ORIGINAL CONTRIBUTION

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Glucocorticoids suppress NF-κB activation induced by LPS and PGN in paranasal sinus epithelial cells*

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SUMMARY

Objectives: *The aim of this study was to examine the innate immune response induced by toll-like receptors (TLRs) in the paranasal sinus epithelial cells in cell culture models and to examine the effect of glucocorticoids (GCs) on the innate immune response.*

Methods: *After stimulation with lipopolysaccharide (LPS) and peptidoglycan (PGN), p50 level was measured as an index of the innate response in the paranasal sinus epithelium. To observe the effect of GCs, the specimens were pre-treated with dexamethasone (DEX) for 48 hours prior to stimulation. On immunocytochemistry GR, TLR2 and TLR4 in the paranasal sinus epithelium were observed.*

Results: *The p50 activity levels increased after stimulation with LPS and PGN in a dose-dependent manner. Pretreatment with DEX significantly suppressed the increase in p50 activity levels induced by LPS and PGN. On immunocytochemistry, TLR2 and TLR4 immunoreactivities were relatively high after 48h DEX pretreatment.*

Conclusion: *The increase in NF-κB activity after LPS and PGN stimulation suggests that stimulation through TLR2 and TLR4 may induce high cytokine expression and inflammatory cell migration in the paranasal sinus epithelial cells. In paranasal sinus epithelial cells GCs not only have anti-inflammatory effects through transcription factor inhibition but also enhance innate host defences.*

Key words: Toll like receptor, NF-κB, glucocorticoids, LPS, PGN, sinus epithelial cells

INTRODUCTION

In the respiratory system, airway epithelial cells play an important role as the first line of immune defence. Recently, epithelial cells including the paranasal sinus epithelial cells have been shown to participate in adaptive and innate immune systems in addition to their role as a physical barrier in the respiratory system to maintain homeostasis and coordinate host defence [1,2].

Toll-like receptors (TLRs) were initially identified as an antifungal defence in Drosophila and are now recognized as being part of the innate immune system in the human airway. In the past few years, 10 different human TLRs have been identified. Among them, TLR2 and TLR4 have been identified as signalling receptors activated by bacterial cell wall components. The TLR2 response is well known to be stimulated by Gram-positive bacterial and mycobacterial cell wall components, such as peptidoglycan (PGN) and lipoteichoic acids. The TLR4 response is mediated by Gram-negative bacterial cell wall components, such as lipopolysaccharide (LPS) [3]. Expression of TLRs has been identified in sinus epithelial cells. Dong et al. [4] reported that TLR2 and TLR4 mRNA in the epithelial cells of human nasal mucosa were upregulated in chronic rhinosinusitis. Lane et al. [1,5] reported that patients with chronic sinusitis expressed TLR2 at significantly higher levels than controls in human paranasal sinus mucosa. They concluded that the stimulation of innate receptors may play an important role in the pathogenesis of chronic sinusitis.

The TLR pathways are known to lead to various gene transcriptions through the activation of various transcriptional factors such as nuclear factor-kappa B (NF-κB) and interferon regulatory factor (IRF) [5]. NF-κB is a representative transcription factor that deals with gene expression of several cytokines and receptors related to various inflammatory states [6]. Various proinflammatory cytokines, chemokines, adhesion molecules, and inducible enzymes are activated through the NF-κB pathway. In the human nose and paranasal sinuses, constitutive NF-κB activation has been detected and may play a role in pro-

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longed inflammatory states in human paranasal sinuses [4]. However, the trigger for NF-κB activation in nasal and sinus epithelial cells has not been clearly delineated, and investigation of this pathway may elucidate the pathogenesis of chronic sinusitis.

In the present study, we have examined the expression of TLR2, TLR4, and GR in human cultured paranasal sinus epithelial cells. Cultured paranasal sinus epithelial cells were stimulated with various concentrations of LPS and PGN. The levels of p50 NF-κB subunit activity were measured using the Trans AM assay™, a novel index to assess the viable transcriptional activity in inflammatory responses. We also evaluated the inhibitory effects of glucocorticoids (GCs) on the NF-κB activation in the cultured cells. GCs have recently been found to inhibit concomitant inflammatory responses while sparing or enhancing the local innate host defence responses. These selective effects of GC are thought to be mediated partially through the activation of transcription [3].

Our results clearly demonstrate an increase of p50 activity in the cultured cells after LPS and PGN stimulation in a dose-dependent manner. On immunocytochemistry, TLR2 and TLR4 immunoreactivity were relatively high after 48h DEX treatment in human cultured paranasal sinus epithelium cells. These results may elucidate the potent anti-inflammatory effects of GCs and inflammatory signal pathways induced though TLRs in paranasal sinus epithelial cells.

MATERIALS AND METHODS

Specimens and culture technique

Specimens of paranasal sinus epithelium were obtained from seven patients (3 men and 4 women; mean age 29.1 years) undergoing endoscopic paranasal sinus surgery at the Hiroshima University Hospital, Hiroshima, Japan. They had all experienced paranasal sinus-related symptoms for longer than 3 months and showed opacities of the paranasal sinuses on CT. None of the patients were given topical or systemic steroids for at least three weeks prior to surgery. None of the patients had lower airway disease, such as bronchial asthma, chronic bronchiolitis, or diffuse panbronchiolitis.

We successfully established cell cultures of sinus epithelial cells by elimination of fibroblast contamination using a trypsinization technique and employment of defined serum-free medium. Briefly, the obtained epithelium was dissected from the underlying submucosal tissue and the cells were collected using a cell scraper. They were then incubated in 0.1% collagenase at 37°C for about 1 hour. Following cell dissociation and centrifugation, the resulting cell pellet was resuspended in culture medium and seeded onto 75 mm^2 cell culture flasks precoated with type-I collagen (Asahi Techno Glass, Tokyo, Japan). The culture medium consisted of Defined Keratinocyte-SFM (Invitrogen, Carlsbad, CA, USA) with growth supplement and antibiotics. The epithelial cells were allowed to adhere to the flasks at 37°C 5% CO_2 for 48 hr, after which medium was changed every 2-3 days. Upon reaching cell confluence, a secondary culture of the epithelial cells was performed.

In order to standardize for both the period and nature of the cultures, the third generation of sinus epithelial cell cultures from each subject was used. Cultured cells that had reached the confluence state were stimulated for 2 hours with LPS (10 g/ml-100 ng/ml) or PGN (1 mg/ml -10 g/ml). Some of the cultured cells were also pretreated with dexamethasone (DEX) 10^{-5}M for 48 hours before stimulation to observe the effects of topical steroids. The study protocols were reviewed and approved by the local ethics committee at the University Hospital, Hiroshima (No. 459).

Evaluation of NF-κB activation

The degree of NF-κB activation was assessed using the Trans AM assay™ (Active Motif, Carlsbad, CA, USA). The Trans AM assay quantifies NF-κB activity using the enzyme-linked immunosorbent assay (ELISA) format [9]. Nuclear extracts of cultured cells were prepared by scraping the cells off the dish and homogenizing the cell pellet in Lysis Buffer. After centrifugation, the supernatant layer with nuclear cell extract was obtained and the protein concentration was measured using Coomassie Protein Assay Reagent Kit (Coomassie protein assay, Pierce, Rockford, IL, USA). An aliquot of the extract was then applied to a 96-well plate well coated with NF-κB consensus oligonucleotides. After incubation with primary and secondary antibodies, a colorimetric reaction was performed. The absorbance of the specimens was measured using Multiskan MS (Thermo Scientific, Yokohama, Japan) at 450 nm, with a reference wavelength of 655 nm. The degree of NF-κB p50 subunit activation was standardized and expressed as OD 450nm-655nm per 10 g cell protein.

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 MgCl2, and 0.5% Triton X-100 for 30 minutes. Rabbit polyclonal anti-p50 antibodies (sc-114; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-TLR2 antibodies (sc-10739, Santa Cruz Biotechnology), rabbit polyclonal anti-TLR4 antibodies (sc-10741, Santa Cruz Biotechnology), and rabbit polyclonal anti-GRα antibodies (sc-1002, Santa Cruz Biotechnology) were applied as primary antibodies in PBS containing 0.1% bovine serum albumin. Specimens were stored overnight at 4°C in a humid chamber and then washed in PBS. The specimens were then incubated with Alexa-Fluor488 conjugated goat anti-rabbit immunoglobulin (5 mg/ml, Molecular Probes, Eugene, OR, USA) for 1 hour in the dark. The stained cells were observed with a Nikon fluorescence microscope (Eclipse E600), and digital photomicrographs were taken for analysis.

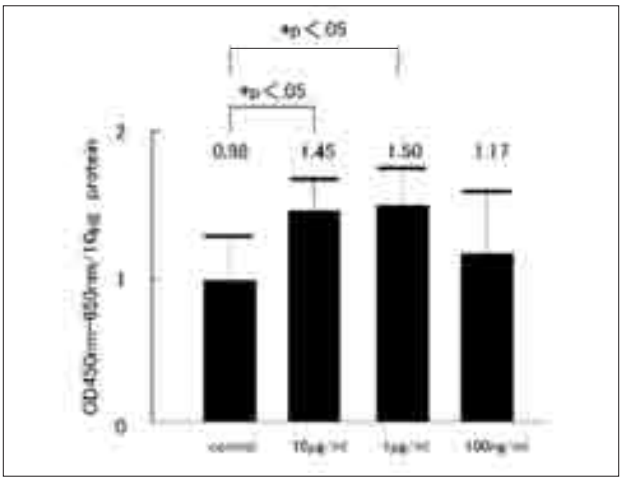

Figure 1. Reactive activation levels of the NF-κB p50 subunit after LPS stimulation in cultured sinus epithelial cells obtained from patients with chronic sinusitis. The cells were treated with LPS for 2 hours and the activity level was expressed as OD 450nm-650nm per 10 g protein. Compared to the control level, the degree of p50 activation increased significantly after LPS stimulation (10 g/ml, 1 g/ml).

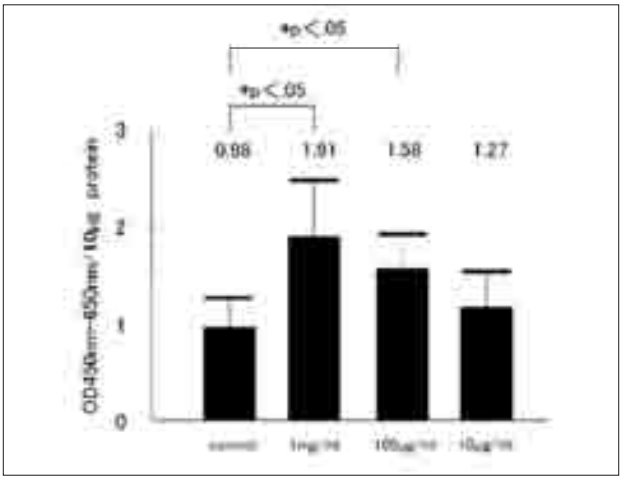

Figure 2. Reactive activation levels of the NF-κB p50 subunit after PGN stimulation in cultured sinus epithelial cells obtained from patients with chronic sinusitis. The levels are expressed as difference in the absorbance per 10 g cell protein. Compared to the control level, the degree of p50 activation increased significantly after LPS stimulation (1 mg/ml, 100 g/ml).

Statistical Analysis

The p50 activity levels were expressed as means ± SEM. Data were compared by the Mann-Whitney U test for between-group analysis. Statistical analysis was carried out with the statistical software package STATMATE (ATMS, Tokyo, Japan) for Windows. Probability values of $p < 0.05$ were accepted as significant.

RESULTS

NF-κB activity levels in cultured sinus epithelial cells

The use of Defined Keratinocyte-SFM- and type I collagen-coated dishes showed superior cell growth of primary sinus epithelial cells while maintaining epithelial morphology and biological markers. Indeed, while the primary cultures showed inter-individual variability in growth rates, cultured cells generally reached confluence within 14 days following isolation.

The NF-κB p50 activity levels of the cultured sinus epithelial cells were measured using the Trans AM assay™. Figure 1 shows the effect of LPS (10 g/ml-100 ng/ml) stimulation on the p50 activity levels in cultured cells. The p50 activity levels increased promptly after LPS stimulation in a dose-dependent manner. Compared to the control group, the p50 activation levels significantly increased from 0.98 to 1.45 and 1.50 after 10 g/ml and 1 g/ml LPS stimulation, respectively. Figure 2 shows the effect of PGN (1 mg/ml -10 g/ml) stimulation on the p50 activity levels in cultured cells. The cells were treated with PGN for 2 hours and the activity level was expressed as stated above. The p50 activity levels increased after PGN stimulation in a dose-dependent manner. Compared to the control group, the p50 levels significantly increased from 0.98 to 1.91 and 1.58 after 1 mg/ml and 100 g/ml PGN stimulation, respectively. Pretreatment with DEX 10^{-5}M for 48 hours effectively suppressed the augmentation induced by 100 ng/ml LPS and 10 g/ml PGN (Figure 3). The p50 activation levels significantly decreased from 1.17 to 0.70 for LPS and from 1.27 to 0.75 for PGN, and both levels remained attenuated compared to that of the control group.

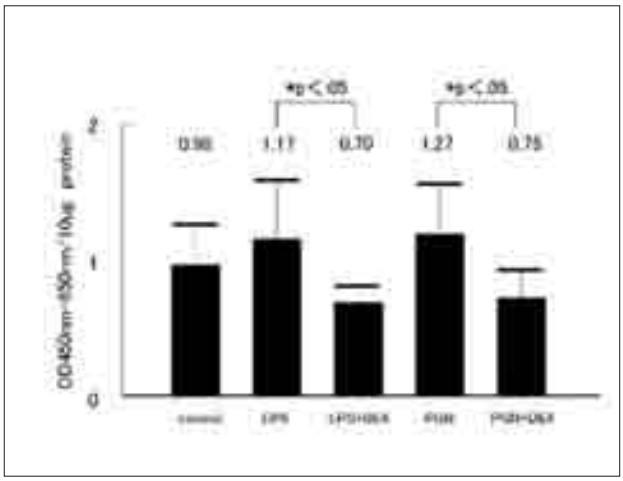

Figure 3. Reactive activation levels of the NF-κB p50 subunit after LPS (100 ng/ml) and PGN (10 g/ml) stimulation with dexamethasone (10 M) pretreatment in cultured sinus epithelial cells obtained from patients with chronic sinusitis. Pretreatment with dexamethasone (10 M) significantly suppressed the p50 activation induced by LPS (100 ng/ml) and PGN (10 g/ml).

Immunocytochemical localization of TLR4, TLR2, and GR

Cellular localizations of GR, TLR2, and, TLR4 in cultured paranasal sinus cells were analyzed using fluorescence microscopy. Figure 4 shows immunocytochemical localization of GR in the cultured cells. We observed uniform expression of GR protein in cultured cells under all conditions.

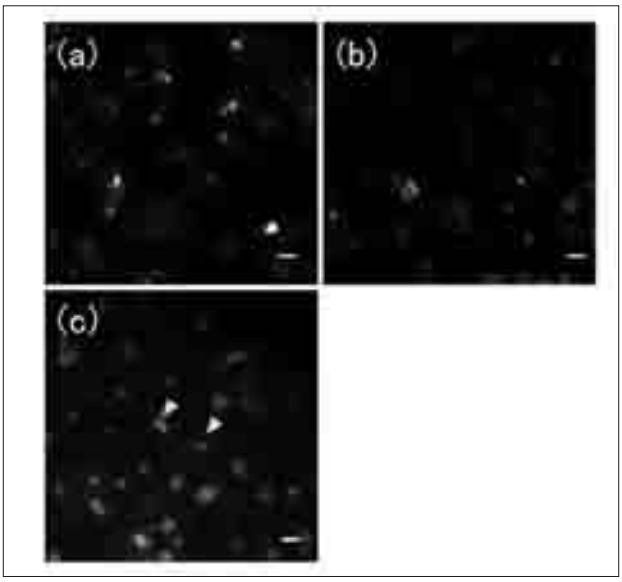

Figure 4. Immunocytochemistry of GR localization in paranasal sinus epithelial cells. All paranasal sinus epithelial cells expressed the GR protein. GR depletion from the cytoplasm was associated with a corresponding increase in GR levels into the nucleus.
(a) Diffuse cytoplasmic GR staining without nuclear translocation observed in the epithelial cells prior to treatment. (b) Diffuse cytoplasmic GR staining without nuclear translocation observed in the epithelial cells 2 hours after LPS (100 ng/ml) stimulation. (c) Dominant nuclear translocation of GR observed 2 hours after LPS (100 ng/ml) stimulation with dexamethasone 10 M pretreatment (arrowheads). Bar = 10 m.

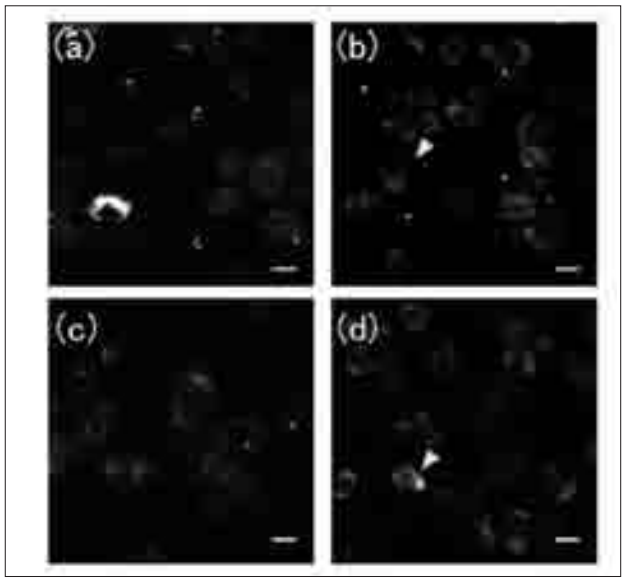

Figure 5. Immunocytochemistry of TLR2 and TLR4 localization in paranasal sinus epithelial cells. Some paranasal sinus epithelial cells expressed TLR2 and TLR4 proteins weakly. TLR2 and TLR4 immunoreactivity was relatively high after 48h DEX treatment. (a) Diffuse cytoplasmic TLR2 staining observed in the cytomembrane of epithelial cells prior to treatment weakly. (b) TLR2 staining upregulated in the cytomembrane of epithelial cells after DEX treatment (arrowhead). (c) Cytoplasmic TLR4 staining observed in the cytomembrane of epithelial cells prior to treatment weakly. (d) TLR4 staining upregulated in the cytomembrane of epithelial cells after DEX treatment (arrowhead). Bar = 10 μm.

Meanwhile, GR depletion from the cytoplasm induced by DEX 10^{-5}M pretreatment was associated with a corresponding increase in GR levels into the nucleus (Figure 4c). Figure 5 shows immunocytochemical localization of TLR2 and TLR4. Some paranasal sinus epithelial cells expressed TLR2 and TLR4 on the surface of the cytomembrane prior to treatment weakly. TLR4 expression is very weak compared with TLR2 expression. TLR2 and TLR4 immunoreactivity was increased after 48h DEX treatment.

DISCUSSION

Chronic sinusitis is a common disease defined as a paranasal sinus inflammation that has persisted for longer than 3 months. Several kinds of inflammatory agents such as microbes, fungi, viruses and their breakdown products are recognized in the diseased cavity. These inflammatory agents come in contact with the epithelial layer, and continuous stimulation occurs. Accordingly, the paranasal sinus epithelial cells have a first line role in the airway's defence against these inflammatory agents. In addition, epithelial cells have recently been found to act as an immunological organ and sustain the release of inflammatory cytokines that respond to these inflammatory agents [1,2,10]. Therefore, it is likely that paranasal sinus epithelial cells have crucial roles with respect to inflammatory responses in human paranasal sinuses.

Recently, it has become known that innate immunity to several microbes is activated through TLRs. To date, 10 kinds of human TLRs have been identified [1,4]. TLR signaling is known to be very diverse and is likely to have a high degree of pathogen specificity [11]. In this study, TLR2 and TLR4 were found to be weakly expressed in cultured paranasal sinus cells. Both of them have been identified as signaling receptors activated by bacterial cell wall components. TLR2 responds to stimulation by Gram-positive bacterial and mycobacterial cell wall components, such as PGN and lipoteichoic acids. TLR4 responds to stimulation by Gram-negative bacterial cell wall components including LPS. Dong et al. [3] reported that TLR2 and TLR4 mRNA were significantly up-regulated in human nasal mucosal epithelial cells of the inferior turbinate under infectious conditions as measured by in situ hybridization. Ooi et al. [12] reviewed recent findings about a role of innate immunity in the paranasal sinuses. In their review nasal epithelial cells also can serve as antigen-presenting cells and amplify the inflammatory response at the local tissue level through an innate immune reaction. Lane et al. [1] reported that the degree of TLR2 mRNA expression in the ethmoid mucosa of patients with chronic rhinosinusitis was higher than that of normal controls. They concluded that the results likely reflect the underlying pathophysiological mechanism of the onset of chronic rhinosinusitis; that is, pathogens including TLR2 ligands may trig-

ger an abnormal immune response at the mucosal surface. Rampey et al. [13] reported that they observed minimal dendritic cell staining of TLR2 and TLR4 in paranasal epithelial surface with increased subepithelial TLR2 and TLR4 stainings. Their results indicated that the robust inflammatory cascade may be driven within the sinonasal mucosa by TLR and dendritic cell activation. Moreover, Schleimer [2] reported that in the nasal cavity, where microbial interaction is frequent, there is likely to be negative regulation of TLR activation and development of tolerance to normal upper flora. Thus TLR expression in paranasal epithelium is thought to be different at each inflammation state of a paranasal sinus. TLR may play an important role in signaling pathways involved in both immunity and inflammation of paranasal sinuses.

In this study, the levels of NF-kB p50 subunit activity were measured as an index of the inflammatory response through the TLR pathway of paranasal sinus epithelial cells. TLRs are known to activate the NF-kB pathway through MyD88, IL-1 receptor-associated kinase, and tumor necrosis factor-associated factor-6 [14]. NF-kB is a representative transcription factor that is involved in the gene expression of several cytokines and receptors related to various inflammatory states. They include proinflammatory cytokines, chemokines, adhesion molecules, and inducible enzymes. In the nose and paranasal sinus mucosa, constitutive expression of NF-kB activity has been reported. Takeno et al. [7] reported that the NF-kB p50 subunit was observed mainly in the epithelial layer of nasal polyps, and the presence was well correlated with the degree of IL-8, IL-16, and eotaxin mRNA expressions. Osada et al. [15] reported that the expression of NF-kB p50 and p65 subunits in cultured human paranasal sinus epithelial cells was upregulated in allergic rhinitis (AR) patients compared to non-AR patients. These reports indicate that augmented NF-kB activation is closely related to the inflammatory state of the paranasal sinus system. In this study, the levels of p50 activation were significantly increased in a dose-dependent manner after stimulation by LPS and PGN. LPS and PGN are mycobacterial cell wall components commonly present in the paranasal fluid obtained from sinusitis patients [10]. Thus, LPS and PGN in paranasal fluid are thought to directly stimulate the paranasal sinus epithelial cells. Our results suggest that LPS and PGN may participate in the high cytokine release in human paranasal sinuses. As a result of this noxious cascade following the onset of chronic sinusitis, continuous expressions of inflammatory cytokines may lead to a prolongation of the disease.

Glucocorticoids (GCs) are widely expressed in various kinds of cells including epithelial cells and infiltrating cells located in the nose and paranasal sinuses. Therefore, the anti-inflammatory effect of GCs directly impacts the nasal mucosa. Glucocorticoids are known to have potent anti-inflammatory and anti-allergic effects based on their ability to regulate transcription factors that initiate gene activation of inflammatory cytokines, adhesion molecules, and inflammatory mediators. So far, there is limited knowledge about the effects of GCs on local innate immunity, and recent reports have shown that GCs inhibit concomitant inflammatory responses while sparing or enhancing local innate host defense responses [8]. Homma et al. [16] reported that the addition of Dexamethasone (DEX) to a combination of TNF-α and IFN-γ synergistically upregulated TLR2 expression in the BEAS-2B cell line. They also found that nontypeable *Haemophilus influenzae* (NTHi) activated both NF-κB and p38 MAPK pathways in the cells, the former acting positively and the latter negatively in regulating TLR2 expression synergistically. DEX upregulated NTHi-induced TLR2 expression via induction of MAPK phosphotase-1. They concluded that these findings suggest the presence of different signalling pathways or transcriptional control mechanisms by which DEX synergistically enhanced cytokine-driven and NTHi-driven TLR2 expression. In another study, Zhang et al. [8] reported that human primary bronchial epithelial cells manifested a local "acute phase response" after stimulation with TLR activation and TNF-α. GC spared or enhanced the epithelial expression of molecules that are involved in host defences, including complement, collectins, and other antimicrobial proteins. They also demonstrated that the selective effects of GCs are mediated through activation of the transcription factor CCAAT/enhancer binding protein (C/EBP) using Western blotting and EMSA. These results suggest that GCs spare or enhance local innate host defence responses in addition to exerting anti-inflammatory actions. In our study, pretreatment with DEX successfully inhibited the inflammatory process at the transcriptional phase in the sinus epithelial cell culture model. TLR2 and TLR4 immunoreactivity was augmented after 48h DEX treatment. These multifunctional aspects of GC in paranasal sinus epithelial cells are consistent with the findings of a previous report [8] and clearly indicate that GCs are capable of not only anti-inflammatory effects via NF-κB transcription factor inhibition but also enhancement of the innate host defense.

In conclusion, we successfully established sinus epithelial cell cultures and examined changes in NF-κB p50 activity levels after LPS and PGN stimulation using the Trans AM assay™. The increase of p50 levels after LPS and PGN stimulation suggests that TLR2 and TLR4 ligands present in paranasal sinus fluid may promote the migration of inflammatory cells into the sinus mucosa through local induction of high cytokine expression. The results also substantiate the possibility that constitutive NF-kB activation plays a key role in both the onset and prolongation of chronic sinusitis along its natural course. Glucocorticoids effectively inhibited the activation response at the transcriptional phase in the cultured epithelial cells. In addition, TLR2 and TLR4 immunoreactivity in the cell was relatively increased after GC treatment. We conclude that in paranasal sinus epithelial cells, GCs not only have potent anti-inflammatory effects but also enhance innate host defences.

Further studies are needed to elucidate the effect of GCs in the human paranasal sinus system at the transcription phase of other factors such as AP-1 and C/EBP.

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