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Changes of Glucocorticoid Receptor Expression in the Nasal Polyps of Patients with Chronic Sinusitis Following Treatment with Glucocorticoid

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Abstract. *Glucocorticoids (GCs) bind to the cellular glucocorticoid receptors (GRs) to exert anti-inflammatory and immunosuppressive actions. We investigated the changes in the expressions of the two GR isoforms, GR-α and GR-β, in nasal polyps treated with GC. Immunofluorescent staining revealed prominent expression of GR-α in the inflammatory cell infiltrate in the polyps obtained from patients with chronic sinusitis and bronchial asthma. Furthermore, while the expression of GR-α was significantly reduced following GC treatment, that of GR-β remained unchanged. The results of real-time PCR also revealed that the prominent expression of GR-α mRNA in the polyps decreased following GC treatment, while the expression of GR-β mRNA remained unchanged. The observations indicate that GR-α may play the major role in the inflammation associated with nasal polyps and the ratio of the expression level of GR-β to that of GR-α may serve as a useful index of the clinical efficacy of GC treatment.*

Chronic sinusitis complicated by bronchial asthma is often refractory to treatment, with a propensity to recur frequently, and many cases of chronic sinusitis complicated by bronchial asthma show nasal polyp formation associated with the inflammation, including the ethmoid sinus (1). It is widely recognized that the membrane of the paranasal sinus in cases of chronic sinusitis with bronchial asthma frequently shows prominent infiltration by eosinophilic leukocytes (2) and that treatment with glucocorticoids (GCs) is effective and produces improvement of the symptoms and clinical findings (3). GCs bind to glucocorticoid receptors (GRs) in the cells, undergo translocation to the nuclei to regulate the expression of genes encoding a variety of inflammatory proteins and exert anti-inflammatory and immunosuppressive actions. Two GR isoforms have been described, GR-α and GR-β; GR-α acts as a transcriptional control factor, whereas GR-β does not. Details of the expression levels of these 2 GR isoforms at the site of pathology in cases of chronic sinusitis and the changes in their expression levels following treatment of these patients with GCs remain unclear. In this study, we determined the expressions of GR-α and GR-β at the tissue and gene levels by immunofluorescent staining and real-time polymerase chain reaction (PCR) assay before and after GC treatment in patients with chronic sinusitis complicated by bronchial asthma showing nasal polyp formation.

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Key Words: Glucocorticoid, glucocorticoid receptor, nasal polyp, chronic sinusitis.

Patients and Methods

Patients. Ten patients (aged 29-67 years, with a mean of 55.9 years) with chronic sinusitis not complicated by bronchial asthma or allergic rhinitis (a non-complication group) and 10 patients (aged 38-68 years, with a mean of 52.9 years) with chronic sinusitis complicated by bronchial asthma (a complication group) were studied. Nasal polyp specimens were surgically resected from the patients who had given their written informed consent according to a study protocol approved by the Ethics Committee of Showa University. Patients who had not been treated with GCs for a minimum of one month before the surgery were enrolled in this study. All the patients underwent endoscopic sinus surgery (ESS) at Showa University Hospital between October 2003 and May 2004. In our hospital patients with severe chronic sinusitis undergo ESS separately on each side at an interval of 1 week, if they wish to undergo surgery under local anesthesia. All of the patients in this study underwent ESS under local anesthesia.

The patients with chronic sinusitis alone did not receive GC treatment. Patients with both chronic sinusitis and bronchial asthma, who had bilateral nasal polyps, underwent surgery twice. The set of polyps on one side was removed during the first operation and the patients received the following postoperative GC treatment after the first operation to prevent bronchial asthma attacks and mucosal edema at the site of the surgical

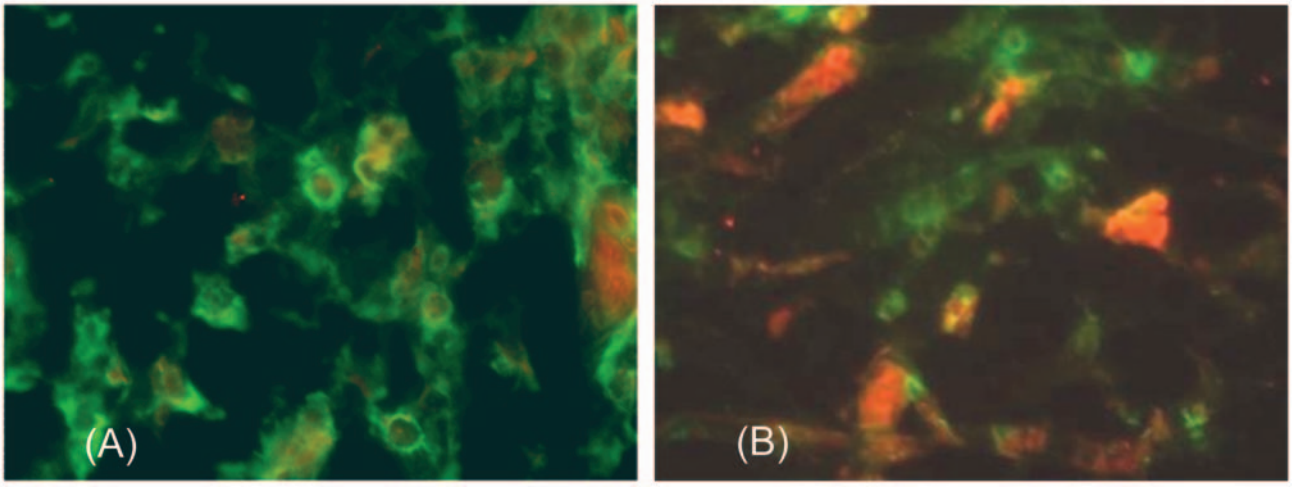

Figure 1. *Double immunofluorescent staining using anti-glucocorticoid receptor (GR)-α antibody and anti-major basic protein (MBP) antibody in a nasal polyp obtained from a patient with chronic sinusitis and bronchial asthma (the complication group). Original magnification x400. (A): Before glucocorticoid administration; and (B): after glucocorticoid administration. Green-colored cells: MBP immunoreactivity-positive cells; red-colored cells: GR-α immunoreactivity-positive cells; yellow-colored cells: MBP and GR-α immunoreactivity-positive cells.*

wound: betamethasone, 2 mg/day, intravenous drip, for the first 3 days, and Celestamine® (betamethasone 0.25 mg – *d*-chlorpheniramine maleate 2 mg/1 tablet; Schering-Plough Pharmaceutical Co. Ltd., Tokyo Japan), 2 tablets/day, for the next 3-4 days. The set of polyps in the nasal cavity on the other side was removed one week later.

Immunofluorescent staining. Tissue specimens were collected from the nasal polyps, embedded in TISSUE-TEK® O.C.T. Compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and immediately stored frozen at –80˚C. The frozen tissue was cut at 5 μm-thick sections with a cryostat. The tissue sections were blow-dried for 30 minutes and fixed in acetone. They were then immersed in 3% H_2O_2 diluted with methanol for 10 minutes for blocking of endogenous peroxidase activity. The primary antibodies used were the anti-glucocorticoid receptor antibodies [PA1-510A (GR-α) and PA3-514 (GR-β); Affinity Bio Reagents Inc., Colorado, USA] and anti-eosinophil major basic protein (MBP) antibody (MNSMON 60081; COSMO BIO Co, Ltd., Tokyo, Japan). After the sections were allowed to react with the antibodies for 24 hours at 4˚C, they were rinsed with phosphate buffer solution (PBS). They were then allowed to react with Alexa Fluor 488 and 546 (Invitrogen Corporation, CA, USA) as the secondary antibodies for 2 hours at room temperature. The sections were then rinsed in PBS and mounted, followed by examination under a fluorescence microscope (AX-70; OLYMPUS Corporation, Tokyo, Japan); 5 visual fields immediately beneath the epithelium were examined in each patient at a magnification x400. The number of GR and MBP immunoreactivity-positive cells were measured in these visual fields and the mean for the 5 visual fields was used for the analysis. The results were statistically analyzed by Mann-Whitney's *U*-test (for comparison of the non-complication group and the complication group before GC administration) and by Wilcoxon's signed rank test (for comparison of the results obtained before and after GC treatment in the complication group).

Table I. *Sequence of primers of GR-α mRNA and GR-β mRNA used for the real-time PCR assay.*

Primer	Sequence
GRα	Sense: 5'-GGGAAGAGGGAGATGGAGAC-3'
	Antisense: 5'-TTGGAATGAGAAGGGTGGTC-3'
GRβ	Sense: 5'-TCCTCAACAGCAACAACAGG-3'
	Antisense: 5'-TCATTCCTTCCAGCACATAGG-3'

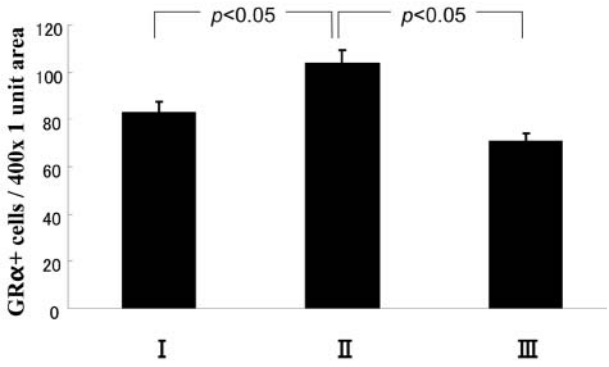

Figure 2. *The count of GR-α immunoreactivity-positive cells. I: Non-complication group; II: complication group (before glucocorticoid administration); III: complication group (after glucocorticoid administration).*

Real-time PCR assay. The tissue specimens collected from the nasal polyps were immediately stored frozen on dry ice at –80˚C. Tissue blocks of the nasal polyp specimens weighing approximately 30 mg

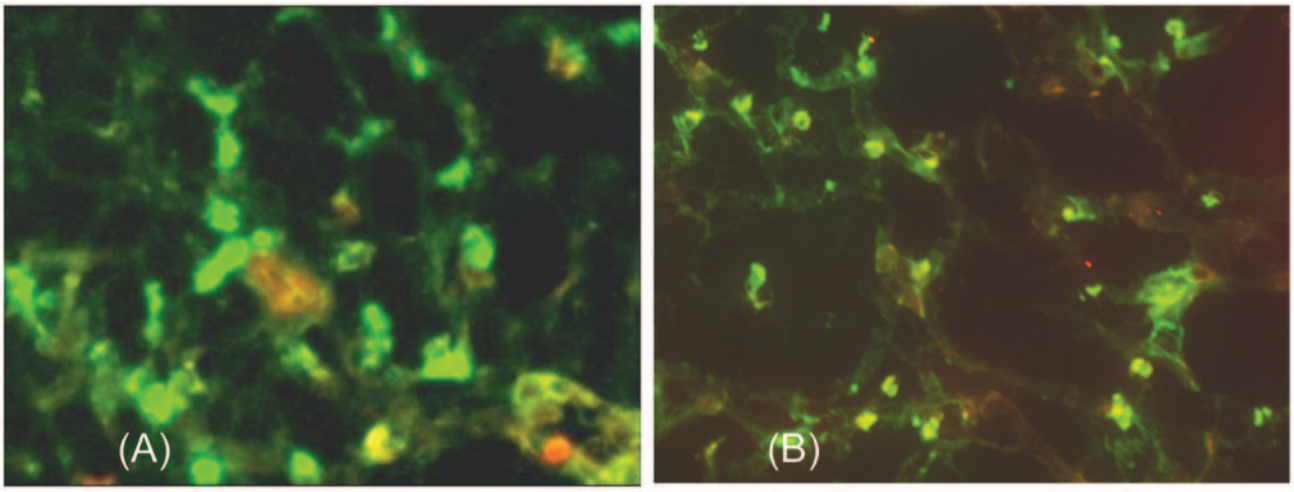

Figure 3. *Double immunofluorescent staining using anti-glucocorticoid receptor (GR)-β antibody and anti-major basic protein (MBP) antibody in a nasal polyp obtained from a patient with chronic sinusitis and bronchial asthma (the complication group). Original magnification x400. (A): Before glucocorticoid administration; and (B): after glucocorticoid administration. Green-colored cells: MBP immunoreactivity-positive cells; red-colored cells: GR-β immunoreactivity-positive cells; yellow-colored cells: MBP and GR-β immunoreactivity-positive cells.*

were excised, so as to include both the epithelium and the stroma. These specimens were homogenized in 1 ml of TRIZOL® Reagent (Invitrogen Corporation), followed by the addition of chloroform to the homogenate. The mixture was centrifuged at 4˚C and at 12000 rpm for 15 minutes. After centrifugation, 500 μl of isopropanol were added to the supernatant fluid. The resultant suspension was further centrifuged at 4˚C and at 12000 rpm for 15 minutes, and 20 μl of RNase-free water was added to the precipitates to obtain total RNA. Reverse-transcription of 1 μg of total RNA was conducted to obtain cDNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Corporation) at 42˚C for 50 minutes and at 70˚C for 15 minutes. The cDNA obtained thus was subjected to real-time PCR with a Light Cycler™ (Roche Diagnostics AG, Rotkreuz, Switzerland). The PCR was conducted at 40 cycles, each cycle consisting of 10 seconds at 95˚C and 15 seconds at 60˚C. Amplification of the target genes (GR-α mRNA and GR-β mRNA) was conducted. The expressions of the target genes were semi-quantitatively determined using GAPDH mRNA as the housekeeping gene. Table I shows the sequence of the primers used for GR-α mRNA and GR-β mRNA [the sequence for GAPDH mRNA was unknown, because the Light Cycler™-Primer Set (SEARCH LC Gmbh, Heidelberg, Germany) was used]. The results obtained were analyzed statistically by Mann-Whitney's *U*-test (for comparison between the non-complication group and the complication group before GC treatment) and by Wilcoxon's signed rank test (for comparison of the results obtained before and after GC administration in the complication group).

Results

Expression of GR by immunofluorescent staining. Figure 1 shows a fluorescent double-staining image of a nasal polyp specimen from the complication group: (A) is the image taken before GC administration, eosinophils (MBP

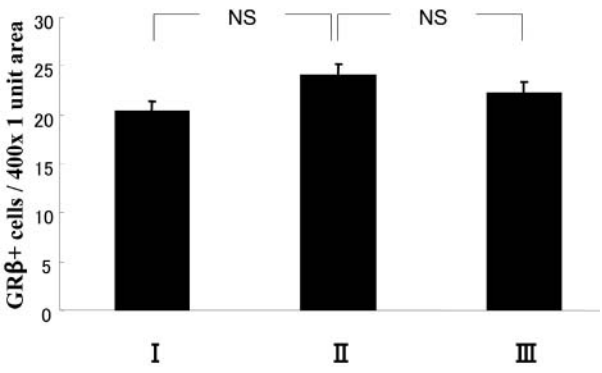

Figure 4. *The count of GR-β immunoreactivity-positive cells. I: Non-complication group; II: complication group (before glucocorticoid administration); III: complication group (after glucocorticoid administration).*

immunoreactivity-positive cells) appear like green rings, and the parts stained red in the rings indicate GR-α expression. (B) is the histological image of a nasal polyp specimen obtained after GC administration. A smaller number of MBP immunoreactivity-positive cells was seen in (B) than in (A). The number of GR-α immunoreactivity-positive cells is also markedly smaller in (B). Figure 2 shows the count of the GR-α immunoreactivity-positive cells. Comparison of the number of GR-α immunoreactivity-positive cells in the nasal polyp specimens between the non-complication group and complication group before GC administration revealed a significantly higher cell count in the complication group. Comparison of the count of GR-α

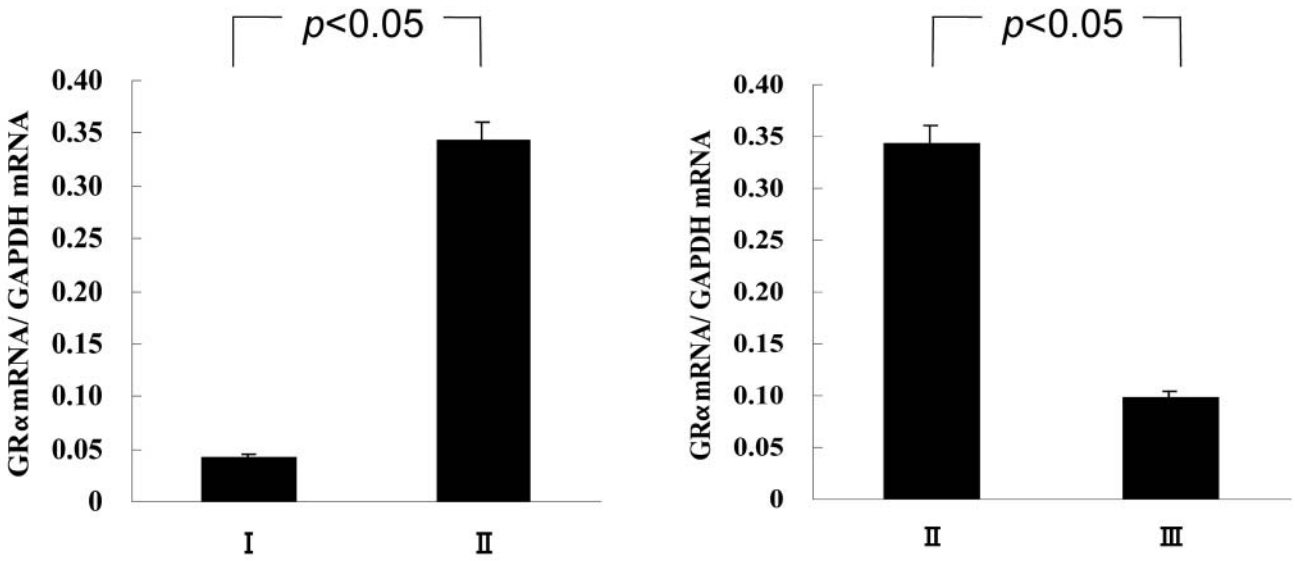

Figure 5. *Expression of GR-α mRNA as assessed by real-time PCR. I: Non-complication group; II: complication group (before glucocorticoid administration); III: complication group (after glucocorticoid administration).*

immunoreactivity-positive cells measured before and after GC administration in the complication group revealed a significant decrease in the cell count after GC administration. Figure 3 shows a fluorescence image of a nasal polyp specimen from the complication group double-stained with anti-GR-β antibody and anti-MBP antibody. The image shows a significantly smaller number of GR-β immunoreactivity-positive cells than GR-α immunoreactivity-positive cells (*cf* Figure 2). Figure 4 shows the count of the GR-β immunoreactivity-positive cells. Unlike the case for the GR-α immunoreactivity-positive cells and MBP immuno-reactivity-positive cells, no significant difference in the number of GR-β immunoreactivity-positive cells was observed neither between the non-complication and complication groups nor between the values measured before and after GC administration in the complication group.

Real-time PCR. Quantitative determination of the GR-α mRNA and GR-β mRNA levels in the nasal polyp specimens by real-time PCR revealed a significantly higher expression level of GR-α mRNA in the complication group than in the non-complication group. Furthermore, a significant decrease in the expression level of GR-α mRNA was observed after GC administration in the complication group (Figure 5). On the other hand, there was no significant difference in the expression level of GR-β mRNA, neither between the non-complication and complication groups, nor between the values measured before and after GC administration in the complication group (Figure 6).

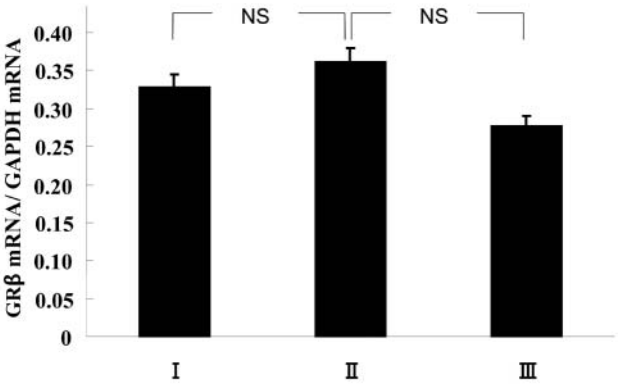

Figure 6. *Expression of GR-β mRNA as assessed by real-time PCR. I: Non-complication group; II: complication group (before glucocorticoid administration); III: complication group (after glucocorticoid administration). NS: not significantly different.*

Discussion

GR-α is a single-strand (binding) protein with a molecular weight of 94,000 Da and a hormone-binding region at its C-terminal, while GR-β shows neither a hormone-binding region nor transcriptional activity. GC binding induces dimerization of GR-α in the nucleus and the dimer binds to the glucocorticoid response element (GRE) in the nucleus to act as a transcriptional control factor (4). Increases in the nuclear GR level and translocation of GR from the cytoplasm to the nucleus have been demonstrated in nasal polyp specimens after GC administration (5). The GRs

mediate anti-inflammatory actions as follows (6): they inhibit the transcription of genes encoding mediators of inflammation, suppress the production of a variety of cytokines and induce production of the anti-inflammatory protein, lipocortin. It is also widely recognized that the GRs which undergo translocation to the nucleus bind as monomers to transcription factors such as activator protein-1 (AP-1) and nuclear factor (NF)-κB to directly inhibit the activities of these factors. Enhanced expression of GR-β has frequently been shown in the nasal polyp specimens of patients with steroid-resistant asthma (7) and it is considered that GR-β forms GR-β/GR-β, or GR-β/GR-α dimers to attenuate the actions of the GR-α/GR-α dimers as a transcriptional control factor (8). In the present study, both the results of immunofluorescent staining and of real-time PCR revealed changes in the expression levels of GR-α, but not GR-β , indicating that GR-α plays the major role in the inflammation associated with nasal polyp formation.

Enhanced expression of the GR protein and GR mRNA has been demonstrated following stimulation of cells, such as peripheral blood mononuclear cells (PBMC), in vitro by inflammatory cytokines, including TNF-α and IL-1. Gagliardo *et al.*, who measured the expression levels of GR mRNA, reported higher levels of expression of GR-α mRNA in the PBMC of patients with bronchial asthma than in healthy people, while no significant difference in the GR-β mRNA expression level was found between the two patient groups (9). Vachier *et al.* showed a reduction in the expression of GR-α mRNA in PBMC following incubation of PBMC with prednisolone (10). Similar to the case for PBMC, a markedly greater degree of infiltration by inflammatory cells, including eosinophils, was observed in the nasal polyp specimens from our complication group as compared with that in the specimens from the non-complication group. Expression of GR-α was augmented by the cytokines and chemokines produced by inflammatory cells; conversely, the suppression of inflammatory cell infiltration following treatment with GCs was associated with a decrease in the expression of GR-α. Another possible reason for the increase in the expression of GR-α is that the receptor, after undergoing translocation to the nucleus following GC binding, may directly control the expression of GR-α mRNA at the transcription and gene levels. That is, the findings suggest the existence of a negative feedback loop within the nucleus.

As described earlier in this report, GR-β, while not functioning itself as a transcriptional control factor, does inhibit the actions of GR-α. The ratio of GR-β/GR-α expression has been shown to be approximately 1/500 (11, 12). In the present assessment by real- time PCR, expression of GR-β mRNA was delayed by approximately 6-10 cycles relative to the expression of GR-α mRNA in the same patient, indicating a GR-β/GR-α expression ratio of approximately 1/100 to 1/1000. This result does not contradict the results reported in the past. Since the expression of GR-β is barely influenced by inflammatory cytokines or steroids, it is considered that the GR-β/GR-α ratio, but not the absolute number of the GR-β immunoreactivity-positive cells, might serve as a valuable clinical index of the sensitivity/resistance to GC treatment. The use of the ratio as an indicator of the efficacy of GC treatment would also allow prediction of the efficacy of steroid treatment, potentially resulting in the avoidance of GC overdosing.

Histologically, nasal polyp specimens from patients with chronic sinusitis complicated by bronchial asthma are characterized by marked infiltration by activated eosinophils of the region immediately beneath the epithelium (2). In the present assessment, marked eosinophilic infiltration was also recognized immediately beneath the nasal polyp epithelium in the complication group. There also appeared to be active infiltration in the deeper layers of the stroma. Following GC administration, scarcely any eosinophils were found in the deeper layer, whereas infiltration could still be recognized immediately beneath the epithelium. Infiltration and activation of eosinophils involve cytokines and chemokines, including IL-5 and eotaxin, which are derived from T-cells and fibroblasts (13, 14). In particular, IL-5 is produced as an autocrine mediator from eosinophils and it is considered that IL-5 suppresses eosinophil apoptosis and promotes eosinophil proliferation (2). It is assumed that GCs decrease the ability of eosinophils to migrate and promote eosinophil apoptosis. Consistent with the report of Watanabe *et al.* (15), in the present assessment also, one possible reason for acidopenia after GC administration was considered to be apoptosis of eosinophils.

Conclusion

Changes of GR expression in the nasal polyps from patients of chronic sinusitis with or without bronchial asthma were assessed. The changes were mainly limited to expression of GR-α. It was considered that a variety of inflammatory cytokines may influence the expression level of GR-α. Our future task is to assess whether modulation of GR-β expression might be useful, particularly in patients showing resistance to GC treatment. It is considered that the GR-β/GR-α ratio may serve as a potentially useful index of the efficacy of GC treatment, in particular, in postoperative treatment, which may allow safer and more secure administration of GCs.

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