EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 38, No. 5, 466-473, October 2006

Expression of glucocorticoid receptor mRNAs in glucocorticoid-resistant nasal polyps

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Accepted 15 June 2006

Abbreviations: GC, glucocorticoid; hGR, human glucocorticoid receptor; hGRβ, human glucocorticoid receptor isoform β

Abstract

Glucocorticoids (GCs) are the most effective group of medications available to treat inflammation. Although most patients with inflammation respond to GC, a small group of patients exhibit persistent GC-resistance with prolonged inflammation. Previously, it was proposed that the GC-resistance is caused by low amount of human GC receptor (hGRα) and/or excessive presence of a GC receptor isoform, hGRβ that was generated from alternative splicing of the hGR message. We have tested this hypothesis by investigating correlation between the expression pattern of hGR mRNAs in patients with inflammatory nasal polyps and the effectiveness of GC treatment. We have performed reverse transcription PCR analysis of mRNAs coding each hGRα and hGRβ in nasal tissues. hGRα mRNA was more expressed in patients with nasal polyps than in normal subjects. However, the elevated hGRα mRNA expression was decreased after GC treatment. Compared with hGRα mRNA expression, level of hGRβ mRNA expression was very low in all groups. In patients, hGRβ mRNA was expressed at a similar level regardless of GC efficacy, indicating that there is no correlation between the GC sensitivity and the expression level of hGRβ mRNA. Thus, persistent GC-resistance is not associated with low expression of hGRα or over-expression of hGRβ.

Keywords: drug resistance; glucocorticoid; glucocorticoid receptors; nasal polyps; reverse transcriptase polymerase chain reaction

Introduction

Tissue inflammation and immune activation play an important role in the pathogenesis of chronic allergic diseases (Oettgen *et al*., 2001; Ruiz-Irastorza *et al*., 2001; Wills-Karp *et al*., 2001). Glucocorticoids (GC) are one of the most potent medications in the treatment of inflammation caused by allergy and autoimmune disorder. Although most patients with allergic diseases respond to GC treatment, a small group of patients demonstrate persistent tissue inflammation despite treatment with high doses of GCs (Leung *et al*., 2003). Since GC resistance complicates the management of these patients, knowledge of the molecular mechanisms responsible for GC insensitivity is critical for the development of effective therapies.

The ability of GC to act on a target tissue and elicit anti-inflammatory effect is mediated by the presence of the α isoform of human GC receptor (hGRα) (Cato *et al*., 1996). hGRα belongs to the superfamily of large steroid-nuclear receptor that also includes receptors for mineralocorticoids, thyroid hormone, retinoic acid, and vitamin D (Oakley *et al*., 1999). hGRα functions as ligand-dependent transcription factor that regulates expression of several inflammation-related target genes. hGRα is expressed in almost all tissues and cells, and in the absence of GC it is mainly located in the cytoplasm of cells as part of a large multiprotein complex (Oakley *et al*., 1999). This complex consists of the

receptor, two molecules of heat shock protein hsp90, and several additional factors (Pratt *et al*., 1993; Smith *et al*., 1993). When GC binds the receptor, the multiprotein complex dissociates, resulting in the release of hGRα. hGRα translocates into the nucleus, where it forms homodimer and specifically binds to glucocorticoid-responsive elements (GREs) located in the regulatory regions of target genes. The bound GR homodimer interacts with the basal transcription factors, which subsequently modulates transcription of the target genes.

In addition to hGRα, an isoform deficient in GC binding has been isolated in humans and termed hGRβ (Hollenberg *et al*., 1985). Human hGRα variant hGRβ is generated by alternative splicing of the human GR (hGR) gene (Figure 1), and hGRβ differs from the wild-type receptor (hGRα) only at the carboxy terminus (Hollenberg *et al*., 1985; Encio *et al*., 1991; Oakley *et al*., 1996). The two isoforms are identical through amino acid 727 but then diverge, with hGRα having an additional 50 amino acids and hGRβ an additional nonhomologous 15 amino acids. It was known that hGRβ resides in the nucleus of cells independent of GC treatment and does not bind GC. Some groups reported that hGRβ functions as a dominant inhibitor of hGRα in transfected cells (Bamberg *et al*., 1995; Oakley *et al*., 1996). However, this observation was not reproduced by others (Bamberger *et al*., 1997; Hecht *et al*., 1997; Brogan *et al*., 1999; de Lange *et al*., 1999).

From the studies of asthmatic patients treated with GC, it has been proposed that resistance to the anti-inflammatory effects of GC could result from an elevated level of hGRβ expression (Leung *et al*., 1997). Glucocorticoid resistance in asthma has also been associated with qualitative or quantitative deficiency in hGRα (Sher *et al.,* 1994). However, it is still controversial about the putative role of hGRβ as a dominant inhibitor of hGRα in the GC- treated asthmatic patients (Gagliardo *et al*., 2000). In contrast to the studies of asthma patients with a respect to hGR gene expression in GC resistance, expression of hGRα and hGRβ in nasal polyps caused by inflammation and its hypothetical correlation with glucocorticoid-resistance has not yet been investigated.

An immunostaining study shows that hGRα and hGRβ isoform was mainly present in ephithelial cells and infiltrating inflammatory cells in subepithelial layer of nasal tissues (Kang *et al*., 2000). The study has suggested that increased number of inflammatory cells infiltrating the nasal mucosa did not amplify the amount of immunostained hGR proteins. Thus, in this study, to evaluate the entire level of hGR messages present in the cells of nasal polyp we have chosen nasal tissues instead of respectively

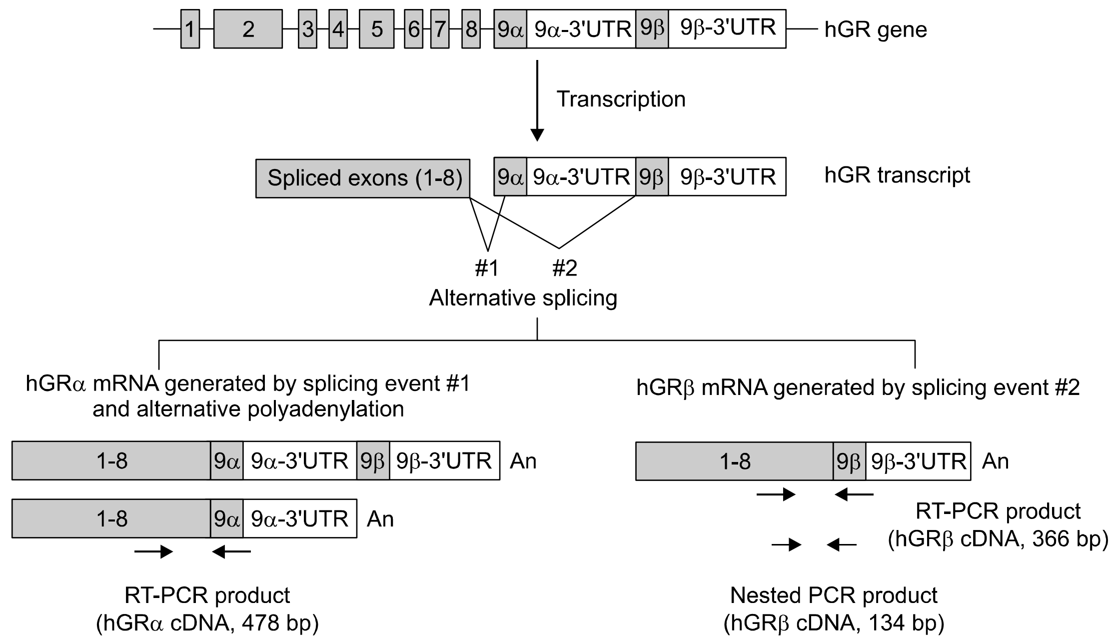

Figure 1. Organization of the hGR gene and gene products. hGR sequences formerly identified as exon 9 were composed of exon 9α, and exon 9β. Alternative splicing event generates multiple hGR messages. The hGRα- and hGRβ- specific cDNA primers for RT-PCR in this study are indicated solid arrows. Note that RT-PCR product for hGRβ message was reamplified through a second round of PCR (nested PCR) and cDNA primers for the nested PCR were indicated as solid arrows below the RT-PCR product. UTR and An represents untranslated sequences and polyadenylated tail sequence, respectively.

cultured cells derived from nasal tissues or peripheral blood mononuclear cells from blood. We analyzed the expression of hGRα and hGRβ at mRNA level in the tissues derived from patients with nasal polyps as well as from the normal subjects to better understand persistent GC-resistance in nasal polyps and assess whether this inflammatory disease is associated with a dysregulation of hGRα or hGRβ.

Materials and Methods

Preparation of nasal tissue samples

Patients with nasal polyps who visited the local hospital (Maryknoll Hospital, Busan, Korea) were selected under a written consent for a participation of this study. They were divided into three patient groups (Table 1). The first patient group (P1) consisted of 21 subjects with nasal polyp who took GC treatment and showed positive efficacy of the medication. The second patient group (P2) consisted of 12 subjects with nasal polyp who showed negative efficacy of the medication with a persistent GC insensitivity, resulting in a required surgical removal of polyp after the GC-treatments for 2 weeks. These patients (P1 and P2) had not been exposed to any GC medication at least for 6 weeks prior to a short course (2 weeks) of oral GC (20 mg/day prednisolone) treatment, and their nasal biopsy specimens were obtained after the medication. Nasal tissues of some patients of group P1 were taken before and after the GC treatment to compare the changes of hGR mRNA expression during the medication. The third patient group (P3) consisted of 21 subjects with nasal polyp who took the operation for a surgical removal of polyp without prior GC treatment. Their nasal tissue specimens were obtained from the removed nasal polyp during the operation. As a control group, nasal tissues from normal subjects were obtained from 10 subjects who underwent maxillofacial surgery after facial trauma. They had not been exposed to the GC treatment and termed as a normal group (group N). Nasal tissues taken from each subject were immediately washed with saline solution and were kept frozen at -80°C. The frozen tissue samples were used within a week for RNA isolation.

Isolation and analysis of total RNA

Total RNA was extracted by lysing 2 to 3 g of isolated nasal tissues prepared as above. RNA extraction was performed by following a standard protocol. Briefly, the extracted RNA in the aqueous phase was obtained after homogenization of the finely sliced tissues in the reaction mixture containing RNA PrepMate™ lysis buffer (Bioneer, Daejeon, Korea) and chloroform, followed by centrifugation at 10,000 *g* for 10 min at 4°C. Supernatant containing RNA was mixed with equal volume of phenol:chloroform (5:1) mixture and centrifuged at 12,000 *g* for 5 min at 4°C. The RNA extract was precipitated with 1 volume of isopropanol at -20°C for 10 min and centrifuged at 10,000 *g* for 10 min at 4°C. The RNA pellet was washed with 80% ethanol, vacuum dried briefly, solubilized in water, and stored at -80°C until subsequent analysis. The quantity of RNA was calculated by spectrophotometry at 260 nm. The integrity of purified RNA was determined by visualization of the 28S and 18S ribosomal RNA bands after electrophoresis of 1 to 2 μg of each RNA sample through a 1.0% formaldehyde agarose gel.

Reverse transcription and polymerase chain reaction

An amount of 1 μg of total RNA was subjected to reverse transcription (RT) for 1 h at 42°C. Reaction mixture contained 1 mM deoxynucleotide mixture (Roche, Germany), 40 U RNase OUT™ (Invitrogen), 1.6 μg oligo dT (Bioneer, Daejeon, Korea), and 50 U of AMV reverse transcriptase (Roche, Germany) in 20 μl supplied buffer. The reaction mixture was initially incubated at 25°C for 10 min for annealing of primer to the RNA template and was then further incubated for 1 h at 42°C for RT reaction. The reac-

Table 1. Characteristics of the patients and normal subjects.

	GC-treated polyp patients		GC-untreated polyp patients	Normal subjects
	GC-effective	GC-ineffective		
Group name	P1	P2	P3	N
Number of cases	21	12	21	10
Gender (M/F)	12/9	7/5	11/10	8/2
Age, yr	44 (15-76)	32 (17-58)	47 (7-65)	35 (19-52)
Oral prednisolone (dose, mg/daily)	20	20	0	0

tion mixture was heated to 98°C for 5 min to stop RT.

The primers used for amplification of hGRα message were as follows (Figure 1): 5'-CCTAAGGACGGTCTGAAGAGC-3' (upstream) and 5'-GCCAAGTCTTGGCCCTCTAT-3' (downstream), corresponding to nucleotides 2158-2178 and 2616-2635 of hGRα complementary DNA (cDNA) (Oakley *et al*., 1996). The primers used for amplification of β-actin were as follows: sense 5'-TGACGGGGTCACCCACACTGTGCCCAT-3', antisense 5'-GAAGCATTTGCGGTGGACGATGGAGGG-3'. Polymerase chain reaction (PCR) was performed with 2 μl and 4 μl of RT reaction mixture to analyze β-actin and hGRα mRNA levels, respectively. The reactions contained 0.5 U *Taq* DNA polymerase, 0.2 μM of each oligonucleotide primer, 0.2 mM deoxynucleotide mixture, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2 mM $MgCl_2$ in a final volume of 20 μl. PCR conditions were 30 cycles of 30 s at 95°C, 30 s at 63°C, and 30 s at 72°C. These were followed by a final extension step at 72°C for 10 min. Amplified DNA fragments were electrophoretically separated on 1.7% agarose gels containing 0.5 μg/ml ethidium bromide and visualized under UV light. hGRα and β-actin PCR products were semiquantified by densitometric scanning using a image report system Fluorochem 5500 (Core Biosystem, Korea) equipped with image analysis software. Amount of amplified hGRα cDNA was normalized to that of β-actin.

Nested PCR

Nested PCR was used to amplify hGRβ due to rare amount of hGRβ mRNA template. The first round of PCR was performed using 2 μl of the cDNA from the reverse transcription. The primers used for the first round were as follows (Figure 1): 5'-CCTAAGGACGGTCTGAAGAGC-3' (upstream) and 5'-CCACGTATCCTAAAAGGGCAC-3' (downstream), corresponding to nucleotides 2158 to 2178 and 2503 to 2523 of hGRβ cDNA (Oakley *et al*., 1996). The PCR mixture contained 0.2 μM of each outer primer or 0.2 μM of the β-actin primers, together with the same reagents as described above. PCR conditions were 40 (hGRβ) or 30 (β-actin) cycles of 30 s at 95°C, 30 s at 54°C (hGRβ) or 63°C (β-actin), and 30 s at 72°C. These were followed by a final extension step at 72°C for 10 min.

Nested PCR was initiated with 4 μl of the first-round PCR products of hGRβ cDNA amplification. The primers were used as follows: 5'-AGCACATCTCACACATTAAT-3' (upstream) and 5'-TATAGTTGTCGATGAGCATC-3' (downstream), corresponding to nucleotides 2338 to 2357 and 2455 to 2471 of hGRβ cDNA (Gagliardo *et al*., 2000). The PCR mixture was as described above. PCR reaction was performed by 30 cycles of 30 s at 95°C, 30 s at 54°C, and 30 s at 72°C. These were followed by a final extension step at 72°C for 10 min. Amplified DNA fragments were analyzed as above. Quantities of hGRβ product from the nested PCR and of β-actin product from the first round of PCR were estimated by densitometric scanning. Amount of hGRβ cDNA amplified was normalized to that of β-actin.

Statistical analysis

Nonparametric tests were used to analyze the data. The Wilcoxon-Mann-Whitney test was used for paired comparisons of each test group with a respect to hGR mRNA expression levels. Statistical significance was set at $P < 0.05$.

Results and Discussion

Quantitative RT-PCR analysis of the hGR messages in nasal tissues

To accurately assess the relative levels of the hGRα and hGRβ mRNA transcripts, we performed quantitative RT-PCR on RNA present in nasal tissues isolated from subjects who have nasal polyp. Figure 2A shows reaction cycle intensity curves for the 478-bp hGRα PCR product and 134-bp hGRβ PCR product that was obtained by a second round of nested PCR with the template amplified at a first PCR amplification of 40 cycles. 30 and 40 cycles of PCR amplifications that do not reach to the saturation of the PCR product was chosen to be appropriate to compare the expression level of hGRα and hGRβ mRNA, respectively (arrows in Figure 2A). At a first round of PCR, the presence of hGRβ cDNA of estimated length of 366 bps was not able to be detected because of rare abundance of the hGRβ message (data now shown). Thus, the hGRβ cDNA was reamplified with an additional round of PCR (nested PCR). Using the amplified hGRβ cDNA at a first round of RT-PCR as template, the hGRβ cDNA was amplified again with an additional 40 cycles of nested PCR. Expression of the housekeeping gene β-actin was also determined to provide an internal control for RT and PCR efficiencies. In order to assess basal expression level of hGR mRNAs in normal nasal tissues, the same RT-PCR for hGR mRNAs was also performed with nasal tissues of normal subjects. Figure 2B shows a representative electrophoretic analysis of the PCR products of hGR mRNAs obtained from the each patient group as well as the normal subject group. cDNA of 478 bp length that was reverse transcribed against hGRα mRNA and the internal control cDNA (661 bps) synthesized from β-actin

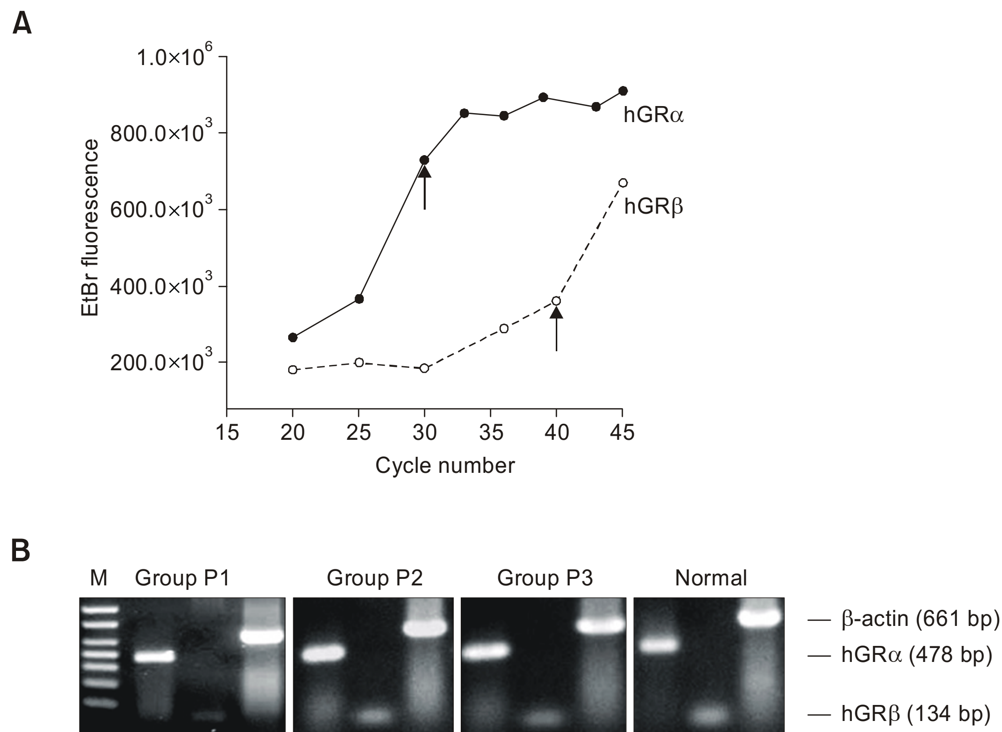

Figure 2. Quantitative RT-PCR analysis of hGRα and hGRβ messages. (A) RNA derived from nasal tissue of group P1 was reverse transcribed, and the resulting cDNA amplified using hGRα- or hGRβ-specific primers. Aliquots of the PCR reaction was removed at 5-cycle intervals and electrophoresed on agarose gels stained with ethidium bromide. Note that RT-PCR product for hGRβ cDNA was the nested PCR products for hGRβ cDNAs; cDNA products obtained at the first round was reamplified with hGRβ-specific cDNA primers. Arrow indicates the cycle number of PCR that was used for quantification of the PCR product in agarose gel electrophoresis as shown in (B). (B) Representative results of RT-PCR for hGRα, hGRβ, and β-actin mRNAs in nasal tissues derived from patient groups and normal subjects. The RT-PCR products were analyzed by 1.7 % agarose gel electrophoresis. Lane M denotes molecular weight markers for DNA, 1000 bp, 800 bp, 600, bp, 500 bp, 400 bp, 300 bp, and 200 bp (top to bottom).

mRNA were detected in each tissue sample. We have also detected PCR product of hGRβ cDNA of 134 bps after the nested PCR (Figure 2B). All of groups have shown that detection of hGRβ cDNA requires the use of nested PCR, indicating that hGRα mRNA level is largely predominant over hGRβ mRNA in nasal tissues irrespective of the presence of inflammatory polyp. Thus, we report herein that hGRα mRNA was more expressed than the hGRβ mRNA in the nasal tissues regardless of polyp or GC treatment because hGRα was revealed by a simple PCR whereas detection of hGRβ required nested PCR.

It has been hypothesized that hGRβ has to be more abundant than hGRα to inhibit hGRα-mediated gene regulation, but conflicting data concerning the relative levels of the two isoforms have been reported. In one study conducted with various human tissues and HeLa cells, the amount of hGRβ was found to be equal or higher than that of hGRα (Castro *et al.,* 1996). In contrast, other studies have shown that level of hGRβ was relatively lower than hGRα in HeLa cells and human lymphocytes (Bamberger *et al.,* 1997; Hecht *et al.,* 1997). In agreement with those latter results, our result demonstrates that hGRβ mRNA was less expressed than that of hGRα in the nasal tissues, which is regardless of GC efficacy or inflammation. Moreover, our observation was consistent with previous reports, in which quantitative RT-PCR experiments have shown that the hGRβ mRNA was 200- to 500-fold less represented than the hGRα mRNA in all human tissues and cell lines (Oakley *et al*., 1996). Clinically, this observation was also observed in GC-dependent asthmatics by showing less expression of hGRβ mRNA and protein relative to those of hGRα (Gagliardo *et al*., 2000).

hGRα mRNA is more expressed in nasal polyps than normal nasal tissues

To compare the expression level of hGRα in nasal polyps and normal nasal tissues, we measured expression level of hGRα mRNA and β-actin as internal control with RT-PCR. Electrophoretic analysis of the RT-PCR products of hGRα mRNAs was performed on RNAs obtained from three patient groups and the normal group. Patient group of P3, which had nasal polyp without GC treatment, was observed to have hGRα mRNA moderately expressed relative to β-actin control. Densitometric scanning of the RT-PCR product bands showed that estimation of expressed hGRα/β-actin cDNA ratio was averaged to be 0.593 (Figure 3A). In contrast, normal nasal tissue without polyp ('N' in Figure 3A) expressed less amount of hGRα mRNA (average hGRα/β-actin cDNA ratio of 0.245) compared with P3 ($P < 0.01$). Thus, nasal tissues without inflammation maintained relatively less amount of hGRα than the patient groups with nasal polyp. Interestingly, patient group P1 who were treated with GC and showed positive GC efficacy exhibited lower level of hGRα mRNA expression (average hGRα/β-actin cDNA ratio of 0. 310, Figure 3A) than the group of GC-untreated nasal polyp (P3) ($P < 0.01$), but is almost close to the level observed in the normal group.

Five patients with nasal polyp were treated with GC (Group P1), and their nasal tissues were obtained twice, before and after the medication. As shown in Figure 3B, hGRα mRNA expression was decreased after a GC treatment ($P < 0.05$), indicating that a short-course of GC treatment downregulated hGRα in conjunction with anti-inflammation effect in nasal tissues. Thus, low level of hGRα expression in P1 was likely to be caused by GC administration to the patients. This observation was consistent with the previous report that hGRα was downregulated after a short-course of oral GC treatment of asthmatics (Gagliardo *et al.,* 2000). GC treatment elicited anti-inflammation effect as well as a decrease of hGRα expression in those asthma patients. This result was partly explained by earlier study with COS1 cells, in which hGR cDNA can downregulate the induction of hGR gene (Burnstein *et al.,* 1990). Thus, increased level of hGRα mRNA in nasal polyp could subsequently be reduced after GC treatment as observed in our study (group P1). Previous studies have suggested that the transcription factor nuclear factor kappa B (NF-κB) plays an important role in the induction of various proinflammatory cytokines (Schreck *et al.,* 1990; Mukaida *et al.,* 1994). These proinflammtory cytokines have been shown to upregulate hGR gene expression (Webster *et al.,* 2001). Hence, we suggest that high level of NF-κB expression in inflamed tissue activates proiflammatory cytokines, which in turn induce expression of hGR gene. Therefore, relatively low level of hGRα mRNA expression in P1 and normal

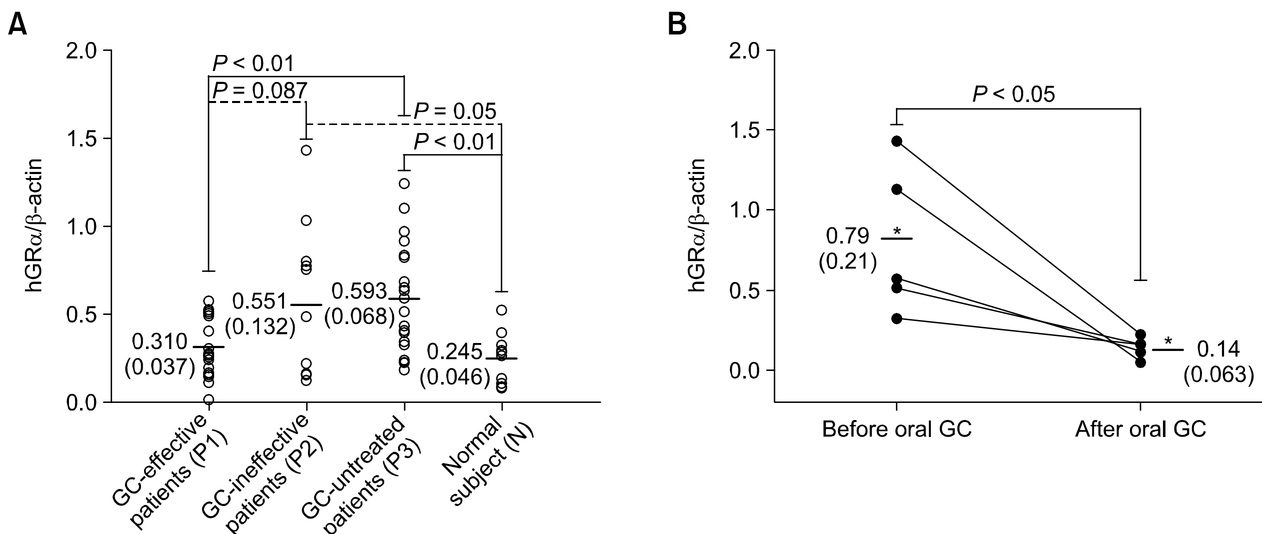

Figure 3. Comparison of hGRα expression in nasal tissues from various groups. (A) RT-PCR products for hGRα mRNA was semiquantified by densitometric scanning and normalized relative to the amount of β-actin. Result of each subject was expressed as spot and the average of the values in each group was indicated as horizontal bar in the middle of graph with a standard error in parenthesis. Statistical analysis for correlation was performed and represented in the graph. (B) Effect of GC-treatment on hGRα expression. Expression of hGRα mRNA in 5 patients was monitored before and after an oral GC treatment (prednisolone at 20 mg/daily for a week). Individual data are shown, and average was indicated with a horizontal bar with an asterisk (a standard error in parenthesis).

group might be caused by the result of low level of the proinflammatory cytokines in nasal tissues. We are currently investigating this issue of whether an excess of NF-κB activity and increased level of proinflammatory cytokines are related with hGRα expression in the tissue and cells.

We also compared hGRα expression level of GC-resistant patients (P2, average hGRα/β-actin cDNA ratio of 0.551) with other groups (Figure 3A). Compared with groups of P1 and N, hGRα mRNA was relatively more expressed in group P2 (P = 0.05-0.09). This result indicates two points; (1) anti-inflammation efficacy of GC and hGRα expression level has no correlation during the GC treatment (P1 *vs.* P2), and (2) inflamed nasal tissues with GC-resistance express more hGRα mRNA than the normal or GC-effective nasal tissues (P2 *vs.* P1 or N). Therefore, the incapacity of GC to inhibit inflammation in nasal tissue of the GC-resistant patients is not due to a low level of hGRα.

Inefficacy of GC treatment is not the result of overexpression of hGRβ

It has been proposed that elevated level of hGRβ might cause glucocorticoid resistance in patients by overriding hGRα activity (Oakley *et al.*, 1996; 1999). This hypothesis on the inhibitory effect of hGRβ in hGRα-mediated transcription has been tested *in vitro* with tissues obtained from corticoresistant asthmatics (Bamberger *et al.*, 1995; Oakley *et al.*, 1996). However, the hypothesis is still controversial with respect to putative role of hGRβ in inhibition of hGRα activity. Thus, to test this hypothesis in the nasal polyp tissue after GC treatment, we have analyzed the amount of hGRβ mRNA in each patient group. hGRβ mRNA expression in nasal tissues of three patient groups and normal subject was analyzed by agarose gel electrophoresis after a second round of PCR (Figure 2B). PCR products were semiquantified by densitometric scanning and amount of hGRβ mRNA was determined by the ratio of hGRβ cDNA relative to the amount of β-actin cDNA (hGRβ/β-actin cDNA in Figure 4). Densitometric analysis of the data indicated that the amount of hGRβ was similar in normal subjects and in the three groups of nasal polyps (Figure 4) without a statistically significant difference ($P > 0.05$). Importantly, we observed that the expression of hGRβ was not related with GC- resistance because hGRβ mRNA expression in group P1 (average = 0.073) was statistically similar to that observed in group P2 (average = 0.103). This finding indicates that incapacity of GC to inhibit inflammation in these patients (group P2) is not due to a high level of hGRβ.

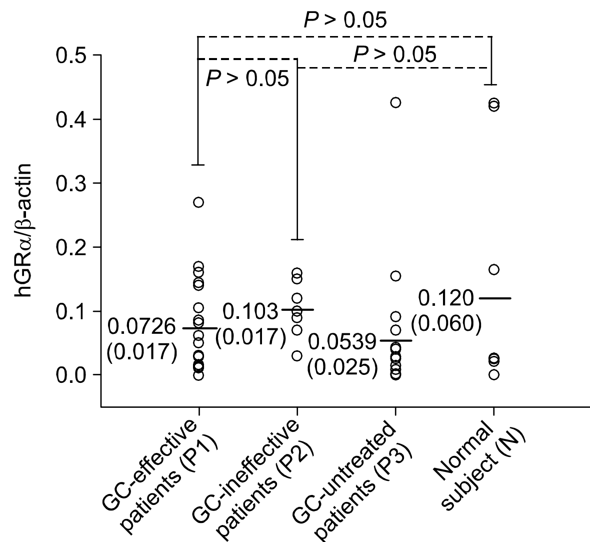

Figure 4. hGRβ mRNA expression in nasal tissues from various groups. Nested PCR products for hGRβ mRNA was semiquantified by densitometric scanning and normalized relative to the amount of β-actin. Result of each subject was expressed as spot and the average of the values in each group was indicated as horizontal bar in the middle of graph with a standard error in parenthesis. Any statistically significant difference between each group was not observed ($P > 0.05$).

It has been reported that in asthma patients increased IL-4 production might cause GC-resistance by demonstrating a close relationship between GC-resistance and IL-4 production, in which enhanced IL-4 production caused by DNA sequence variant was positively correlated with GC-resistance in asthmatic patients (Burchard *et al.*, 1999; Rosenwasser *et al.*, 2001). Thus, yet undefined factors *in vivo* are likely responsible for preventing activity of hGRα with glucocorticoid in those patients showing the GC-resistance. In conclusion, in nasal tissues with polyp the persistent GC-resistance and resulted inflammation despite GC treatment is not the result of low expression of hGRα or overexpression of hGRβ.

Acknowledgment

This work supported by the Dong-Eui University Research Grant (2005AA182) endowed to D-E Kim.

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