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Expression and localization of nuclear factor-kappa B subunits in cultured human paranasal sinus mucosal cells*

Rika Osada, Sachio Takeno, Katsuhiro Hirakawa, Tsutomu Ueda, Kyosuke Furukido and Koji Yajin.

Department of Otolaryngology, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan

SUMMARY

The purpose of this study was to compare the expression of cytokines and nuclear factor-kappa B (NF-κB) subunits in cultured sinus mucosal cells by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) in chronic sinusitis patients with allergic rhinitis (abbreviated as AR patients) versus patients without AR (abbreviated as non-AR patients). The localization of p50 in cultured sinus mucosal cells was also observed by immunocytochemistry. The expression of messenger RNAs (mRNA) encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, IL-8, p50 and p65 subunits, and inhibitory kappa B-alpha (IκB-α) were analyzed by RT-PCR. The proportion of active NF-κB-positive cells in the epithelial layer was analyzed using a laser-scanning confocal microscope image system. The levels of GM-CSF, IL-6, IL-8, and p50 mRNAs in AR patients were significantly higher than those in non-AR patients ($p<0.01$, $p<0.01$, $p<0.01$, and $p<0.001$, respectively). Immunocytochemical reaction for p50 in sinus mucosal cells in AR patients showed more intense nuclear staining compared to non-AR patients. These findings could support the hypothesis that the increase of cytokines from sinus mucosal cells in AR patients was associated with augmented NF-κB mRNA expression, resulting in the modification of the cytokine network.

Key words: chronic sinusitis, allergic rhinitis, primary cell culture, transcription factors, cytokines

INTRODUCTION

Chronic sinusitis is pathologically characterized by basement membrane thickening, subepithelial edema, goblet cell hyperplasia, and persistent inflammatory cell infiltration (Hamilos, 2000). Currently we do not fully understand the factors responsible for the development of sinusitis in a given individual. A panel of cytokines has been found to be intimately involved in the inflammatory processes of chronic sinusitis. Increased expression of inflammatory cytokines, such as IL-1ß, tumor necrosis factor-alpha (TNF-α), GM-CSF, IL-6, and IL-8, is commonly observed in the chronic sinusitis mucosa (Hamilos, 2000; Min and Lee, 2000). In addition, it has been pointed out that the presence of allergic rhinitis (AR) may be a risk factor for the development and persistence of the disease (Benninger, 1992). However, little is known about the difference in the molecular regulatory pathways between chronic sinusitis patients with AR and such patients without AR that lead to local cytokine production.

It has been well recognized that the respiratory epithelium of the paranasal sinuses is capable to modulate inflammatory and immunological events during sinusitis. The epithelium could promote and sustain the local inflammatory processes through the release of these cytokines (Polito and Proud, 1998; Hamilos, 2000). For example, Kenny et al. (1994) have investigated the synthesis of several cytokines from cultured nasal epithelial cells obtained from turbinate tissues and demonstrated that amounts of cytokines released were in the order IL-8 > IL-6 > IL-1ß. The mechanisms underlying the regulation of such cytokine production are complex, but recent evidence indicates that activation of various transcription factors plays a central role in the induction of multiple genes involved in the inflammatory responses.

The transcription factor nuclear factor-kappa B (NF-κB), firstly identified by Sen and Baltimore (1986), is now considered to be a crucial component of the host's response against external stimuli. This transcription factor is formed by several different

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dimers, including p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel. The most prevalent NF-kB dimer consists of the two subunits, p50 and p65, and is present in the cytosol in an inactive form bound to inhibitory kappa B-alpha (IκB-α) (Siebenlist et al., 1994). Increased expression levels of NF-κB subunits are commonly seen in patients with various chronic inflammatory diseases such as rheumatoid arthritis and adult respiratory distress syndrome (Handel et al., 1995; Christman et al., 2000). Also in patients with bronchial asthma, Hart et al. (1998) observed that the proportion of NF-κB-positive epithelial cells was increased as compared with that in normal subjects.

Although it is generally assumed that all cell types ubiquitously express NF-κB, the exact expression patterns of this transcription factor remain to be studied for most human tissues. The role of NF-κB activation and its regulation of related cytokine production in the paranasal sinus system are poorly understood. In this study we used a method of culturing mucosal explants that are obtained from sinusitis patients. We examined levels of mRNA expression of cytokines and NF-κB subunits, including GM-CSF, IL-6, IL-8, p50, p65, and IκB-α by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), and we tried to determine whether the levels differed between chronic sinusitis patients with AR and without AR patients. We also investigated the immunocytochemical localization of the NF-κB subunits in cultured sinus mucosal cells in order to find a possible correlation between the NF-κB activity and the gene expression related to those transcription factors. The present study indicates that increased expression of the p50 subunit plays a role in the onset of paranasal sinus inflammation through the regulation of the relevant cytokine gene expression.

MATERIALS AND METHODS

The study was carried out on a group of 58 patients with chronic sinusitis at the ENT Department, Hiroshima University Hospital, Hiroshima, Japan. They all had exhibited sinus-related symptoms for more than 3 months and showed opacities of the paranasal sinuses as revealed by CT scans. The patients were subdivided into two groups based on the presence (abbreviated as AR patients) or absence of AR (abbreviated as non-AR patients); these groups consisted respectively of 23 AR patients (15 men and 8 women; mean age 46.9 years) and 35 non-AR patients (24 men and 11 women; mean age 47.9 years). The criteria for AR included perennial AR with a positive skin test for a house dust mite allergen, a positive radioallergosorbent test (RAST) score of 2 or more for *Dermatophagoides farinae,* and the presence of subjective allergic symptoms. Patients with lower respiratory tract diseases such as bronchial asthma, chronic bronchitis and diffuse panbronchiolitis were excluded from the study. Antibiotic therapy was discontinued 2 weeks before the surgery, and the allergic patients did not receive any anti-allergic medication in the 30 days before the surgery.

Specimens were obtained from ethmoidal mucosa on the occasion of the sinus surgery. Each patient gave informed consent before the surgery. The study was carried out in compliance with the principles approved by the Institutional Review Board at the University of Hiroshima School of Medicine.

Primary culture of sinus mucosal cells

Sinus mucosal cells were cultured by using the explant cell culture technique according to the methods of Devalia et al. (1990). Briefly, the epithelium was dissected from the underlying submucosal tissue, and was then cut into smaller sections of approximately 1 mm^2. The explants were harvested onto sterile 35 mm diameter cell culture plastic dishes precoated with poly-L-lysine (Asahi Techno Glass, Tokyo, Japan), and an aliquot of culture medium (500 μL) was added to enable cell attachment. The culture medium consisted of Dulbecco's-modified Eagle's medium (D-MEM, Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, L-glutamine (1 μg/mL), Insulin-Transferrin-Selenium supplement (Gibco BRL), and antibiotics. These mucosal explants attached to the plastic dishes at 37°C in a humidified 5% CO_2 in air atmosphere for 48 hours, after which medium was replaced gently so as not to dislodge the tissue. The identity of the outgrowing cells of the cultured epithelial layer and the presence of ciliated cells were confirmed in all cultures by phase contrast microscopy. In order to standardize for both the culturing time and size of cultured cells, 5 to 7 day-old cell cultures from each subject were used for the study. This experimental model has the advantage of allowing epithelial cell to express cytokines in the absence of de novo inflammatory cell recruitment.

Analysis of mRNA expression in cultured cells

The steady-state levels of the cytokine mRNA and NF-κB subunit mRNA, encoding for p50, p65, and IκB, were investigated by semiquantitative RT-PCR. Cells were washed in ice-cold Hanks balanced salt solution (HBSS) and flash frozen in liquid nitrogen. Total RNA was isolated by the TRIZOL reagent (Gibco BRL, Rockville, MD), which is based on the method of Chomczynski and Sacchi (1987). The RNA pellet was precipitated in cold isopropanol, washed in 75% ethanol, vacuum-dried, and resuspended in 20 μL of RNase-free water. Contaminating DNA was removed using deoxyribonuclease I (amplification grade; Gibco BRL) at 2 U/20 μL reaction for 30 minutes at 37°C. The cellular RNA was then reverse-transcribed to complementary DNA (cDNA) in the presence of Moloney murine leukemia virus reverse transcriptase (2 U/μL, Toyobo, Tokyo, Japan), 1 mmol/L of deoxyribonucleoside triphosphates (dNTPs), ribonuclease (RNase) inhibitor (0.5 U/μL), 50 pmole of random primers, and RT buffer to a total volume of 30 μL. The RT mixture was incubated for 10 minutes at 30°C for initial annealing, followed by 20 minutes at 42°C for RT, and 5 minutes at 99°C for inactivation.

Table 1. Oligonucleotide primers used for PCR amplification.

Target gene	Sequences (5' → 3')	Product size, bp
ß-actin	AAGAGAGGCATCCTCACCCT (S)	218
	TACATGGCTGGGGTGTTGAA (AS)	
GM-CSF	GGCTGCAGAGCCTGCTGCTCTTGGGCACTG (S)	196
	CTGGAGGTCAAACATTTCTGAGATGACTTC (AS)	
IL-6	ATGAACTCCTTCTCCACAAGCGC (S)	628
	GAAGAGCCCTCAGGCTGGACTG (AS)	
IL-8	ATGACTTCCAAGCTGGCCGTGGCT (S)	289
	TCTCAGCCCTCTTCAAAAACTTCTC (AS)	
p50	CACTTATGGACAACTATGAGGTCTCTGG (S)	406
	CTGTCTTGTGGACAACGCAGTGGAATTTTAGG (AS)	
p65	AGCCATGGTATCAGCTCTGG (S)	461
	TAGGAGCTGATCTGACTCAGC (AS)	
IκB-α	GCCTGGACTCCATGAAAGAC (S)(AS)	253
	CAAGTGGAGTGGAGTCTGCTGCAGGTTGTT	

An aliquot of the RT product was then used for PCR amplification using an MJ Research PTC-100 thermal cycler (Watertown, MA). Each reaction mixture (50 μL) consisted of PCR buffer, 1.5 mmol/L of $MgCl_2$, 0.2 mmol/L of dNTPs, 1.25 U of rTaq polymerase (Gibco BRL), and specific paired primers (10 pmol of each). The oligonucleotide primers used for RT-PCR and their expected product sizes are summarized in Table 1 (Krishnaswamy et al., 1993; Jobin C et al., 1997). Primers for the housekeeping gene ß-actin were also used as internal controls in each experiment. The PCR conditions were as follows: denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 90 seconds for ß-actin, GM-CSF, IL-6, and IL-8, and for 120 s for p50, p65, and IκB-α. Kinetic analysis based on cycle number was used to ensure that sample-to-sample comparisons were made in the linear range. The number of cycles was determined to be 40 for p50 and 35 for the others. For semiquantitative analysis, PCR products (10 μL) were analyzed in 1.5% agarose gels containing 1 μg/ml ethidium bromide in Tris acetate ethylenediamine tetra-acetic acid (EDTA) (TAE) buffer solution. The intensity of the luminescence was measured with a CCD image analyzer (AE-6915, Atto, Tokyo, Japan). The intensity of the target genes in each sample was expressed as a ratio relative to the intensity of ß-actin in the same sample.

Immunocytochemistry

The culture dishes were fixed in 4% paraformaldehyde solution for 30 minutes. After being rinsed several times in phosphate-buffered saline (PBS), they were permeabilized with a solution of 20 mmol/L HEPES (pH 7.4), 300 mmol/L sucrose, 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, and 0.5% Triton X-100 for 30 minutes. After being rinsed in PBS, anti-p50 antibodies (1:200, Santa Cruz Biotechnology, CA) were applied in PBS containing 0.1% bovine serum albumin (BSA). Specimens were stored overnight at 4°C in a humid chamber and then washed in PBS. The specimens were then incubated with Alexa Fluor 488 conjugated goat anti-rabbit immunoglobulins (5 mg/ml, Molecular Probes, Eugene, OR) for 1 hour in the dark. On the control slides, the primary and secondary antibodies were omitted and an appropriate immunoglobulin control was used. The proportion of active NF-κB-positive cells in the epithelial layer was analyzed using the laser scanning confocal microscope (LSCM) (Raychaudhuri et al., 1999). Fluorescence images were recorded with a Leica TCS-NT (Leica Microsystems, Heidelberg, Germany) equipped with an argon-krypton laser at a wavelength of 488 nm.

Statistical Analysis

Data were analyzed with STATISTICA for Windows (Ver. 5.0J, StatSoft Inc., Tulsa, OK). The comparison of mRNA expression levels between the allergic group and the non-allergic group was assessed with the Mann-Whitney U test. Differences were considered significant if p-values were less than 0.05.

RESULTS

Cytokine mRNA expression in cultured cells

We employed semiquantitative RT-PCR analysis to examine steady-state levels of mRNA expression of cytokine and the NF-κB subunits in cultured mucosal cells. Figure 1 shows representative profiles of the mRNA transcripts derived from an AR patient (a) and from a non-AR patient (b). Expression levels of ß-actin mRNA from each sample were found to be almost the same. Messenger RNA transcripts of GM-CSF, IL-6, IL-8, p50, p65, and IκB-α were demonstrated in both groups of patients with amplification products seen at the predicted size. Figure 2 shows the levels of mRNA expression in cultured mucosal cells in the (a) and (b) patients. The raw intensity data for ß-actin did not differ by more than 1.5-fold throughout the cultures. The levels of mRNA expression of GM-CSF

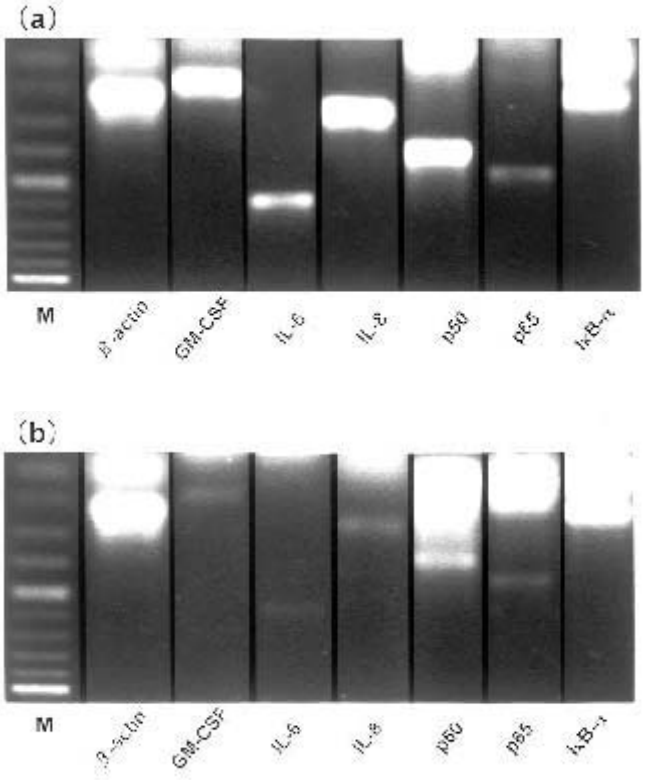

Figure 1. Representative images of RT-PCR analysis in the cultured sinus mucosal cells obtained from an AR patient (a) and a non-AR patient (b). Specific amplification products for ß-actin, GM-CSF, IL-6, IL-8, p50, p65 subunits, and IκB-α were demonstrated.

($p < 0.01$), IL-6 ($p < 0.01$), IL-8 ($p < 0.01$), and the p50 subunit ($p < 0.001$) in AR patients were significantly higher than those in non-AR patients. On the other hand, there was no significant difference between the two groups in the levels of p65 or IκB-α mRNA expression. No significant correlation was observed between the degree of AR and the levels of cytokines expression.

Immunocytochemical localization of NF-κB p50 subunit

As semiquantitative RT-PCR analysis indicated an increased mRNA expression of the NF-κB p50 subunit in AR patients, we then investigated the localization of the NF-κB p50 subunit in the outgrowthal cells of cultured epithelial layer by comparing fluorescence imaging between the two groups (Figure 3a, b). The AR patients generally showed intense cytoplasmic staining, and p50 immunoreactivity was extremely high not only in the cytoplasm but also in the nucleus, indicating increased levels of NF-κB activation (arrowheads). On the other hand, lower degrees of p50 staining of the cytoplasm as well as nuclei were expressed in the non-AR patients. Image analysis by LSCM has indicated that the mean value of the fluorescence intensity in AR patients were nearly two-fold greater than in non-AR patients.

DISCUSSION

A panel of cytokines has been found to be intimately involved in the inflammatory processes of chronic sinusitis (Hamilos, 2000; Min and Lee, 2000). Recently, substantial evidence has emerged that mucosal epithelial cells are able to liberate a number of these cytokines fundamental to both inflammatory and immune responses. Thus, through a paracrine mechanism, cytokines secreted by the epithelial cells may be involved in the initiation and progression of chronic sinus inflammation (Polito and Proud, 1998). In addition, the presence of AR has been proposed to be a risk factor in the development and persistence of sinusitis Benninger, 1992. Therefore, comparison of cytokine release from sinus mucosa in AR and non-AR patients would be of great interest.

It is not fully understood whether the expression of these cytokines in sinus mucosal cells is influenced by the presence of local allergic responses. Calderón et al. (1997) reported that explant cultured nasal epithelial cells obtained from atopic

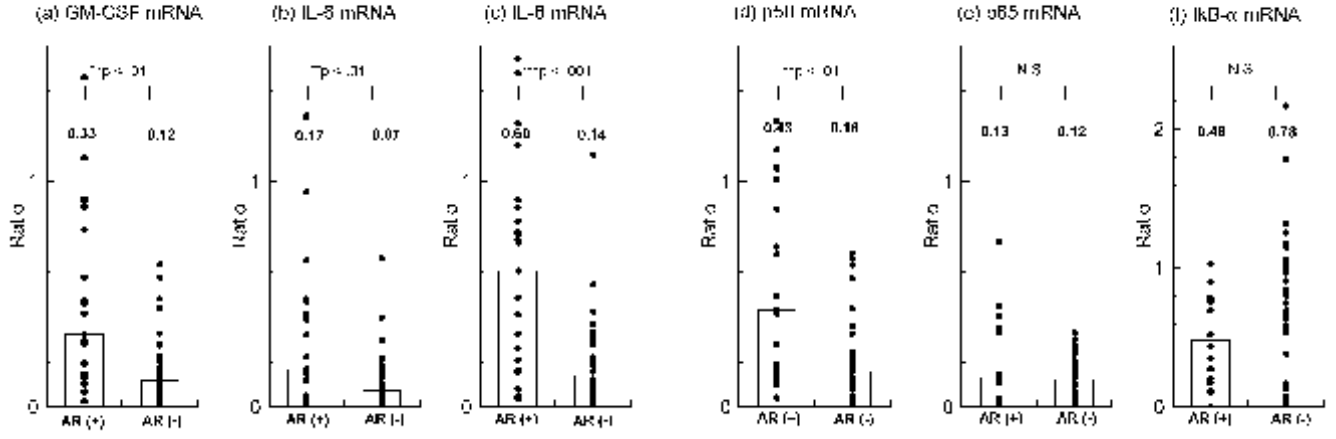

Figure 2. Relative mRNA expression levels of GM-CSF (a), IL-6 (b), IL-8 (c), p50 (d), p65 (e), and IκB-α (f) in the cultured sinus mucosal cells from AR patients (n=19) and non-AR patients (n=35). The levels were expressed as ratios relative to those of ß-actin using RT-PCR. Columns represent median.

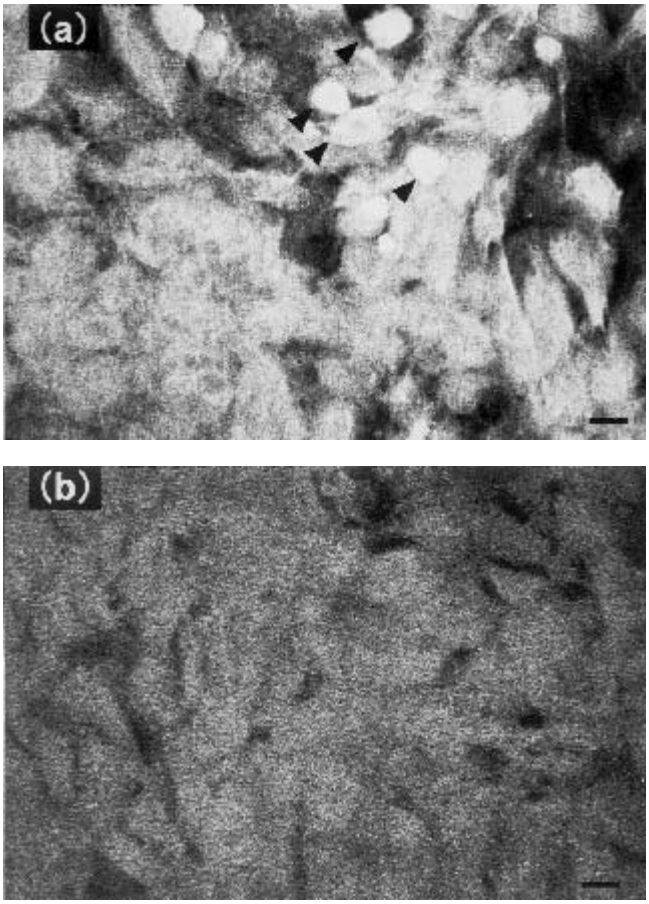

Figure 3. Immunocytochemical localization of the NF-κB p50 subunit in cultured sinus epithelial cells. (a) In the AR patients, distinct immunoreactivity was observed in the epithelial layer. Nuclear translocation of the subunit was also noted in some cells (arrowheads), indicating the site of NF-kB activation. (b) In the non-AR patients, the immunoreactivity of the p50 subunit was relatively weak and was mainly localized to the cytoplasm. Bar = 10 m.

patients release significantly greater quantities of IL-6, IL-8, GM-CSF, IL-1ß and TNF-α than those from normal subjects. Their observation is in consistent with *in vivo* findings that elevated levels of these cytokines are commonly found in the nasal mucosa of AR patients (Christodoulopoulos et al., 2000). In the present study, cultured paranasal sinus mucosal cells also showed significant differences in cytokine expression levels between the two populations. We employed the primary explant culture method and 5 to 7 day-old cell cultures were used for RT-PCR analysis. Using this method, at the earlier culture stages, a high proportion of the cultured cells usually exhibited features of mature cilitated cells with small amounts of fibroblast out-spreading. The mucosal explant partially contains the non-epithelial cell population and the results may not be solely attributable to the epithelial nature. However, we consider that this simple method can reflect inflammatory conditions of the original host sinus mucosa more directly than established cell culture methods, leading to obtain the difference between AR and non-AR patients.

The NF-κB family of transcription factors is of particular interest because it induces many genes that are involved in immune and inflammatory responses in the airways. The initiation of the transcriptional pathway of the cytokines examined in this study is known to be closely related to NF-κB activation (Blackwell and Christman, 1997). Kim et al. (2000) evaluated the role of NF-κB in rhinovirus-induced IL-6, IL-8, and GM-CSF production by the electrophoretic mobility shift assay (EMSA). They detected maximal NF-κB binding to the consensus oligonucleotides as soon as 30 min after infection, which was consistent with the rapid induction of mRNA for IL-8 and IL-6. Miyanohara et al. (2000) reported that NF-κB was activated in nasal epithelial cells by *H. influenzae* endotoxin. Takeno et al. (2002) reported that the activation of NF-kB in the nasal polyp epithelium is responsible for the recruitment of inflammatory cells, particularly eosinophils, through the initiation of the transcriptional pathway of related cytokines. In this study, we demonstrated that the levels of inflammatory cytokines and p50 mRNA expression in AR patients were significantly higher than those in non-AR patients. These results indicate that the increase of cytokines from sinus mucosal cells in AR patients is associated with augmented NF-κB mRNA expression and leads to modifications of the pathological condition in sinus infection.

Immunocytochemical observation of the cultured cells also supports the idea that persistent sinus infection is influenced by the existence of AR. In AR patients, the cytoplasm of most epithelial cells remained positive in spite of the increased translocation of p50 to the nucleus, which indicates that a large proportion of the NF-κB complex is replenished in the cytoplasm. Induction of the NF-κB DNA-binding activity involves not only the release of cytoplasmically stored factor from IkB but also new expression of the NF-κB gene. The mechanism of NF-κB activation and its regulatory role in cytokine production in the paranasal sinus system is poorly understood. Ramis et al. (2000) reported that constitutive NF-κB activity was detected by EMSA in the nasal mucosa, independent of the inflammatory state of the tissue. According to their study, the nasal mucosa had a specific pattern of subunit composition in which p50 was the predominant subunit in the activated NF-κB complexes, whereas p65 was present in lesser amounts in the whole tissue. This is consistent with our immunocytochemical observations.

The role of NF-κB activation in allergic inflammation has been previously reported in asthmatic patients (Donovan et al., 1999). Immunoreactivity of the NF-κB subunit was also confined to the epithelium rather than to submucosal inflammatory cells in bronchial biopsy specimens. Hart et al. (1998) observed that the proportion of NF-κB-positive epithelial cells increased in patients with asthma as compared with that in normal subjects (45.1% versus 20.7%), indicating that persistence of the NF-κB activity could be of particular importance

in the pathogenesis of the chronic inflammation in asthma. In addition, mice deficient in p50 are incapable of mounting eosinophilic airway inflammation after an allergen challenge, and this incapacity is concomitant with a lack of IL-5 and eotaxin production (Yang et al., 1998). Stacey et al. (1997) found that Der p1 promotes activation of NF-κB by interfering with the function of cytoplasmic IκB-α. Yamashita et al. (1999) observed that stimulation of eosinophils with GM-CSF and TNF-α induced a significant increase in the synthesis and secretion of IL-8 that was associated with the translocation of NF-κB p50 into the nucleus, indicating that activation and translocation of NF-κB plays a crucial role in the signal-transduction pathway leading to the synthesis and release of IL-8 by eosinophils. These findings in combination with ours indicate that NF-κB plays an important role in the pathogenesis of the allergic reaction.

It is difficult to determine the exact mechanisms of the increased steady-state level of p50 mRNA in cultured sinus mucosal cells in AR patients, because the activation of this transcriptional factor represents a highly regulated process (Cogswell et al., 1993; Siebenlist et al., 1994). However, there are several possible explanations for the level. First, the production of IL-1ß and TNF-α in explant mucosa from AR patients was reportedly higher than in that from non-AR patients. This production stimulates NF-κB activity, and increased expression levels of p50 are seen. NF-κB is activated by diverse stimuli such as viral infection, lipopolysaccharides, and pro-inflammatory cytokines (e.g., TNF-α, IL-1ß) (Tan X et al., 1994). The interaction of regulatory transcription factors and cytokines may represent an autoregulatory loop responsible for perpetuating the chronic airway inflammatory process (Blackwell and Christman, 1997). Second, the increased NF-κB activity concomitant with a high cytokine gene expression in AR patients may reflect a hypersensitivity to a given stimulus. For instance, the allergen may directly promote the NF-κB activity (Stacey et al., 1997). Third, the mucosal explant culture may reflect an inflammatory condition of the original host sinus mucosa more directly, leading to obtain the difference between AR and non-AR patients. Fourth, one should also consider multiple pathways for the regulation of p50 gene expression, given the existence of different binding sequences for NF-κB, AP-1, NF-IL6, and other regulators identified in the promoter region of this gene (Cogswell PC et al., 1993; Siebenlist et al., 1994; Blackwell and Christman, 1997). In addition, the human NF-κB p50 gene itself is regulated by other members of the NF-κB/Rel family (Cogswell et al., 1993). These transcription factors can be activated within the cell at the same time and may exert influence on p50 gene transcription.

In conclusion, our present results clearly indicate that sinus mucosal cells are susceptible to allergic conditions. In addition, the state of enhanced activation of NF-κB in AR patients is primarily responsible for the increased expression of inflammatory cytokines, leading to the perpetuation of chronic sinus inflammation. Further studies are needed to elucidate the extraction of nuclear proteins and performance of EMSA and real-time PCR (Blaschke et al., 2000). In addition, further studies should clarify the factors responsible for the regulation of NF-κB, not only at the post-transcriptional level but also at the gene-expression level in allergic conditions. A better understanding of the molecular mechanisms underlying transcriptional regulation in inflammatory cytokine production could lead to new and more effective treatment options for chronic sinusitis.

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Rika Osada, MD
Department of Otolaryngology
Hiroshima University School of Medicine
Kasumi 1-2-3, Minami-ku
Hiroshima 734-8551
Japan

Tel: +81-82-257-5252
Fax: +81-82-257-5254
E-mail: osadam@ff.iij4u.or.jp.