

High levels of glucocorticoid receptors in patients with active Crohn's disease may predict steroid resistance

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Summary

Up to 20% of Crohn's disease (CD) patients respond poorly to glucocorticoids (GC). A product of an alternative splicing of the glucocorticoid receptor (GR) premRNA, GR β , may play a role as a dominant inhibitor of the glucocorticoid response. Increasing evidence suggests that inflammatory cytokines such as interleukin (IL)-18 alternate the splicing of the primary transcript between the two isoforms GR β and GR α in hGR gene of CD patients. The aim of this study is to assess the expression of GR α and GR β in patients with CD and to look for a possible correlation between these receptors and the response to glucocorticoid treatment. Forty-two CD patients and 17 healthy volunteers were studied. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using real-time PCR techniques. Serum IL-18 protein levels were measured by enzyme-linked immunosorbent assay (ELISA). The amount of hGR α -mRNA in patients in remission was significantly lower than in controls ($P < 0.05$). The amount of hGR β -mRNA was significantly higher in GC-resistant patients in the active stage of disease compared with all other groups ($P < 0.05$). Patients in the active stage of the disease had higher levels of IL-18 than patients in remission and both had higher levels than controls ($P < 0.05$). The amounts of IL-18 were directly correlated with the amount of hGR β mRNA in GC-resistant patients with an active disease. High levels of hGR β might be connected to GC resistance. IL-18 might participate in the alternative splicing of the hGR preliminary mRNA of CD patients. The results support the theory that augmented hGR β mRNA expression level in PBMC is connected with GC-resistance of CD patients.

Keywords: Crohn's disease, glucocorticoid receptor alpha, glucocorticoid receptor beta, glucocorticoid receptor mRNA, IL-18

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Introduction

Glucocorticoids (GC) have a known immunosuppressive effect and are used widely in the treatment of chronic inflammatory disease such as Crohn's disease (CD) [1,2]. However, up to 20% of CD patients are resistant to glucocorticoid from the onset of treatment [3]. Because the administration of glucocorticoid can cause a wide range of side effects such as Cushingoid features, osteoporosis, cataract and other toxicities [4,5], it is important to avoid treatment in those who will not benefit from its use.

GC anti-inflammatory response is mediated through glucocorticoid receptor α (GR α), which is a ligand-dependent transcription factor [6]. Binding of the GR α to the steroid

causes translocation of the hormone-receptor complex from the cytoplasm to the nucleus and the activated complex binds to GC response element (GRE) on the DNA.

Two human glucocorticoid receptor isoforms have been described, GR α and GR β [7]. Both isoforms are produced by alternative splicing of the same gene and share the first 8 exons but differ in exon 9. Due to this difference they do not share the same carboxy terminus, and GR β is unable to bind the hormone and to transactivate the glucocorticoid responsive promoter [8].

The molecular mechanism underlying steroid resistance is still unknown. Recently it was suggested that hGR β might serve as a dominant negative inhibitor of the GR α activity. A relatively high endogenous expression of the GR β isoform in

a cell-type-specific pattern might be responsible for the GR β -mediated anti-glucocorticoid activity [8–11]. Honda *et al.* [12] have reported that hGR β mRNA expression in peripheral lymphocytes in ulcerative colitis (UC) patients have a close relationship to their GC response.

Increasing evidence suggests that cytokines and/or growth factors might participate in the control of GR β expression. Combination of the cytokines interleukin (IL)-2 and IL-4 in peripheral blood of glucocorticoid-resistant asthma patients was found to enhance the expression of GR β [13]. IL-18, an early signal in the development of T lymphocyte helper type 1 (Th-1) response, was seen to be increased in CD patients [14,15]. Moreover, in *in-vitro* culture experiments of human peripheral blood mononuclear cells (PBMC) in inflammatory bowel disease (IBD) patients, IL-18 was found to induce the expression of hGR β mRNA [16].

The aim of the study was to determine if levels of hGR α and hGR β can predict a patient's response to glucocorticoid therapy. IL-18 levels in the serum of CD patients were also measured in order to investigate a possible connection between IL-18 and alternative splicing of the GR gene. We also attempted to develop a reliable, reproducible and rapid technique for the detection and quantification of hGR α and hGR β expression.

Materials and methods

Patients and materials

Blood from 42 CD patients and 17 healthy volunteers was examined. All patients with CD had been diagnosed by accepted criteria, i.e. typical clinical symptoms, histological manifestation, colonoscopy, computerized tomography or small bowel radiology [17,18]. Patients were divided into subgroups according to their Crohn's disease activity index (CDAI) at the time of blood donation: active stage of disease (CDAI \geq 150) and inactive stage of disease (CDAI $<$ 150); and according to their previous GC-response. Patients who went into remission on 40 mg/day dose of prednisone (or an equivalent medication) within 4 weeks were considered GC-responsive. Patients who did not respond to GC therapy received either azathioprine or anti-tumour necrosis factor

(TNF). GC-resistant patients who were in remission were maintained in remission by either azathioprine (four patients) or methotrexate (two patients); one patient entered remission spontaneously. Patients whose CDAI did not decrease below 150 or who required surgery were considered GC-resistant. Characteristics of patients in each subgroup and healthy volunteers are summarized in Table 1.

Isolation of peripheral blood mononuclear cells

Ten ml of heparinized blood was donated by the CD patients and by the healthy volunteers. PBMC were isolated by Ficoll gradient centrifugation using Histopaque 1077 (Sigma, St. Louis, MO, USA). Informed consent was obtained from each patient included in the study. The protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Detection and analysis of hGR α and hGR β mRNA

Expression of hGR α and hGR β mRNA in PBMC was determined by reverse transcription of total RNA followed by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from PBMC was isolated by EZ-RNA total RNA isolation kit (Biological Industries, Beit Haemek, Israel). cDNA was synthesized by extension of random hexamer primer with mouse mammary leukaemia virus (MMLV) reverse transcriptase (EZ-first strand cDNA synthesis kit for RT-PCR, Biological Industries). The transcript of human porphobilinogen deaminase (hPBGD) was amplified from each of the cDNA samples to serve as an internal standard and for normalizing the results as described by Wang *et al.* (19) (LightCycler-hPBGD housekeeping gene set; Roche Diagnostics GmbH, Mannheim, Germany). This method allows simultaneous reverse transcription and real-time PCR amplification of two mRNAs of specific genes of interest (analyte genes) and mRNA of constantly transcribed genes (housekeeping genes) in a single-tube reaction. Serial dilutions of hGR α and hGR β RT-PCR products were used as external standards in each run.

Amplification of the target transcript was monitored using labelled hybridization probes that hybridize to an

Table 1. Characteristics of patients with Crohn's disease (CD)

	No. of cases	Gender (F/M)	Age (years)	CDAI ^a (at blood donation)
Inactive disease				
GC-responsive	6	4/2	40 \pm 21	79 \pm 37
GC-resistant	7	2/5	40 \pm 17	80 \pm 46
Active disease				
GC-responsive	14	5/9	31 \pm 14	243 \pm 64
GC-resistant	15	10/5	36 \pm 15	259 \pm 80
Control	17	13/4	46 \pm 18	

Data are presented as mean \pm s.d. ^aCDAI, Crohn's disease activity index. GC: glucocorticoid receptor.

Table 2. Primers and hybridization probes used to detect hGR α and hGR β transcripts.

	Oligonucleotide ^a	Position in message
hGRα		
Forward primer ¹²	CCTAAGGACGGTCTGAAGAGC	2158–2178
Reverse primer ¹²	GCCAAGTCTTGGCCCTCTAT	2616–2635
Donor probe	TTGTCAGTTGATAAAACCGCTGCCAGTTCT-flu	2290–2261
Acceptor probe	LC Red640-GCTGGAGTTTCCTTCCTCTTGACAATG	2259–2230
hGRβ		
Forward primer ¹⁹	ACACAGGCTTCAGGTATCTT	2091–2110
Reverse primer ¹²	CCACGTATCCTAAAAGGGCAC	2503–2523
Donor probe	TGTGCACTTCGTGTGCAATAATAAGTCAACT-flu	2421–2451
Acceptor probe	LC Red640-ATGCTCATCGACAACACTATAGGAGGCTTT	2453–2480

^aOligonucleotide sequences are 5'–3'.

internal sequence of the amplified target. Data analysis of the LightCycler results was performed with the second derivative maximum method (LightCycler software version 3.5, Roche Diagnostics GmbH, Mannheim, Germany).

PCR conditions and reagents

Quantitative real-time PCR was performed with the LightCycler instrument (Roche Applied Science, Penzberg, Germany) using fluorescent resonance energy transfer (FRET) chemistry. Each 10 μ l of PCR reaction contained 1 μ l cDNA and final concentrations of: 0.5 μ M each primer, 0.2 μ M of each probe and 2 mM Mg²⁺ and 1 μ l 'FastStart' PCR reaction mix (LightCycler-FastStart DNA Master Hybridization Probes kit; Roche Diagnostics GmbH). Primers and hybridization probes are summarized in Table 2. Each of the PCR programmes consisted of 10 min at 95°C for activation of the FastStart *Taq* DNA polymerase followed by 45 PCR cycles of denaturing at 95°C for 10 s, annealing at 60°C for 10 s (hGR α) or 55°C for 10 s followed by 50°C for 10 s from the 11th cycle (for hGR β) and elongation at 72°C for 20 s. For hPBGD the transcript was amplified according to the manufacturer's instructions (Roche Diagnostics GmbH).

Detection of IL-18 in serum

IL-18 concentration in patients' serum as well as healthy volunteers were measured by enzyme-linked immunosorbent assay (ELISA) (MBL, Naka-Ku Nagoya, Japan) in only 31 CD patients and 10 healthy volunteers, due to technical problems with the serum specimens.

Statistical analysis

Values are given as mean \pm s.e.m. unless otherwise stated. The data were analysed using SPSS statistical software (SPSS Inc. Chicago, IL, USA). Statistical differences in the amounts of hGR α and hGR β mRNA between the different groups were analysed using the non-parametric Mann–Whitney two-sample test and the Kruskal–Wallis test. Correlation

Table 3. Mean expression of hGR α mRNA, hGR β mRNA in Crohn's disease (CD) patients and controls.

	No. of cases	hGR α /PBGD ($\times 10^{-3}$)	hGR β /PBGD ($\times 10^{-4}$)*
Controls	17	81.1 \pm 21	6.18 \pm 1.0
CD patients	42	44.4 \pm 7.2	13 \pm 5.3
Inactive disease	13	8.2 \pm 2.2	3.8 \pm 5.3
GC-responsive	6	9.12 \pm 3.7	6.14 \pm 3.7
GC-resistant	7	7.47 \pm 2.7	1.88 \pm 0.5
Active disease	29	63.6 \pm 17	15.8 \pm 7.0
GC-responsive	14	77.1 \pm 25	5.69 \pm 2.2
GC-resistant	15	51.0 \pm 23	23.8 \pm 13

*The amounts of hGR α mRNA and hGR β mRNA in peripheral blood mononuclear cells (PBMC) of CD patients and controls were normalized using the result of quantitative reverse transcription-polymerase chain reaction of human porphobilinogen deaminase (hPBGD) mRNA in each individual case. GC: glucocorticoid receptor.

between GR mRNA values and IL-18 content were analysed with Pearson's correlation coefficient. Statistical significance was based on a two-sided *P*-value of less than 0.05.

Results

The quantitative real-time PCR assay we used proved to be extremely sensitive in comparison to previously reported assays, which used conventional PCR products that were run on an agarose gel [7] or used Syber green fluorescence [8]. We used hybridization probes that are separated by one nucleotide and are complementary to the sequence within the central region of exon 9 α for hGR α or exon 9 β for hGR β . HGR expression was normalized using specific primers and hybridization probes for the PBGD gene. PBGD was used in our assay because it belongs to the low abundance class of mRNAs. To achieve absolute quantification of hGR mRNA we used serial dilutions of *in-vitro* transcribed hGR α or hGR β RNA as an external standard.

The amounts measured for hGR α mRNA and hGR β mRNA in PBMC of CD patients and healthy volunteers are summarized in Table 3. The mean amount of hGR α mRNA

expression in PBMC was lower in CD patients compared with healthy volunteers. The amounts were significantly lower in patients in remission ($P < 0.05$) compared to patients with active disease.

The mean amount of hGR β mRNA in GC-resistant active patients was significantly higher than in all other groups (patients as well as healthy volunteers) ($P < 0.05$) hGR β was significantly decreased in GC resistant compared to GC responsive patients with inactive disease. Our assay allowed us to measure hGR β mRNA expression in all samples (patients and controls).

IL-18 protein levels in CD patients and healthy volunteers

The levels in CD patients and healthy volunteers are summarized in Table 4. ELISA of patients' serum showed that CD patients, whether active or inactive, had up to sixfold higher amounts of IL-18 protein levels compared to healthy volunteers. Patients in the active stage of disease (CDAI ≥ 150) had up to twofold higher amounts of IL-18 protein than CD patients in remission (CDAI < 150). That serum IL-18 levels rise together with the CDAI was also shown in an as yet unreported study of ours in 120 patients with CD. The serum concentrations of IL-18 showed a significant correlation to the amounts of hGR β mRNA in GC-resistant patients in an active stage of disease ($r = 0.774$, $P < 0.05$) (Fig. 1). These results indicate that IL-18 might play a role in the alternative splicing of the hGR preliminary mRNA of CD patients.

Discussion

In the present study we have analysed the expression and regulation of hGR α and hGR β isoforms in human PBMC, and we have presented a fast and reproducible method for quantification of GR mRNA expression levels. In contrast to current end-point quantification methods, such as the competitive PCR assay, the fluorometric method for cycle-to-cycle quantification of PCR product proved to be a fast and reliable quantification method.

We found that the expression of hGR α mRNA in PBMC of CD patients is significantly higher in patients with active dis-

ease and in control subjects than in patients with inactive disease, whether steroid-resistant or steroid-responsive. This is an interesting and intriguing finding, for which we as yet have no satisfactory explanation. Other authors have also described low levels of hGR in autoimmune diseases [20]. Hori *et al.* [21] found a lower amount of GR α mRNA in PBMC of CD patients compared with healthy volunteers. Most of the patients in their study were in remission, therefore their finding of lower levels of GR α in patients compared to controls are in agreement with our group of patients in remission and our control group. Andreae *et al.* [22] found that children with autoimmune disease had lower amounts of GR on PBMC than healthy children. Schlaghecke *et al.* [23] have also described a reduced number of GR in mononuclear leucocytes of rheumatoid arthritis patients compared with healthy volunteers. All these studies point to down-regulation of the hGR α in patients with autoimmune disease during remission. In contrast, Raddatz *et al.* [32] did not find a difference in GR levels between patients with inflammatory bowel disease and healthy subjects; they did, however, find that GR expression was decreased in biopsies from ulcerative colitis patients. The significance of this observation is not yet clear and further studies of GC receptor regulation are required.

We chose to measure IL-18 levels because IL-18 was found to be a part of hGR regulation [16,30]. Levels of hGR β showed a significant correlation with IL-18. However, the discrepancy we found between IL-18 levels, which rise in all CD patients, whether in remission or during an exacerbation of disease, and hGR α levels, which are lower in CD patients during remission than in controls, suggests that other mechanisms and inflammatory mediators such as TGF α may also play a role in the regulation of GR α levels.

The other significant finding of our study was that the level of hGR β was significantly higher in steroid resistant CD patients during an exacerbation but not during a remission.

Table 4. Mean levels of cytokine interleukin (IL)-18 in Crohn's disease (CD) patients and controls.

	No. of cases	IL-18 (pg/ml)*
Inactive disease		
567 \pm 100	6	GC-responsive
652 \pm 193	6	GC-resistant
Active disease		
963 \pm 188	10	GC-responsive
1227 \pm 542	9	GC-resistant
218 \pm 46	10	Controls

*Results are reported as mean \pm s.e. GC: glucocorticoid receptor.

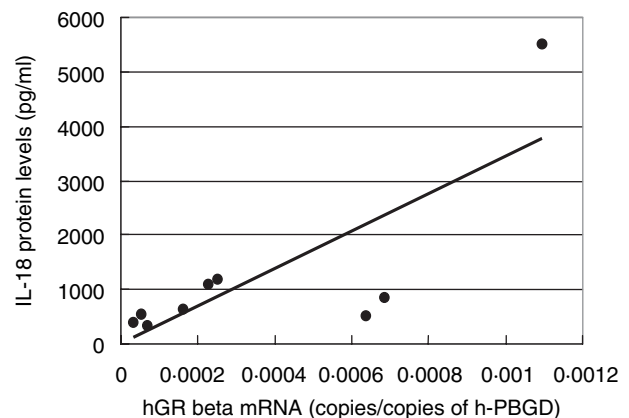


Fig. 1. Positive correlation between the amounts of hGR β mRNA in glucocorticoid (GC)-resistant patients in an active stage of disease and their interleukin (IL)-18 protein levels ($n = 9$) ($r = 0.774$, $P < 0.05$).

That the augmented level of hGR β could not be due to GC treatment has been shown in other studies [25,26]. Korn *et al.* showed that in bronchial cells *in vivo*, exposure to GC did not cause augmented levels of hGR β mRNA [27]. Honda *et al.* [12] showed that high doses of GC are not the inducers for the alternative splicing of the hGR gene in PBMC of UC patients.

The precise role of hGR β is not known. The level of this form of the GC receptor is 200–500 times lower than the level of hGR α [28,29], so it could not directly block hGR α activity by competitively binding to the same ligand [25]. On the other hand, higher levels of hGR β were found to be connected to GC resistance in ulcerative colitis patients [12] and in asthma patients [13]. It was also found that within tissues, hGR β is expressed at high levels in a cell type-specific manner [10], therefore it may inhibit hGR α by another, still unknown, mechanism. Metabolism of hGR β in patients resistant to glucocorticoids was different than in those who were glucocorticoid-sensitive both during exacerbation of disease, where it was significantly higher, and during remission, when it was significantly lower. Our findings support the possible role for hGR β as a predictor of steroid resistance. Further study is needed to confirm the precise role of the hGR β molecule.

It is conceivable that IL-18 and other proinflammatory cytokines contribute to the alternative splicing of the hGR gene by inducing an imbalanced expression of the hGR β isoform in PBMC of CD patients. Indeed, our results support previous hypotheses that high hGR β isoform expression in PBMC can be used to predict glucocorticoid resistance during the active phase of the disease [20,21]. Further studies are, however, required as to the function and regulation of steroid receptors.

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