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Glucocorticoid receptor beta splice variant expression in patients with high and low activity of systemic lupus erythematosus

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Abstract: The glucocorticoid receptor (GR) occurs mainly in two alternative splice variants encoding GRα and GRβ. The GRβ variant does not contain a GC binding domain and cannot mediate anti-inflammatory GC effects. Peripheral blood mononuclear cells (PBMCs) were isolated from venous whole blood of twelve patients with SLE. Ten of the SLE patients exhibited low disease activity while two patients displayed highly active stage of the disease. The quantitative analysis of GRα and GRβ transcripts in PBMC was performed by reverse transcription and real-time quantitative PCR SYBR Green I system. The protein level of GRα and GRβ isoforms in PBMCs was determined by western blotting analysis. We found that the two SLE patients with high disease activity exhibited significantly elevated GRβ transcript levels and corresponding protein levels in PBMCs. These preliminary findings suggest that increased expression of GRβ isoform may be associated with relatively more severe clinical presentation of SLE syndrome.

Key words: Glucocorticoid receptor α and β - Alternative splicing - Systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by enhancement of humoral and impaired cellular immune response. Autoreactivity of the $CD4^+$ T and B cells is suspected to be responsible for abundant autoantibody production directed against self antigens [1,2]. The excessive production of autoantibodies causes immune complex formation, which can be deposited in various tissues resulting in dysfunction of organs and systems and clinical manifestation of SLE [1,2].

Standard treatment of patients with SLE includes reduction of lymphocyte autoreactivity with synthetic glucocorticosteroids (GCs) or other immunosuppressive agents [3-6]. Biological actions of GCs are mediated by glucocorticoid receptor that is a member of steroid/thyroid/retinoic acid receptor [3-6]. GCs and receptor complex binds to DNA and regulates gene transcription in a ligand-dependent manner. This leads to a reduction of the cellular pool of NF-kappa B and AP-1 transcription factors, and a decreased expression of genes encoding proteins contributing to autoreactivity of the T and B cells [3-6].

The GR occurs mainly in two alternative splice variants of nine exons encoding functional GRα and nonfunctional GRβ. GRα and GRβ variants consist of 777 and 742 amino acids, respectively. They are identical from amino acid residue 1 to 727, and are distinct only in the carboxyl terminal region. GRα contains an additional 50 amino acids, encoding helices 11 and 12 to form a complete carboxy terminus ligand-binding domain (LBD) [7,8]. However, GRβ does not contain helix 12 of LBD but possesses a distinct amino acid sequence in helix 11 compared to GRα (Fig. 1) [7,8]. The shorter LBD of GRβ is found constitutively in the

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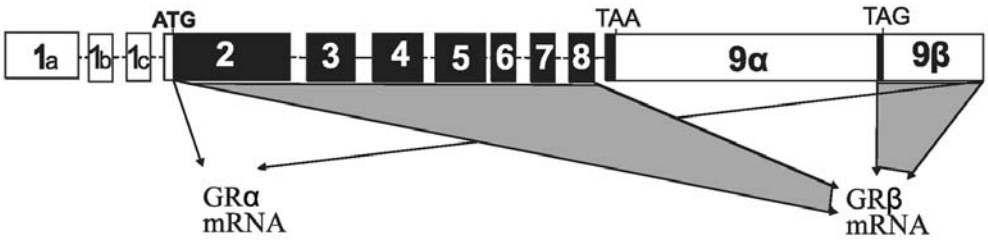

Fig. 1. Diagram of GR gene map and composition of GRα and GRβ transcript isoforms. Boxes and dashed lines represent exons and introns, respectively. Exons are numbered at the middle of the corresponding box. The exons 1a, 1b and 1c correspond to multiple promoters leading to transcription of several GR mRNAs with unique 5'-untranslated region in exon 1. The black areas of exons 2 to 9 include an open reading frame. The white boxes correspond to 5'-untranslated and 3'-untranslated regions. Alternative processing of the GR primary transcript at exon 9 generates GRα and GRβ splice variants, which differ by the presence of 9α and 9β sequences [7,8]. GRα and GRβ proteins are composed of 777 and 742 amino acids, respectively. They are identical from amino acid residue 1 to 727, and are distinct only in the carboxyl terminal region. GRβ does not contain helix 12 of carboxy terminus ligand-binding domain (LBD) but has a different amino acid sequence in helix 11 compared to GRα. The shorter LBD of GRβ cannot bind ligand, and is not able to mediate anti-inflammatory GCs effects [7,8].

nucleus and is not able to bind ligand or to mediate anti-inflammatory GC effects [7]. Three mechanisms responsible for negative activity of GRβ have been proposed [7,8]. GRβ competes with GRα for binding to GC response element of DNA, GRβ and GRα form non-transactivating heterodimers, or GRβ may also bind limiting numbers of coactivators for GRα [7,8].

Glucocorticoid resistance due to an imbalance in the GRα/GRβ ratio was found in various autoimmune diseases [7,9]. However, the increased expression of GRβ receptor in SLE patients was never determined.

In our study, we compared the GRα and GRβ transcript and protein levels in peripheral blood mononuclear cells (PBMCs) from ten patients with low disease activity and two patients who exhibited high disease activity, both groups confirmed as SLE patients.

Materials and methods

Patients. Twelve patients fulfilling the American College of Rheumatology Classification criteria for SLE were randomly chosen for this study at the Institute of Rheumatology in Warsaw, Poland (Table 1) [1,2]. All patients were orally treated with methylprednisolone >0.5 mg/kg/day. Two of them, with the most active disease according to scoring calculated by Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (Table 1) received as much as 4 mg/kg/day of methylprednisolone over 3 days, and intravenous pulses of cyclophosphamide. The protocol of the study was approved by the Local Ethical Committee. Written informed consent was obtained from all participating subjects. Patients were asked not to take any medication for at least 24 h before drawing the blood. Blood samples were collected with EDTA-based blood sampling syringes Monovette (Sarstedt, Stare Babice, Poland).

Antibodies. Rabbit polyclonal anti-GRα antibodies (Ab) (ab3580), rabbit polyclonal IgG anti-GRβ Ab (ab3581) were purchased from Abcam (Cambridge, MA). Goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (Ab) and goat polyclonal anti-actin HRP-conjugated Ab (clone I-19) were provided by Santa Cruz Biotechnology (Santa Cruz, CA).

Real-time quantitative PCR (RQ-PCR) analysis of GRα and GRβ transcript level in PBMCs. The PMBCs were isolated from venous whole blood by centrifugation over Ficoll-Hypaque (d=1.077 g/cm^3). Total RNA was isolated according to the method of Chomczynski and Sacchi [10]. RNA samples were treated with DNase I, quantified, and reverse-transcribed into cDNA. Quantitative analysis of GRα and GRβ cDNA was performed by RQ-PCR SYBR Green I system with Light Cycler, Roche Diagnostics GmbH, (Mannheim, Germany). The quantity of GR variant transcripts in each sample was normalized with polymerase II (POLR2A) transcript level. The GRα, GRβ, and POLR2A cDNA were amplified employing pairs of primers: (5'TCA ACT GAC AAA ACT CTT GG3'), (5'TGA TTG GTG ATG ATT TCA GC3'),

Table 1. Demographic, organ system involvement of patients with low and high disease activity (DA).

Features	Low DA	High DA	
Number of patients(Female)	10	2	
Median[b] age (years)	27 (range 21-40)	23	36
Central nervous system[a]	0/10	+	-
Vascular[a]	0/10	-	+
Renal[a]	6/10	+	-
Musculoskeletal[a]	3/10	+	+
Serosal[a]	0/10	+	-
Dermal[a]	3/10	+	-
Immunologic[a]	7/10	+	+
Constitutional (fever) [a]	0/10	-	-
Hematologic[a]	3/10	+	+
Median[b] of SLEDAI scores	6 (range 2-9)	30	17

[a]As defined by SLEDAI score index [2]. [b]regards low DA patients.

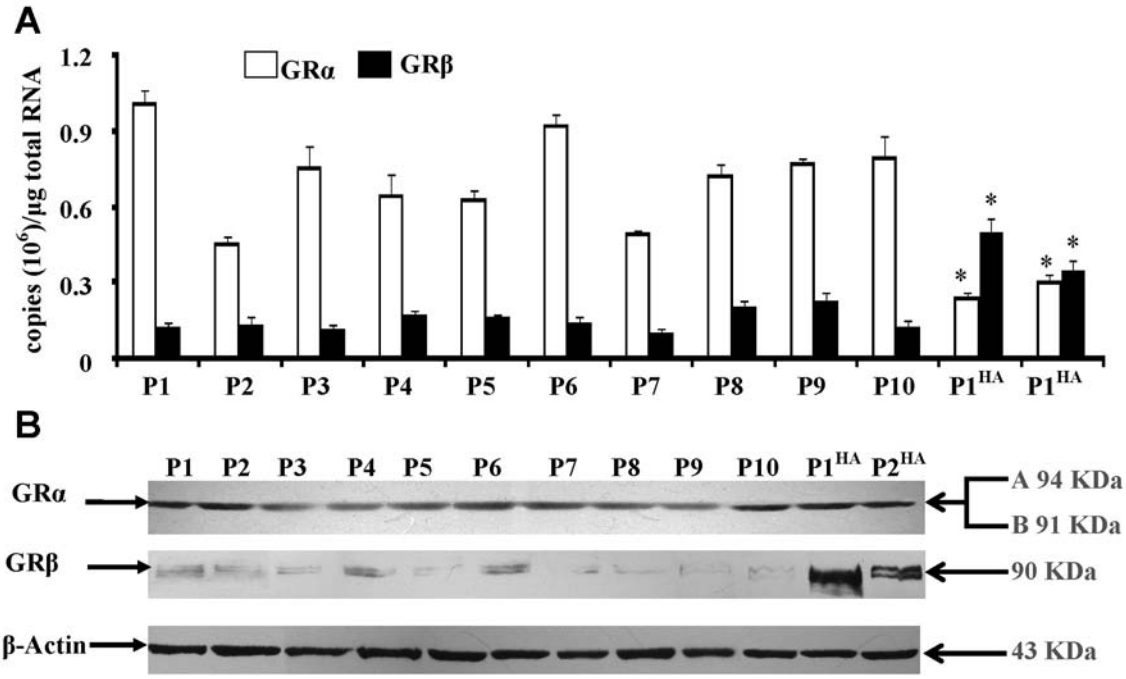

Fig. 2. Levels of GRα and GRβ transcript (**A**) and protein (**B**) in PBMCs from SLE patients with low disease activity (P1-P10) and high disease activity ($P1^{HA}$, $P2^{HA}$) PMBCs were used to isolate total RNA and proteins for RQ-PCR and western blotting analysis of GR variants. RQ-PCR results were standardized by POLR2A cDNA levels and were expressed as copies per 1 RNA μg. Results are representative of triplicate value means with SEM <15%; * $p<0.05$. For western blotting analysis, cell proteins were separated by SDS-PAGE and transferred to a membrane that was then immunoblotted with rabbit polyclonal anti-GRα and anti-GRβ Abs, and secondary goat anti-rabbit HRP-conjugated Ab. To equalize protein loading we reblotted with anti-actin HRP-conjugated Ab. The GRβ amounts were determined based on the band intensity in the autoradiogram. A and B correspond to 94 and 91 KDa GRα isoforms, respectively.

(5'AGC GGT TTT ATC AAC TGA C 3'), (5'TGA GTT CTA TTTT TTG AGC G3') and (5'CTG GAG ACA GCA AGG TCG TCC -3'), (5'CCA GCT TCT TGC TCA ATT CC3'), respectively. For amplification, 2 μl of total (20 μl) cDNA solution was added to 18 μl of QuantiTect® SYBER® Green PCR Master Mix QIAGEN GmbH (Hilden, Germany) and primers. Since the amplification efficiency of target and references genes slightly differed, quantification of copy number of these genes was respectively derived from a different standard curve for target and references genes. One RNA sample of each preparation was processed without RT-reaction to provide a negative control in subsequent PCR. RQ-PCR results were expressed as copies per 1 μg of RNA.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis of GRα and GRβ proteins content in PBMCs. PBMCs were lysed in lysis buffer and twenty micrograms of protein was resuspended in sample buffer and separated on 10% Tris-glicyne gel using SDS-PAGE. Gel proteins were transferred to nitrocellulose, which was blocked with 3% milk in Tris buffered saline/Tween. Immunodetection was performed with rabbit anti-GRα Ab, rabbit anti-GRβ Ab and HRP-conjugated goat anti-rabbit Ab. The membranes were reblotted with anti-actin HRP-conjugated Ab to equalize protein loading of the lanes. Bands were revealed using ECL kit and Hyperfilm ECL Amersham (Piscataway, NJ). The quantities of GRα and GRβ were determined based on the band intensity in the autoradiogram.

Statistical analysis. Data groups were analyzed by ANOVA to evaluate similarity between the groups (significant difference was recognized at $p<0.05$). For all experimental groups, which satisfied the initial ANOVA criteria, individual comparisons were done with the use of post hoc Newman-Keul's test with the assumption of two-tailed distribution and two samples with equal variance at the $p<0.05$ level.

Results and discussion

GCs are both naturally occurring and synthetic steroid hormones that contribute to a wide spectrum of human physiological processes and participate in the development of many organs and systems [11-13]. GCs are effective immunomodulatory and anti-inflammatory agents in the treatment of autoimmune diseases and some lymphoid malignancies [5,9].

We found that two patients with high SLE activity exhibited elevated GRβ transcript and protein levels in PBMCs (Table 1, Fig. 2). The average GRβ transcripts and proteins amount in PBMCs of these two patients was several times higher compared to patients with low disease activity, respectively (Fig. 2). The levels of expression of GRβ in low disease activity patients and healthy individuals were similar (results not shown).

Our findings suggest that increased expression of GRβ in SLE patients may be associated with high disease activity. Previous studies have demonstrated that GRβ could function as a dominant inhibitor of GRα

erasing the anti-inflammatory effect of GCs in inflammatory cells [7,9]. Overexpression of GRβ in inflammatory cells was found in GC-resistant patients with asthma, ulcerative colitis, nasal polyposis, Crohn's disease, and some lymphoid malignancies [9,14-16]. Lymphoblastic/lymphocytic leukemia patients with high GRβ/GRα proteins ratio were also less sensitive to the apoptosis induced by GC treatment [15,16].

We did not find sequence alterations in GR cDNA and in DNA of 5'-regulatory region, nine exons, and ~300 bp of 3' region of the GR gene in PBMCs from GC-resistant patients (result not shown). This finding is consistent with those of Shahidi *et al.* [16], who have not indicated sequence alterations in GR cDNA and GR gene in a single case of GC-resistant chronic lymphocytic leukemia [16].

Our preliminary report is the first to demonstrate a possibility of association between increased expression of GRβ isoform and more severe manifestation of SLE. This also supports the significance of GRβ expression in the development of certain severe cases of other autoimmune diseases.

Increased expression of GRβ can be responsible for glucocorticoid resistance and loss of immunosupresive effect of GCs. Recently we observed that epigenetic modifiers trichostatin A, sodium butyrate, and 5-aza-2'-deoxycytidine are able to cause a decrease in GRβ expression, which can be of importance for treatment possibilities in patients with increased expression of GRβ [17].

However, further investigations must be conducted to confirm these findings and to determine a mechanism of GR mRNA splicing regulation and the role of post-transcriptional/translational regulation of GR expression in increased productivity of GRβ expression in highly active SLE disease.

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