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Superantigen-Induced Glucocorticoid Insensitivity in the Recurrence of Chronic Rhinosinusitis with Nasal Polyps

Mingming Wang, PhD^{1*}, Peng Shi, PhD^{2*}, Bei Chen, PhD³,
Guanggang Shi, MD¹, Hong Li, MS¹, and Haibo Wang, MD¹

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Abstract

Objective. To investigate a potential mechanism by which superantigens could induce glucocorticoid insensitivity in chronic rhinosinusitis (CRS) patients.

Study Design. Prospective cohort study.

Setting. Tertiary medical center.

Subjects and Methods. Sinonasal polyps were obtained from CRS patients with nasal polyps (CRSwNP; 20 without recurrence, 18 with recurrent NP followed for 1.5–2.0 years) and nasal mucosa from 16 CRS patients without nasal polyps (CRSsNP). Specimens were tested by enzyme-linked immunosorbent assay for staphylococcal exotoxins (SEs) including SEA, SEB, SEC, SED, and toxic shock syndrome toxin type-I (TSST-I) and assessed by immunohistochemistry for glucocorticoid receptor (GR) α and β , and the GR β /GR α ratio was analyzed.

Results. In CRSwNP, 13 of 18 (72.22%) subjects with subsequently recurrent NP, 11 of 20 (55.00%) subjects without NP recurrence, and 1 of 16 (6.25%) CRSsNP subjects with positive reactions for SEs were obtained. There were no positive results in controls. The expressions of GR β in 3 CRS groups and controls were significantly different (all $P < .05$), and a similar increasing tendency of the GR β /GR α ratio was found among groups besides the comparison of CRSwNP versus recurrent NP groups ($P = .053$). Furthermore, there was a clear trend of increased GR β expression in the enzyme-linked immunosorbent assay (ELISA)-positive samples compared with ELISA-negative samples. Concerning GR α , the expression was enhanced significantly just in toxin-positive recurrent NP versus controls ($P = .048$), but the relative induction of GR β was much higher, thereby leading to a higher GR β /GR α ratio.

Conclusions. Bacterial superantigens may contribute to glucocorticoid insensitivity through induction of GR β , which appears to be a marker of steroid insensitivity in CRSwNP.

Keywords

chronic rhinosinusitis, nasal polyps, superantigens, glucocorticoid resistance, glucocorticoid receptor

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Chronic rhinosinusitis (CRS) is the most common chronic disease. In addition to economic burden, it imposes considerable impact on the quality of life of patients. Clinically, CRS can be broadly categorized into CRS without or with nasal polyps (CRSsNP or CRSwNP). CRSsNP with poor disease control would develop to CRSwNP, and CRSwNP with recurrence potential is considered to be a more severe form of the disease and tends to be more refractory to both medical and surgical intervention. Glucocorticoid treatment is widely advocated to control CRS inflammation and growth. However, a subset of patients does not respond to the maximal treatment, showing progression of disease despite steroid treatment. Clinical improvement with potent glucocorticoids is observed only after prolonged periods of treatment. Given these observations, there is a need to decipher the mechanisms and molecular basis of glucocorticoid insensitivity in CRS.

More recently, studies on glucocorticoid resistance have proposed that the abnormal expression level of glucocorticoid receptor (GR), especially the overexpression of the GR β subtype that acts as a dominant inhibitor of functional GR α to inhibit glucocorticoid action, is functionally involved in

¹Department of Otorhinolaryngology—Head and Neck Surgery, Provincial Hospital affiliated to Shandong University, Jinan, China

²Department of General Surgery, Provincial Hospital affiliated to Shandong University, Jinan, China

³Department of Otorhinolaryngology—Head and Neck Surgery, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania, USA

*Drs Wang and Shi contributed equally to this article.

Corresponding Author:

Haibo Wang, MD, Department of Otorhinolaryngology—Head and Neck Surgery, Provincial Hospital affiliated to Shandong University, 5th Longitude Road #324, Jinan 250021, China
Email: shipwmm@mail.sdu.edu.cn

steroid resistance.¹⁻⁴ However, there is controversy regarding the putative role of GR β .^{5,6} Furthermore, the mechanisms that induce the overexpression of GR β remain poorly understood. Staphylococcal exotoxins (SEs), known as superantigens, bind directly without antigen processing to MHC class II molecules on antigen-presenting cells and elicit a potent T-cell activation. Recently, T-cell receptor repertoire analysis of peripheral blood mononuclear cells (PBMCs) and in vitro cells from local chronic inflammation demonstrated SEs, as disease modifiers, contributed to the pathogenesis of severe bronchial asthma and CRSwNP.^{7,8} We hypothesized that a similar overexpression might be present in nasal polyp (NP) inflammatory cells and associated with steroid insensitivity. Thus, to better understand persistent glucocorticoid resistance in nasal polyps as well as to assess whether this inflammatory disease is associated with the microbial superantigens and the dysregulation of GR α or GR β , we analyzed directly the presence of SEs and the entire expressions of GR α and GR β at the protein level in the NP instead of explant culture or peripheral blood model.

Methods

Study Population

A total of 54 consecutive adult patients with CRS undergoing endoscopic sinus surgery and 12 normal controls were recruited in this study. Only patients who met the published criteria for CRSwNP or CRSsNP⁹ with confirmed postoperative histopathologic diagnosis and accepted regularly postoperative glucocorticoid medication (flixonase for at least 3 months) followed up to 1.5 to 2.0 years were included in the study. The control group consisted of 12 adult volunteers in generally good health with no history of nasal or sinus disease. All subjects failed adequate trials of conservative medical therapy (prolonged antibiotic regimens, nasal and oral steroids, saline irrigations, and decongestants) for control of symptoms. All medications were ceased at least 6 weeks before biopsies. Subjects with anatomic abnormal sinus or any immunodeficiency were excluded. This study was approved by the Clinical Otorhinolaryngology Division of Provincial Hospital affiliated to Shandong University, and informed consent was obtained from all subjects.

Polyp tissue obtained from patients with CRSwNP and sino-nasal mucosa samples from the middle meatus with CRSsNP were collected at the time of sinus surgery. Nasal mucosal biopsies from the middle turbinate served as the control tissue from patients in the control group. Duplicate tissue samples were obtained from each patient. A portion was processed for toxin detection using an enzyme-linked immunosorbent assay (ELISA) technique. The second surgical biopsy was immediately fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin, and sectioned into 4- μ m slices for the routine hematoxylin and eosin (H&E) staining and immunohistochemical staining.

ELISA Procedures

Freshly obtained tissue specimens from 66 subjects were weighed, and 1 mL of 0.9% NaCl solution was added per 0.1 g of tissue. The tissue was then homogenized with a mechanical

homogenizer (B. Braun, Melsungen, Germany) at 1000 rpm (120g) for 5 minutes on ice. After homogenization, the suspensions were centrifuged at 3000 rpm (1560g) for 10 minutes at 4°C, as noted by Bachert et al.¹⁰ All supernatants were assayed for SEs (SEA, SEB, SEC1-C3, SED, and toxic shock syndrome toxin type-1 [TSST-1]) by means of ELISA with commercially available kits (Gene Tex Inc, San Antonio, Texas) as described by Wang et al.¹¹ The absorbance was read on a plate reader at 450 nm. Samples with an absorbance more than twice that of the negative control were considered positive for that toxin. The negative control was made of 0.05% PBS-Tween. All control and test samples were run in triplicate.

Immunohistochemistry

Slides were deparaffinized in xylene and then rehydrated through graded levels of ethanol. Endogenous peroxidase activity was neutralized with 3% hydrogen peroxide. The slides were then added to normal goat serum in 1% bovine serum albumin to block nonspecific antibody binding. Commercially available rabbit polyclonal antibodies specific for human GR α or GR β and no cross-reactivity against GR β or GR α (Abcam plc, Cambridge, UK) were each added at a concentration of 10 μ g/mL, respectively. Slides were incubated overnight at 4°C. After washing in phosphate-buffered saline, the sections were blocked with biotin-labeled anti-rabbit IgG (Zymed Laboratories, San Francisco, California) for 15 minutes at 37°C. The avidin-biotin complex method (Zymed Laboratories) was then used for secondary antibody labeling. Antigen-antibody complexes were visualized using 0.075% 3',3'-diaminobenzidine tetrachloride and 0.002% hydrogen peroxide as a chromagen. Slides were lightly counterstained with Harris hematoxylin, dehydrated through graded ethanol, and then coverslipped. Negative control was also evaluated with the use of secondary antibody alone on the study specimens. All of the preparations were counterstained with hematoxylin and mounted prior to examination. One section from each paraffin-embedded specimen was stained with H&E.

To avoid observer bias, slides were coded before analysis and examined in a blinded fashion by 2 independent pathologists using an Olympus microscope (Tokyo, Japan). The graticule (0.2 mm²) was oriented beneath the epithelial basement membrane, and cells positive for GR α or β were counted along the whole length of the biopsy. At least 2 sections in 10 randomly selected fields were counted (final magnification, 400 $\times$) for each patient at each time point, and the results were expressed as the mean number of positive cells per mm² $\pm$ the standard error of the mean (SEM).

Statistical Analysis

All statistical analyses were completed using SPSS version 16.0 statistical software (SPSS Inc, Chicago, Illinois). Measurements of GR α - and GR β -positive cells among the study groups were analyzed by 1-way analysis of variance, and the least significant difference (LSD) multiple comparisons were used for testing the differences between each of the 2 groups. SE-positive results among groups were analyzed by χ^2 test. *P* values of less than .05 were considered significant.

Table 1. Baseline Characteristics of Subjects and ELISA Results^a

	CRSsNP (n = 16)	CRSwNP (n = 20)	Recurrent NP (n = 18)	Control (n = 12)
Demographics				
Mean age, y	37.44 (17-68)	41.85 (16-60)	42.50 (17-65)	28.42 (17-45)
Male sex	9 (56.25%)	14 (70.00%)	13 (72.22%)	7 (58.33%)
Allergies	6 (37.50%)	9 (45.00%)	10 (55.56%)	0
Asthma	2 (12.50%)	3 (15.00%)	4 (22.22%)	0
ELISA				
Positive proportion	6.25% (1/16)	55.00% (11/20)	72.22% (13/18)	0.00% (0/12)
SEA	0	2	2	0
SEB	1	6	6	0
SEC1-C3	0	0	0	0
SED	0	1	2	0
TSST-I	0	4	4	0

Abbreviations: CRSsNP, chronic rhinosinusitis patients without nasal polyps; CRSwNP, chronic rhinosinusitis patients with nasal polyps; ELISA, enzyme-linked immunosorbent assay; NP, nasal polyps; TSST-I, toxic shock syndrome toxin type-I.

^aIn the CRSwNP group, 2 of those 11 subjects who tested positive for the presence of toxin were positive for 2 toxins (TTST-I and SEB, SEA and SED). In the recurrent NP group, 1 of those 13 subjects was positive for SEB and SED simultaneously.

Results

Baseline Findings

Fifty-four adult patients with CRS were followed for 1.5 to 2.0 years, and 12 healthy individuals were recruited for this study. These 66 subjects were divided into 4 groups: (1) CRSsNP group (n = 16), in which patients remained without NP until the end of the follow-up period; (2) CRSwNP group (n = 20), in which patients remained without recurrence of NP for the duration of the follow-up period of 1.5 to 2.0 years; (3) recurrent NP group (n = 18), in which patients initially presented with CRSwNP and had recurrence of their NP within the follow-up period; and (4) control group (n = 12), in which all subjects were healthy. **Table 1** summarizes the demographics of patients in the study.

ELISA Results

A positive response to staphylococcal toxins was detectable in tissue homogenates of 13 of 18 patients (72.22%) with NP recurrence in the subsequent follow-up period. In 11 of 20 patients (55.00%) in the CRSwNP group, and in 1 of 16 (6.25%) patients in CRSsNP group, positive reactions were obtained. Moreover, 3 of those 25 subjects who tested positive for the presence of toxins were positive for 2 toxins simultaneously. There were no positive findings in the control group (**Table 1**). The positive rate of detectable SEs was significantly higher in the CRSwNP and recurrent NP groups compared with the CRSsNP group (both $P < .05$). Although the positive rate of SEs was higher in the recurrent NP group compared with the CRSwNP group, the difference did not reach statistical significance ($P > .05$).

GR α and GR β Analysis

GR β expression in the 3 groups of CRS patients with a different prognosis of disease and controls was quantified. Positive

staining for GR β could be observed in all samples in the cytoplasm and nucleus of epithelium, glandular, and inflammatory cells, especially in the cytoplasm of epithelium and glandular cells (**Figure 1**). Analysis of the variants by LSD multiple comparisons demonstrated that the numbers of GR β^+ cells were significantly different among the 4 groups (all $P < .05$; **Figure 2**). The subjects with CRSwNP and recurrent NP were divided into 2 subgroups respectively on the basis of the results of ELISA: those with toxin-positive results and those with toxin-negative results. LSD multiple comparisons were performed on the variable expression of GR β^+ cells, which demonstrated that the numbers of GR β^+ cells in toxin-positive recurrent NP tissue were significantly higher compared with that in toxin-negative CRSwNP subjects ($P = .009$). The GR β expression showed an increasing tendency in patients with toxin-positive CRSwNP paralleling that of toxin-negative CRSwNP, although the difference did not reach statistical significance ($P = .099$), and a similar increasing tendency was found in the toxin-positive and -negative subgroups of those with recurrent NP (**Figure 3**). Concerning GR α , except for negative controls, the immunoreactivity could be found in the cytoplasm and nucleus of epithelium, glandular, and inflammatory cells, especially in the cytoplasm of epithelium and glandular cells in all subjects (**Figure 4**), and there were no statistically significant differences among these 4 groups (all $P > .05$). However, GR α expression was enhanced significantly in toxin-positive recurrent NP versus controls ($P = .048$; **Table 2**).

Analysis of the variable GR β /GR α ratio of the 4 groups demonstrated that the mean ratios in the CRSsNP and control groups were significantly different compared with that of the CRSwNP or recurrent NP group (all $P < .05$), and there was no significant difference between CRSwNP versus recurrent NP ($P = .053$). Further analysis of the variable GR β /GR α ratio was performed to test the differences between each of the

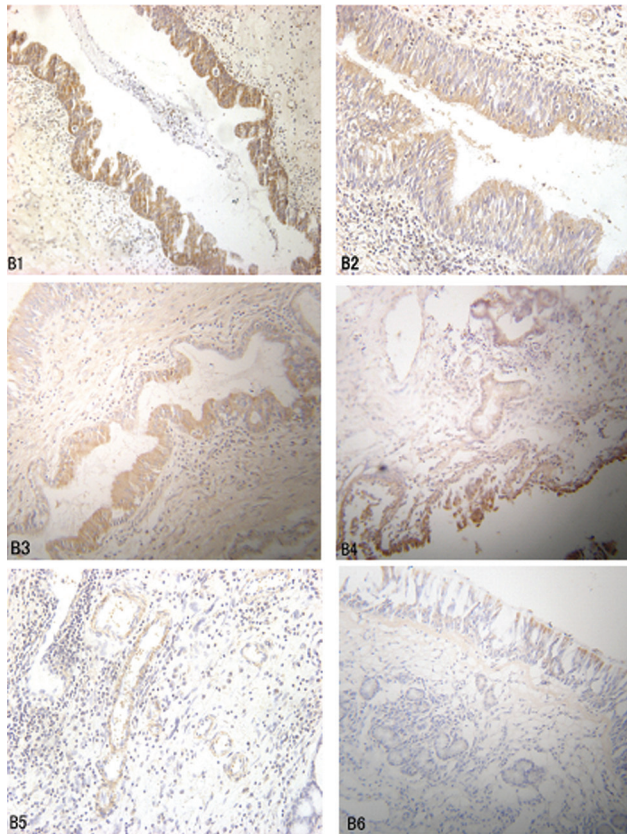


Figure 1. Immunohistochemical staining for glucocorticoid receptor (GR) β^+ cells in the nasal tissue in recurrent nasal polyps (NP) with superantigen presence (B1) and superantigen-negative (B2), chronic rhinosinusitis (CRS) patients with nasal polyps (CRSwNP) without recurrence with superantigen-positive (B3) and superantigen-negative (B4), respectively, CRS patients without nasal polyps (CRSsNP) without NP occurred followed up to 1.5 to 2.0 years (B5), and in the controls (B6). Note the increased cells expressing GR β mainly in the kytoplasm of the epithelium and glandular cells presenting brown grains (original magnification 200 $\times$).

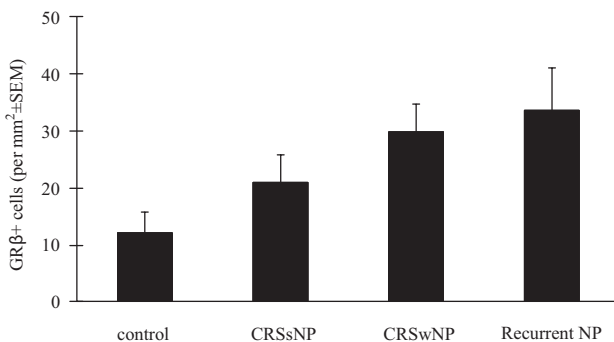


Figure 2. Glucocorticoid receptor (GR) β immunoreactivity in nasal tissue. The numbers of GR β^+ cells were significantly different among the 4 groups.

2 groups among the 6 groups, including toxin-positive and -negative subgroups of CRSwNP and recurrent NP. The mean ratio in toxin-positive recurrent NP was significantly higher

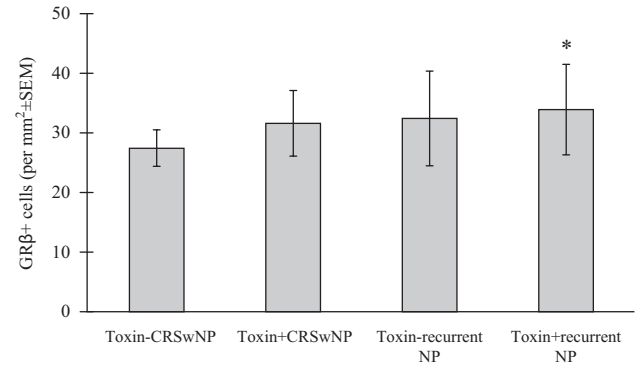


Figure 3. Glucocorticoid receptor (GR) β immunoreactivity in subgroups of chronic rhinosinusitis (CRS) patients with nasal polyps (CRSwNP) and recurrent NP. * $P < .05$ versus toxin-negative CRSwNP.

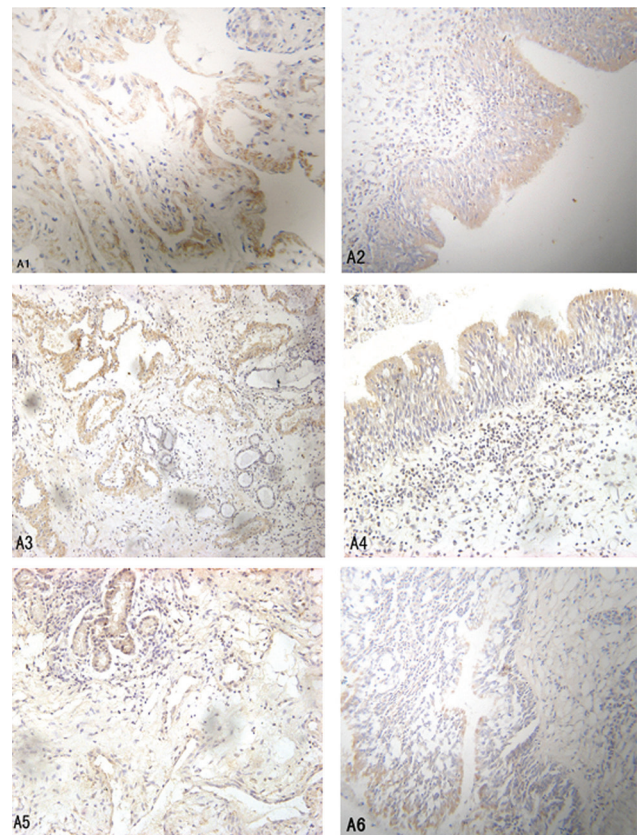


Figure 4. Immunostaining for glucocorticoid receptor (GR) α mainly expressing in the kytoplasm of the epithelium and glandular cells with brown grains (original magnification 200 $\times$). A1, recurrent nasal polyps (NP), superantigen positive. A2, recurrent NP, superantigen negative. A3, superantigen-positive chronic rhinosinusitis (CRS) patients with NP (CRSwNP) without recurrence. A4, superantigen-negative CRSwNP without recurrence followed up to 1.5 to 2.0 years. A5, CRS patients without NP occurred up to follow-up period end. A6, an example of normal controls.

than that in toxin-negative CRSwNP ($P = .03$). However, there were no significant differences between the toxin-positive and toxin-negative subgroups of CRSwNP ($P = .173$), as well as

Table 2. Means of GR-Positive Cells in Samples (per mm² ± SEM)^a

Group	n	GRα ⁺ Cells	GRβ/GRα
Control	12	16.47 ± 3.83	0.76 ± 0.28 ^b
CRSsNP	16	16.86 ± 4.63	1.27 ± 0.24 ^b
CRSwNP	20	19.70 ± 4.15	1.54 ± 0.34
Toxin negative	9	19.09 ± 3.24	1.44 ± 0.29
Toxin positive	11	20.20 ± 4.87	1.63 ± 0.37
Recurrent NP	18	19.72 ± 4.92	1.74 ± 0.34
Toxin negative	5	18.71 ± 4.63	1.75 ± 0.31
Toxin positive	13	20.11 ± 5.15 ^c	1.73 ± 0.36 ^d

Abbreviations: GR, glucocorticoid receptor; CRSsNP, chronic rhinosinusitis patients without nasal polyps; CRSwNP, chronic rhinosinusitis patients with nasal polyps; NP, nasal polyps.

^aPositive cells with GRα or GRβ were determined by immunohistochemical analysis by using the specific polyclonal antibodies and no cross-reactivity against GRβ or GRα. *P* values of less than .05 were considered significant.

^bThe mean difference compared with all groups is significant.

^cThe mean difference compared with the control group is significant.

^dThe mean difference compared with the toxin-negative CRSwNP subgroup is significant.

that in 2 subgroups of recurrent NP (*P* = .901). In addition, there was no statistical difference between CRSsNP and toxin-negative CRSwNP subjects (*P* = .192; **Table 2**).

Discussion

The anti-inflammatory and immunosuppressive properties of glucocorticoids are their major pharmacologic benefits and make them the most widely prescribed class of drugs. However, the response and sensitivity to glucocorticoids vary among individuals, tissues, and cell types.¹² In a subgroup of patients, clinical management of CRSwNP and other inflammatory conditions such as asthma and rheumatoid arthritis is becoming a major challenge, considering the resistance to glucocorticoid treatment. Hamilos et al¹³ reported an association between glucocorticoid insensitivity and the increased expression of GRβ. However, the molecular basis of glucocorticoid resistance and the mechanisms that trigger the overexpression of GRβ are not well understood. To date, only 3 reports have appeared in the literature¹⁴⁻¹⁶ that examined an explant culture and PBMC models to analyze indirectly the relationship between microbial superantigens and GRβ in the induction of glucocorticoid insensitivity. In this study, through analyzing directly the presence of superantigens and the expression level of GRβ in the nasal polyp, we investigated a potential mechanism by which microbial superantigens contribute to steroid insensitivity.

Understanding the mechanism by which superantigens contribute to poor disease control in superantigen-triggered chronic inflammatory diseases would be of great interest. In the current study, we first investigated directly whether SEs, instead of toxin-producing *Staphylococcal aureus*, presented in sinonasal polyp tissue and mucus of patients with an insensitivity to glucocorticoid treatment. Our data demonstrated that SEs were detectable in 72.22% of patients with recurrent NP and insensitivity to glucocorticoids, which was significantly higher than that in other patients in whom the development of the disease

could be controlled by glucocorticoid treatment. In addition, the positive rate (55.00%) in CRSwNP patients without recurrence was more than that in CRSsNP patients (6.25%). This study was designed to detect the 7 most common staphylococcal toxins (ie, SEA, SEB, SEC1-C3, SED, and TSST-1). The incidence of toxins in NP was likely to be higher considering there were at least 19 known staphylococcal superantigens. Our results revealed that glucocorticoid resistance was apt to occur in superantigen-stimulated patients.

Recent studies suggest that GRβ, as an endogenous inhibitor for the classic GRα, might play an important role in tissue sensitivity to glucocorticoids.^{17,18} GRβ, lacking the ability to bind glucocorticoids, can form heterodimers with hormone-bound GRα and thereby inhibit glucocorticoid action.⁴ High-level expression of GRβ would inhibit GRα binding affinity and transcription of glucocorticoid-responsive genes in a dose-dependent manner.³ The present study confirmed that GRβ expression was significantly higher in the recurrent NP versus the CRSwNP or CRSsNP group, suggesting that steroid resistance seems to be associated with induction of GRβ cells. To investigate a potential mechanism by which SEs induce glucocorticoid resistance, we assessed the expression of GRβ in SE-positive versus SE-negative nasal tissues, respectively, with glucocorticoid insensitivity or sensitivity. Our results demonstrated that the expression of GRβ exhibited an increasing tendency in toxin-positive patients with recurrent NP or CRSwNP paralleling that of toxin-negative recurrent NP or CRSwNP, respectively, although the differences did not reach statistical significance. Furthermore, the expression level of GRβ in superantigen-positive tissue of recurrent NP was significantly higher compared with that in superantigen-negative CRSwNP subjects. These findings from subjects with both sensitivity and insensitivity to glucocorticoids indicate that the expression of GRβ-positive cells would increase relatively in the nasal tissue with superantigens compared with superantigen-negative tissue. This may suggest a potential that microbial superantigens induce the increased GRβ, resulting in more insensitivity to the effects of glucocorticoids. Further studies comprising abundant cases will be required to identify the presumption.

In addition, previous studies have shown different findings about GRα expression levels in cases of glucocorticoid resistance.^{1,19-21} Therefore, we related the expression of GRα in an immunostaining study. Our results showed that expression of GRα was not significantly different among these groups. However, GRα expression was enhanced significantly in toxin-positive recurrent NP versus controls. We subsequently analyzed the variable GRβ/GRα ratio of the same sample in different groups. The results demonstrated that the increase of the ratios was similar to the variable GRβ. The highest increase of the mean ratio was observed in toxin-positive recurrent NP compared with that of toxin-negative CRSwNP, and there were no significant differences between toxin-positive and toxin-negative subgroups of CRSwNP, as well as in 2 subgroups of recurrent NP.

At present, glucocorticoids represent the most effective therapy to control CRS inflammation and NP growth. However, we

have observed a subgroup of patients whose illness conditions cannot be controlled by glucocorticoids. For these patients, glucocorticoid insensitivity appears to have merit and warrants further research into the potential mechanisms of molecular pathways. Further studies are needed to determine the correlation between GR β /GR α and superantigens in the model of glucocorticoid insensitivity and to elucidate the mechanisms of inducing steroid insensitivity in inflammatory cells.

Conclusion

The present study demonstrates that the role of microbial toxins is more likely to be found in patients with CRSwNP, especially in patients insensitive to glucocorticoids. This indicates that induction of the up-regulated GR β activity is a potential mechanism by which superantigens may cause glucocorticoid resistance in nasal tissue. Our findings shed new light on the issue relevant to steroid insensitivity in CRSwNP, which may, in turn, offer a promising therapeutic strategy for this disorder.

Author Contributions

Mingming Wang, study conduct; **Peng Shi**, data analysis; **Bei Chen**, revising the article; **Guanggang Shi**, data acquisition; **Hong Li**, data acquisition; **Haibo Wang**, corresponding author, conception and design.

Disclosures

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