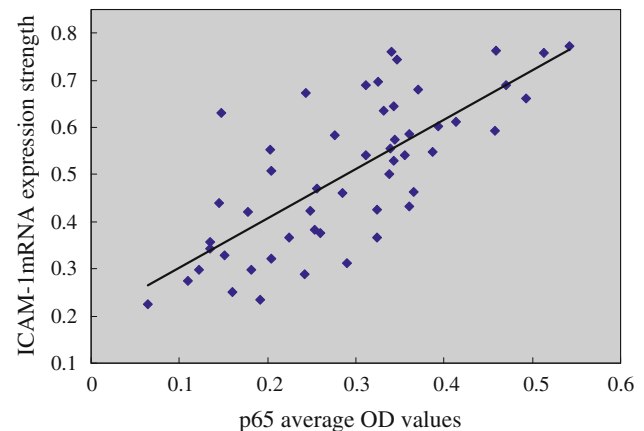


Fig. 4 Expression of p50 and p65 in the nasal mucosa cells of simple nasal septum deviation by immunohistochemical staining, $\times 400$

Table 1 Image analysis results of middle turbinate mucosa p50 and p65 expression in two groups ($X \pm S$)

Groups	p50		p65	
	Average OD values	Positive nuclei (%)	Average OD values	Positive nuclei (%)
AR	$0.6484 \pm 0.0809^*$	$53.4056 \pm 2.4523^{**}$	$0.3569 \pm 0.0744^*$	$22.5329 \pm 2.9921^{**}$
Control	0.3240 ± 0.0577	12.2011 ± 2.6002	0.1977 ± 0.0712	12.3568 ± 1.1002

* $P < 0.01$, ** $P < 0.01$, versus simple nasal septum deviation group

**Fig. 5** Gel electrophoresis of ICAM-1 mRNA in middle turbinate mucosa detected by RT-PCR 1 mark group 2 control group 3 experimental group**Fig. 7** p50 and ICAM-1 mRNA expression tendency chart**Fig. 6** Gel electrophoresis of internal standard β -actin mRNA detected by RT-PCR 1 mark group 2 control group 3 experimental group**Fig. 8** p65 and ICAM-1 mRNA expression tendency chart**Table 2** ICAM-1 mRNA expression strength in two groups ($X \pm S$)

Groups	ICAM-1 mRNA expression strength
AR	$0.7569 \pm 0.0647^*$
Control	0.1982 ± 0.0705

* $P < 0.01$, versus simple nasal septum deviation group

Discussion

Allergic rhinitis is a chronic inflammatory disease of the nasal mucosa with IgE-mediated mediator release and a

variety of immuno-competent cells and cytokines involved after atopic individuals are in contact with allergens. The pathogenesis of allergic rhinitis involves a complex network structure of a variety of inflammatory cell interactions. Recent studies have shown that the activation of NF- κ B might be a central part of this complex network.

NF- κ B is composed of two Rel family protein forms of homo- or heterodimers, and among them the p50-p65 heterodimer is the most common, which is the main form of its activity. NF- κ B exists in many types of cells, and regulates target genes including immune-related receptors, cytokines, inflammatory factors, adhesion molecules, and

acute phase reactive protein [4]. NF- κ B is a multidirectional nuclear transcription factor, both constitutive and inducible. As a constitutive nuclear transcription factor, NF- κ B in low level sustained activity in the tissue cells plays an important role in the growth and development of immune cells and their normal immune function. As an inducible nuclear transcription factor, the activity of NF- κ B abnormally rises and leads to excessive release of large number of pro-inflammatory cytokines because of the failure to be timely cleared, and further expands and enhances stimulation, resulting in inflammation [5].

Our study showed that NF- κ B was expressed in the mucous membrane of the nose in both the allergic rhinitis and the simple nasal septum deviation groups, which indicated that NF- κ B played a role in transcriptional regulation of these parts. NF- κ B was weakly expressed in the deflection of nasal septum groups and slightly expressed in the cytoplasm, which indicated that under normal circumstances NF- κ B was constantly activated and deactivated. Compared with control groups, NF- κ B was positively and strongly positively expressed in allergic rhinitis groups, which indicated that NF- κ B was being continuously activated and over-expressed in the nasal mucosa of allergic rhinitis, which is the basis of the cytokine network imbalance and the related inflammatory factors and adhesion molecule expression disorder in allergic rhinitis. NF- κ B activity of bronchial epithelial cells, sputum eosinophils and macrophages increased in asthma patients, showing that NF- κ B is involved in the occurrence of asthma [6]. Allergic rhinitis and bronchial asthma are not only closely linked in epidemiology, pathophysiology and immunopathology, but have common methods of treatment, showing that the upper and lower respiratory tract inflammatory response is consistent [7]. NF- κ B over-activity might also be the basis of many high inflammatory gene expressions and nasal mucosa hyperergy in allergic rhinitis.

The important step in the formation of airway allergy inflammation is that leukocytes adhere to the vascular wall, go through the vessel wall into the airway, then chemotaxis occurs and finally infiltrate into inflammation areas. With ICAM-1 and its ligand interaction, inflammatory cells adhere to vascular endothelial cells and transmigrate to the bronchi of asthma patients, ICAM-1 plays a decisive role in this airway inflammation and hyper-responsive process [8]. Soluble ICAM-1 levels in nasal secretions are significantly increased in perennial allergic rhinitis patients from the period of onset. The levels of soluble ICAM-1 in the sera of seasonal allergic rhinitis patients are highly increased. In the nasal mucosa and conjunctiva of patients sensitized by mites, leukocytes infiltrate and ICAM-1 is expressed [9–11]. In our study, RT-PCR results also showed that in the allergic rhinitis group the ICAM-1 electrophoretic band brightness was significantly increased and the band was

clear compared with the control group, but the band expression was weak or there was no expression in normal tissues, which showed that expression of ICAM-1 mRNA is unusually increased in the nasal mucosa of allergic rhinitis, and ICAM-1 plays an important role in the pathogenesis of allergic rhinitis. ICAM-1 leads to local inflammatory cells especially eosinophils aggregation and activation, releases inflammatory mediators, causes nasal tissue damage, and thus brings about a series of clinical symptoms of allergic rhinitis.

The ICAM-1 gene promoter contains binding sites with NF- κ B. Activated NF- κ B goes into the nucleus and binds the sites, leading to the transcription increase of ICAM-1 mRNA, and accordingly promotes expression of ICAM-1. But in turn, ICAM-1 further activates NF- κ B, thus forming a positive feedback regulation of continued inflammation and enlargement, in which, NF- κ B plays a key role [12]. Activation of NF- κ B in lung tissue starts an intra-cytoplasm free NF- κ B rapid shift to the nucleus, binds the adhesion molecule gene promoter or enhancer κ B sequence, and up-regulates transcription and protein synthesis of ICAM-1 mRNA [13].

Conclusion

Our study showed that expression of NF- κ B p50 and p65 corresponding to expression of ICAM-1 mRNA had a significant positive correlation, also showed that the NF- κ B played an important role for ICAM-1 transcription, thus indicating that NF- κ B played a significant role in allergic rhinitis generation and development.

Conflict of interest The authors declared that they have no conflicts of interest.

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