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Main Article

A correlative study of NF-κB activity and cytokines expression in human chronic nasal sinusitis

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Abstract

A growing body of literature suggests that cytokines play an important part in the pathogenesis of chronic nasal sinusitis. However, the mechanism by which the expression of cytokines in chronic nasal sinusitis is upregulated has not been well documented. The present study investigated the role of nuclear factor-kappa B (NF-κB) activation in upregulating the expression of interleukin-5, -6 and -8 (IL-5, IL-6 and IL-8). We titrated the levels of IL-5, IL-6 and IL-8 in nasal mucosa in 52 cases of chronic nasal sinusitis and 12 normal subjects using enzyme-linked immunosorbent assay. According to whether allergic rhinitis was associated or not, we subdivided the patients into the AR group (with allergic rhinitis) and the NAR group (without allergic rhinitis). Semi-quantitative reverse transcription-polymerase chain reaction and immunohistochemical staining were used to evaluate expression and activation of NF-κB P50 and P65 subunits in nasal mucosa. The correlation between activities of P50 and P65 and cytokines expression was analysed. Our results showed that IL-5, IL-6 and IL-8 in both the AR and NAR groups were strikingly elevated in comparison with the control group (all $p < 0.01$ for AR group; $p < 0.05$, 0.05, 0.01, respectively, for NAR group); and they were even higher in the AR group than those in the NAR group ($p < 0.01$, 0.05, 0.01, respectively). P50 and P65 mRNA levels in both AR and NAR groups were markedly greater than those in the control group (all $p < 0.01$); and the AR group had further higher levels as compared with the NAR group (both $p < 0.05$). Immunohistochemical study revealed that nucleus-positive rates of P50 and P65 in both AR and NAR groups were significantly higher than those of the control group (all $p < 0.01$), and they were much greater in the AR group in comparison with the NAR group (all $p < 0.01$). Pearson correlation analysis demonstrated that P50 and P65 nucleus-positive rates were closely correlated with IL-6 and IL-8 levels, but not IL-5, with a correlation coefficient of 0.49 for P50 and IL-6, 0.54 for P50 and IL-8, 0.61 for P65 and IL-6, and 0.66 for P65 and IL-8 (all $p < 0.01$). In conclusion, upregulated expression and activation of NF-κB P50 and P65 might be one of the mechanisms for induction of IL-6 and IL-8 expression in chronic nasal sinusitis. Association of allergic rhinitis with chronic nasal sinusitis further enhanced NF-κB activity, and subsequently lead to even stronger expression of IL-6 and IL-8. IL-5 expression appeared to be independent of NF-κB pathway in chronic nasal sinusitis.

Key words: NF-kappa B; Sinusitis; Rhinitis, Allergic, Perennial; Cytokines

Introduction

The pathogenesis of chronic nasal sinusitis still remains unclear. Previous studies implied that multiple cytokines, including IL-5, IL-6 and IL-8, played central roles in development of the disease.[1–2] However, the mechanisms for inducing expression of these cytokines in the diseased nasal mucosa have not been well elucidated.

Nuclear factor-kappa B (NF-κB) is an important transcription factor. It comprises P50 and P65 subunits. In inactive status, NF-κB exists as a heterodimer in cytoplasma and binds with inhibitor-kappa B(IκB). Once the cells are subject to various stimulations, such as inflammation, IκB becomes dephosphorylated and dissociated from NF-κB. The latter is then translocated into the nucleus, where it binds with some specific genes and regulates their expression.[3–6] Previous studies have demonstrated that activation of NF-κB could upregulate expression of many different genes.[7]

The present study detected NF-κB activity and cytokines level in human chronic nasal sinusitis tissue, and aimed to evaluate the role of NF-κB activation in upregulating cytokines expression.

Materials and methods

Clinicopathological data

Fifty-two patients with chronic nasal sinusitis who had undergone nasal endoscopic surgery at our

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hospital between March 2004 and May 2004, were recruited into the study. The diagnosis of chronic nasal sinusitis was made on the basis of symptoms, signs, nasal endoscopic findings, computed tomography (CT) scanning and detection of allergen-specific antibodies. Patients with lower respiratory tract diseases, including bronchial asthma, chronic bronchitis and diffused bronchiolitis, were excluded. Based on whether allergic rhinitis (AR) was associated or not, the patients were categorized into chronic nasal sinusitis with AR (AR group) and chronic nasal sinusitis without AR (NAR group).[8]

There were 29 patients in the AR group, 17 males and 12 females, with an average age of 39.2 ± 14.7 years; and 23 patients in the NAR group, 19 males and four females, with an average age of 36.9 ± 11.1 years. Normal ethmoid sinus mucosa from 12 patients, who suffered from nasal sinus trauma and underwent debridement and reduction, decompression of the optical nerve tube, and repair of nasal leakage of cerebrospinal fluid, were used as a control. All patients in the control group had no history of AR, bronchial asthma or bronchitis.

Ethmoid sinus mucosa was obtained from each patient during nasal endoscopic surgery. Each sample was divided into three portions. Two of them were stored at −80°C for ELISA detection of cytokines and RT-PCR detection of NF-κB, and the remaining one was fixed in 10 per cent formaldehyde for immunohistochemical staining.

ELISA detection of cytokines in nasal mucosa

After the frozen nasal mucosa was thawed, 100 mg of tissue was weighed and placed into 1 ml cold phosphate-buffered saline. The tissue was then homogenized by grinding on ice. The homogenate was centrifuged at 3000 rpm, 4°C for 10 min. The supernatant was saved and stored at −80°C. IL-5, IL-6 and IL-8 levels in the supernatant were detected by using ELISA kits (R&D Co, Minneapolis, USA).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to evaluate mRNA levels of NF-κB P50 and P65 subunits. Fifty mg of frozen nasal mucosa tissue was weighed and placed into the pre-chilled mortar. Afterwards the tissue was ground into a powder with the addition of liquid nitrogen, total RNA was extracted with 1 ml of Trizol (MRC Co, Cincinnati, USA). The RNA concentration was determined with a spectrophotometer (Eppendoff Co, Hamburg, Germany) and stored at −80°C.

RT-PCR was done by using a Revert Aid RT-PCR kit (Fermentas Co, Vilnius, LITHUANIA). Briefly, cDNA was synthesized from 1 μg of total RNA, and then PCR was proceeded by using the cDNA as a template. Primers were as follows: P50: 5′-CAC TTATGGACAACTATGAGGTCTCTGG-3′(sense), 5′-CTGTCTTGTGGACAACGCAGTGGAATTTT AGG-3′(anti-sense); P65: 5′-AGGCTGGGGGAA GGAACGCTGTC-3′ (sense), 5′-CCTCTGGGCCC CTGTCACTA-3′(anti- sense); GAPDH: 5′-GTCA GTGGTGGACCTGACCT-3′(sense), 5′-AGGGG AGATTCAGTGTGGTG-3′(anti-sense). PCR was run for 40 cycles for P50 and P65, and 35 cycles for GAPDH. Each cycle consisted of denaturing at 94°C for 60 sec, annealing at 60°C for 45 sec and primer extension at 72°C for 60 sec.

An aliquot of 10 μl PCR product was run electrophoresis on 1.5 per cent agarose gel, and was visualized by ethidium bromide on a transilluminator (Bio-Rad Co, Hercules, CA, USA). Discovery Series Quantity One 1-D Analysis Software (Bio-Rad Co, Hercules, CA, USA) was used to assess the band intensity of P50, P65 and GAPDH. The relative intensity of P50 and P65 to GAPDH was calculated and used as a semi-quantitative parameter for P50 and P65 mRNA levels, respectively.

Immunohistochemical staining

The distribution of NF-κB P50 and P65 in nasal mucosa epithelial cells was evaluated by immunohistochemical staining. Briefly, 5 μm thick sections of paraffin-embedded nasal mucosa tissue were prepared. After routine deparaffinization, the tissue sections were boiled in 0.01 M citric acid buffer (pH = 6.0) for 5 min for antigen retrieve. Then they were incubated with 3 per cent H_2O_2 at room temperature for 5 min for depletion of endogenous peroxidase. After washing three times with PBS, the tissue sections were incubated with non-immune animal sera at room temperature for 10 min to block non-specific binding. The sera were then removed, and rabbit anti-human P50 IgG (dilution 1:100, NeoMark Co, Fremont, USA) or P65 IgG (dilution 1:200, NeoMark Co, Fremont, USA) was added and incubated at 37°C for 30 min. The tissue sections were then washed with PBS three times, and incubated with biotinylated goat anti-rabbit IgG (NeoMark Co, Fremont, USA) at 37°C for 15–20 min. Following washing three times with PBS, HRP-conjugated streptavidin (NeoMark Co, Fremont, USA) was added and visualized with DBA. The tissue sections were then counter-stained with haematoxylin-eosin. After being dehydrated, they were covered with cover glasses and sealed. At each staining, a negative control was set by replacing primary antibodies with PBS.

Under microscopy, the presence of brown-yellowish granules in the nucleus plus cytoplasma was considered as nucleus-positive, while the presence of brown-yellowish granules in cytoplasma alone was regarded as cytoplasma-positive. Ten vision fields were observed for each slide under magnification of 400 × and nucleus-positive and cytoplasma-positive cells were counted, respectively. The nucleus-positive rate was calculated according to the following formula and used as a parameter of NF-κB activity: Nucleus-positive rate = number of nucleus-positive cells/(number of nucleus-positive cells + cytoplasma-positive cells) × 100 per cent.

Statistical analysis

The results are given as mean ± standard deviation. Statistical analysis was performed using commercially available SPSS software package version 10.0.

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TABLE I

COMPARISON OF IL-5, IL-6 AND IL-8 AMONG GROUPS (X ± S, PG/MG)

Group	Cases	IL-5	t value	IL-6	t value	IL-8	t value
AR	29	11.24 ± 12.18	4.093*	22.94 ± 21.73	4.531*	417.29 ± 465.98	4.437*
NAR	23	3.28 ± 1.83	3.469#	10.48 ± 10.93	2.688△	79.65 ± 62.25	3.859#
Control	12	1.93 ± 0.84	2.996□	4.17 ± 3.27	2.561□	32.70 ± 17.44	3.373↖

Compared with control group, $^{*}p < 0.01$;
Compared with AR group, $\#p < 0.01$, $\Delta p < 0.05$;
Compared with NAR group, $\square p < 0.05$, $\nwarrow p < 0.01$

ANOVA was used to compare the inter-group difference. Correlation between the two variables was evaluated by Pearson test. Statistical significance was considered at two-tailed $p < 0.05$.

Results

Cytokines in ethmoid sinus mucosa

IL-5, IL-6 and IL-8 levels in ethmoid sinus mucosa are shown in Table I. Compared with the control group, both the AR group and the NAR group had significantly greater levels of IL-5, IL-6 and IL-8 (all $p < 0.01$ for the AR group; $p < 0.05$, 0.05, 0.01, respectively for the NAR group). All three cytokines in the AR group were strikingly higher than those in the NAR group ($p < 0.01$, 0.05, 0.01, respectively).

Expression and activity of NF-κB in ethmoid sinus mucosa

Semi-quantitative RT-PCR showed that expression of both NF-κB P50 and P65 in both the AR and NAR groups was markedly stronger than that in the control group, and the AR group had significantly greater levels in comparison with the NAR group (Figure 1). The relative intensity of P50 and P65 is displayed in Table II. It clearly demonstrated that both AR and NAR groups had remarkably higher mRNA levels of both P50 and P65 than the control group (all $p < 0.01$), and they were significantly greater in the AR group than those in the NAR group (both $p < 0.05$).

Immunohistochemical staining illustrated that expression of P50 and P65 was predominantly localized in mucosa epithelial cells (Figures 2 and 3). The infiltrated eosinophils, neutrophils, lymphocytes and plasma cells in the submucosa layer scarcely expressed P50 and P65.

Since NF-κB exerted a regulatory effect on gene expression only after nuclear translocation, we adopted nucleus-positive rates of P50 and P65 as its activity parameter. Our results clearly demonstrated that both AR and NAR groups had significantly greater nucleus-positive rates of P50 and P65 compared with the control group (all $p < 0.01$); and the AR group had even higher nucleus-positive rates of P50 and P65 in comparison with the NAR group, being 18.94 ± 16.72 per cent versus 2.02 ± 1.79 per cent for P50 ($p < 0.01$) and 30.1 ± 16.09 per cent versus 13.81 ± 1.0 per cent for P65 ($p < 0.01$).

TABLE II

COMPARISON OF MRNA LEVELS OF P50 AND P65 (RELATIVE DENSITY) AMONG GROUPS

Group	Cases	P50	t value	P65	t value
AR	29	0.74 ± 0.49	4.854*	0.44 ± 0.28	6.689*
NAR	23	0.53 ± 0.21	2.159△	0.25 ± 0.25	2.525△
Control	12	0.22 ± 0.20	4.167↖	0.08 ± 0.04	3.336↖

Compared with control group, $^{*}p < 0.01$;
Compared with AR group, $\Delta p < 0.05$;
Compared with NAR group, $\nwarrow p < 0.01$

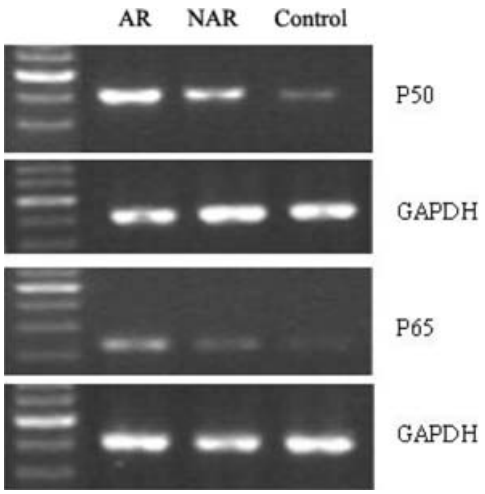

FIG. 1
Semi-quantitative RT-PCR.

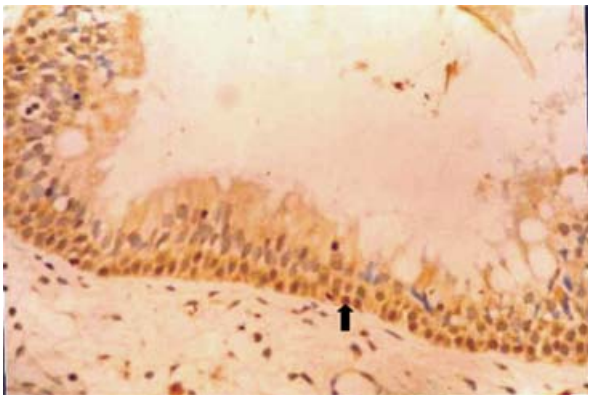

FIG. 2
Immunohistochemical staining for P50 in ethmoid sinus mucosa from patients with chronic nasal sinusitis. P50 was predominantly expressed in the nucleus of epithelial cells (black arrowhead). Magnification of 400.

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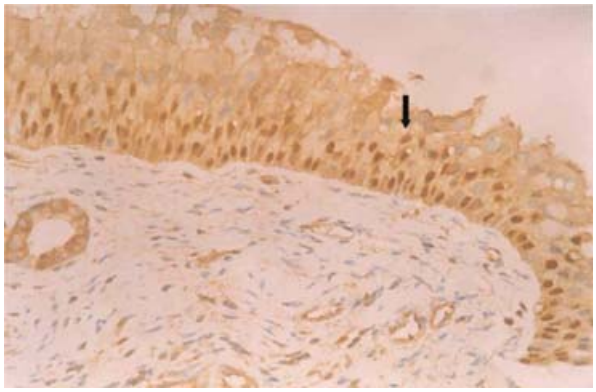

FIG. 3

Immunohistochemical staining for P65 in ethmoid sinus mucosa from patients with chronic nasal sinusitis. P65 was primarily localized in the nucleus of epithelial cells (black arrowhead). Magnification of 400.

Correlation between NF-κB activity and cytokines level

Pearson correlative analysis revealed that nucleus-positive rates of P50 or P65 were closely correlated with both IL-6 and IL-8 (Figure 4), with a correlative coefficient of 0.49 for P50 and IL-6, 0.54 for P50 and IL-8, 0.61 for P65 and IL-6, and 0.66 for P65 and IL-8 (all $p < 0.01$). There existed no remarkable correlation between nucleus-positive rates of P50 or P65 and IL-5.

Discussion

It was believed that cytokines released from mucosa epithelial cells of the respiratory tract participated in development of local inflammation via the paracrine mechanism.[9] Recent studies implied that multiple cytokines, including IL-5, IL-6 and IL-8, played essential roles in the pathogenesis of chronic nasal sinusitis.[1–2] However, the mechanism by which the expression of cytokines was upregulated in the disease has not been well documented.

The present study shows that the mucosa from chronic nasal sinusitis, with or without AR, had strikingly higher levels of IL-5, IL-6 and IL-8 as compared with normal mucosa. The results imply that all three cytokines might play a role in development of chronic nasal sinusitis. Furthermore, we compared levels of IL-5, IL-6 and IL-8 in patients with chronic nasal sinusitis with AR and without AR, and discovered that the AR group had significantly

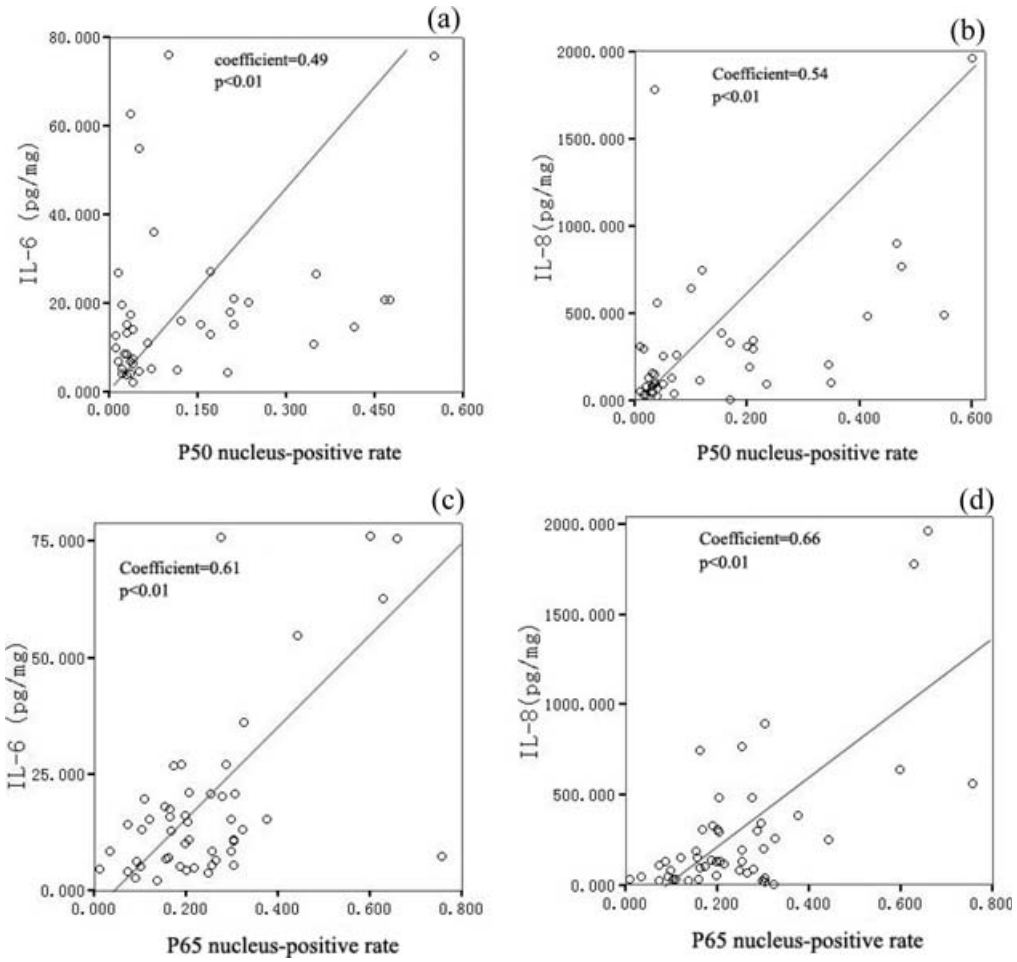

FIG. 4

Correlation between IL-6 (a), IL-8 (b) and percentages of P50 nuclear-positive cells and correlation between IL-6 (c), IL-8 (d) and percentages of P65 nucleus-positive cells.

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greater levels of all three cytokines in comparison with the NAR group. It suggests that AR itself can induce nasal mucosa to release cytokines. Our results were consistent with those reported by Calderón *et al.*,[10] in which nasal mucosa from atopic persons were found to have remarkably greater levels of IL-6, IL-8, GM-CSF, TNF-α and IL-1β as compared with normal nasal mucosa. The upregulatory effect of AR on cytokine expression in nasal mucosa might be one of the reasons for which chronic nasal sinusitis with AR usually has more diffused lesions, a less satisfactory therapeutic outcome and higher recurrence rates.

- **Cytokines may play an important part in the pathogenesis of chronic rhinosinusitis. The mechanism by which the expression of cytokines in chronic rhinosinusitis is upregulated has not been well documented**
- **This study detected NF-κB activity and cytokines level in human chronic rhinosinusitis tissue, and evaluated the role of NF-κB activation in upregulating cytokines expression**
- **Upregulated expression and activation of NF-κB P50 and P65 might be one of the mechanisms for local induction of IL-6 and IL-8**
- **Local expression of IL-5 might be regulated via an NF-κB-independent pathway**

NF-κB is an important transcription factor, and can be activated by many factors, such as endotoxin, viral proteins mitogen, ultraviolet, chemicals, TNF-α and IL-1β.[11–12] The activated NF-κB can induce expression of multiple cytokine genes. Recently, Takeno and his associates reported that activation of NF-κB in the epithelial cells of nasal polyps was closely correlated with local levels of IL-8, IL-16 and eotaxin.[13] In another study conducted by Yamashita *et al.*,[14] it was found that release of IL-8 from eosinophils could be dramatically suppressed by NF-κB inhibitors (FK506, MG-132). They suggested that activation of NF-κB played a pivotal role in IL-8 expression in eosinophils. In the present study, semi-quantitative RT-PCR revealed that both the AR and NAR groups had a significantly higher mRNA level of NF-κB P50 and P65 as compared with the control group. Meanwhile, immunohistochemical staining illustrated that nucleus-positive rates of P50 and P65 were markedly greater in both the AR and NAR groups than those in the control group. These results clearly indicated that in chronic nasal sinusitis NF-κB not only had an elevated expression, but was activated as well. Furthermore, correlative analysis revealed that nucleus-positive rates of P50 and P65 were closely correlated with IL-6 and IL-8 levels in nasal mucosa, but not IL-5. Taken together, our results support the view that in chronic nasal sinusitis upregulated expression and activation of NF-κB P50 and P65 might be one of the mechanisms for local induction of IL-6 and IL-8.

On the other hand, the AR group had significantly greater mRNA levels of P50 and P65, and nucleus-positive rates of P50 and P65 in comparison with the NAR group. This suggests that activity of NF-κB is even more elevated in patients with chronic nasal sinusitis with AR than those without AR. The underlying mechanisms still remain unclear, but some factors might be contributory. Osada *et al.*[15] discovered that nasal mucosa of AR patients contained much higher levels of IL-1β and TNF-α than that of healthy persons. It is well known that both IL-1β and TNF-α are activators of NF-κB. In addition, allergens themselves can also activate NF-κB.[16] In a study conducted by Hart *et al.*,[17] it was found that patients with asthma had many more NF-κB-positive epithelial cells than healthy persons. These results support the view that activation of NF-κB may play an important role in allergic response.

In conclusion, our results revealed that upregulated expression and activation of NF-κB P50 and P65 might be one of the mechanisms for local induction of IL-6 and IL-8 in chronic nasal sinusitis. Coexistence of AR could further enhance the activity of NF-κB, and subsequently local expression of cytokines. Local expression of IL-5 in chronic nasal sinusitis might be regulated via NF-κB-independent pathways.

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