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ORIGINAL ARTICLE

Low expression of glucocorticoid receptors in children with steroid-resistant nephrotic syndrome

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Abstract
Background About 10–20 % of children with idiopathic nephrotic syndrome (NS) are steroid-resistant (SR). Low expression of glucocorticoid receptors (GRs) has been associated with poor response to steroids in a variety of autoimmune diseases. This study was done to assess the expression of cytoplasmic GRs for CD3 and CD14 in children with NS.
Methods Expression of cytoplasmic GRs in lymphocytes ($CD3^+$/GR) and monocytes ($CD14^+$/GR) in the peripheral blood were assessed in 51 children with NS before the start of therapy by flow cytometry. Patients were divided into two groups: 30 children who were steroid-sensitive (SSNS) and 21 children who had initial steroid resistance (SRNS). Twenty age- and sex-matched healthy children served as controls.
Results Expression of $CD3^+$/GR was significantly lower in SRNS in comparison to SSNS patients and controls ($p<0.0001$). Similarly, expression of $CD14^+$/GR was significantly lower in SRNS in comparison to SSNS patients ($p<0.0001$) and controls ($p=0.002$). $CD3^+$/GR and $CD14^+$/GR expression were not significantly different in SSNS patients compared with controls ($p=0.06$ and 0.07 respectively).
Conclusions Patients with initial SRNS showed decreased GR expression in peripheral blood mononuclear cells (PBMC) before starting therapy, and this low expression may be one of the pathophysiological mechanisms of steroid resistance in these children.

Keywords Gene expression · Nephrotic syndrome · Steroid-sensitive nephrotic syndrome · Steroid-resistant nephrotic syndrome · Glucocorticoid receptors · CD3 · CD14

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Introduction

Glucocorticoids (GCs) are the main treatment for nephrotic syndrome (NS). They have been used to treat idiopathic NS since the early 1950s [1]. The International Study of Kidney Disease in Children (ISKDC) defines steroid-resistant nephrotic syndrome (SRNS) by the persistence of proteinuria after a 4-week course of oral prednisone (60 mg/m^2/day) [2]. About 10–20 % of children with nephrotic syndrome have SRNS. Treating such patients is a great challenge since they are not only at risk of complications of unremitting disease with subsequent progressive renal disease, but also the side effects of treatment with immunosuppressive medications [3]. GCs easily diffuse through the cell membrane and bind to their cytoplasmic glucocorticoid receptors (GRs) [4]. GR-induced gene activation or inhibition is the main rationale for GC therapy in immune-mediated diseases [5]. Activation of GRs results in their translocation to the nucleus and activation of anti-inflammatory gene expression. Furthermore, activated GRs suppress pro-inflammatory genes, possibly through inhibiting transcription factors such as nuclear factor kappa beta (NFkB) [6].

It is well established that GC resistance may be due to defects in the number, distribution, and expression of specific receptors [7]. Defects in GR expression and their relationship with the response to steroid therapy have been studied in a variety of diseases. It has been found that defective response

of peripheral blood mononuclear cells (PBMC) to GC could be correlated with clinical resistance to GC treatment in bronchial asthma [8], systemic lupus erythematosus [9], rheumatoid arthritis [10], lymphoma, and leukemia [11].

Although relationships between GRs and steroid response in NS have been previously studied, the results obtained were equivocal. Immunoassays of GRs revealed increased numbers in monocytes of adult patients with steroid-sensitive nephrotic syndrome (SSNS) in comparison to those with SRNS [12]. Moreover, in SSNS, peripheral lymphocyte GR expression was considered a possible predictor of early response to steroids [13]. On the other hand, the density and binding affinity of GRs as determined by dexamethasone binding assays in mononuclear leucocytes was not different in children with SRNS and SSNS [14].

Flow cytometry (FCM) is a sensitive method for differentiating cells by size and cytoplasmic granularity. It can also detect the expression of different receptors using fluorochromes conjugated to antibodies against the proteins of interest [15, 16]. Thus, FCM is a good method for detecting the expression and binding of GRs by using anti-GR monoclonal antibody (mAb) and fluorescein isothiocyanate (FITC)-labeled dexamethasone (Dex) probes. The present study used FCM to assess the expression of cytoplasmic GR on lymphocytes (CD3) and monocytes (CD14) in the peripheral blood of children with SSNS and SRNS before starting therapy.

Patients and methods

This cross-sectional study included 51 children diagnosed with their first attack of idiopathic NS before receiving any immunosuppressive medications. Patients were recruited consecutively from the Nephrology Unit in Mansoura University Children's Hospital in the period from January 2009 to September 2011. Diagnosis of NS depended on the presence of edema, heavy proteinuria (≥40 mg/m^2/h or a urine protein/creatinine ratio ≥2.0) and hypoalbuminemia (≤2.5 g/dl) [17]. None of the patients had a family history of NS or consanguineous parents. All patients were treated by daily prednisolone at a dose of 60 mg/m^2/day and children who failed to achieve remission after continuous therapy for 4 weeks were diagnosed as having SRNS [18]. Any patients with elevated serum creatinine, gross hematuria, low serum C3 level or history of recent steroid therapy of any cause in the preceding 6 months were excluded. Remission was defined by the absence of proteinuria (urine albumin nil or trace for 3 consecutive days) [17]. Patients were retrospectively divided into two groups: group I, which included 30 children (19 male, 11 female) who were steroid-sensitive, and group II, which included 21 patients (14 male, 7 female) who had SRNS. Renal biopsy was done for group II patients and revealed focal segmental glomerulosclerosis and minimal change disease in 16 and 5 patients respectively. Twenty age- and sex-matched healthy children with no evidence of renal or immune-mediated disease or recent histories of steroid intake for any reason were used as a control group.

Informed consent was obtained from the parents of patients and controls prior to the study after the research protocol was approved by the local ethics committee.

GR-mAb-FCM analysis for the expression of GR

EDTA-k2-containing blood samples were obtained from all patients before the start of therapy by density gradient centrifugation, using Ficoll-Hypaque to isolate all mononuclear cells, and then stored at 4 °C in absolute ice-cold alcohol until stained. FITC-labeled CD3 mAb (catalog no. abs24947; Abcam, Cambridge, UK) and FITC-labeled CD14 mAbs (Biolegend, catalog no. 325604) were used for cell surface staining. Anti-mouse IgG-FITC (catalog no. 345815, Becton Dickinson, Franklin Lakes, NJ, USA) was used for the detection of GR. FITC-labeled mouse IgG1 isotype control was used as an isotypic control for the staining. Samples of 100 μL of isolated mononuclear cells with an appropriate concentration of anti-CD3-FITC (10 μL) or anti-CD14-FITC mAb (10 μL) or FITC-mouse IgG1 were incubated for 20 min at room temperature in the dark. Cells were then washed twice with PBS/BSA and fixed in 100 μL of 4 % buffered paraformaldehyde (fixation buffer; eBioscience, San Diego, CA, USA) for 30 min at 4 °C. Nonspecific antibody binding was blocked with 5 % normal goat serum containing permeabilization buffer for 30 min. Samples were then incubated with 100 μL of permeabilization buffer (Nodite No-40, Sigma, N-3516) containing anti-GR mAb or mouse IgG1 at 4 °C for 60 min, washed twice in permeabilization buffer, and then pellets were resuspended in 300 μL of fixation buffer. Cell samples were run on a FACS Caliber flow cytometer (Becton Dickinson) and analyzed by CELL Quest software. At least 20,000 events in the light-scatter (SSC/FSC) lymphocyte and 8,000 events in the light-scatter (SSC/FSC) monocyte region were acquired. $CD3^+$ and $CD14^+$ populations were identified and gated on FITC plots.

Statistical methods

The data were statistically analyzed by SPSS (Statistical Package for Social Science, version 17). Data were found to have a nonparametric distribution using the Kolmogorov–Smirnov test. Data are expressed as median and interquartile range (IQR). Differences in the levels of $CD3^+$/GR and $CD14^+$/GR between groups were assessed by the Mann–Whitney U test. P value of <0.05 was considered significant.

Table 1 Demographic and baseline laboratory parameters of the study groups

	Group I	Group II	Controls
Age (years)	5.3 (4–8)	6.5 (4–7.6)	6.5 (4.3–8)
Male/female	19/11	14/7	12/8
Creatinine (mg/dL)	0.6 (0.5–0.8)	0.7 (0.6–0.8)	0.7 (0.6–0.8)
Total cholesterol (mg/dL)	265 (199–288)	268 (208–310)	_
Serum albumin (g/dL)	2 (1.7–2.2)	1.8 (1.5–2)	_
24 h urinary protein (g/m^2)	1.2 (1.1–1.3)	1.2 (1.1–1.5)	_
Urinary protein/creatinine	3.2 (3–3.4)	3.2 (3–3.6)	_

Results

Demographic and baseline laboratory data of the studied patients and controls are summarized in Table 1. Lymphocyte expression of glucocorticoid receptor ($CD3^+$/GR) was significantly lower in group II in comparison to group I and controls (median [IQR]=17.6 % ([3.5–18.4] vs 56.3 % [51.6–67.9], $p<0.0001$ and vs 51.7 % [43.6–60.6], $p<0.0001$ respectively). Similarly, monocyte expression of glucocorticoid receptor ($CD14^+$/GR) was significantly lower in group II in comparison to group I and controls (median [IQR]=17.3 % [11.6–19.4] vs 41.5 % [38.9–46.2], $p<0.0001$ and vs 35.5 % [15.2–51.4], $p=0.002$ respectively). Lymphocyte and monocyte GR expression were not significantly different in group I compared with controls (median [IQR]=56.3 % [51.6–67.9] vs 51.7 % [43.6–60.6], $p=0.06$ and 41.5 [38.9–46.2] vs 35.5 % [15.2–51.4], $p=0.07$ respectively; Table 2).

Discussion

Glucocorticoids represent the standard of treatment for NS. Steroid responsiveness is the major determinant of prognosis in NS. It was reported that 50 % of SRNS patients progress to end-stage renal failure within 1–4 years [2]. PBMCs are among the most important targets for immunosuppressive therapy. Several studies have shown abnormalities in T lymphocytes of patients with SRNS [19, 20]. Furthermore, cytokines may play a role in steroid resistance [21]. Recently, it was reported that glucocorticoids can have effects directly on the kidney, independent of their effects on the immune system. Clement et al. showed that the glomerular expression of angiopoietin-like 4, a secreted glycoprotein, is glucocorticoid-sensitive and is highly up-regulated in podocytes in experimental and human minimal change NS independent of the immune system [22]. This present study was based on the rationale that resistance to GC treatment may be mediated by defective expression of GR in the PBMC.

Equivocal data about the relationship between GRs and steroid response in NS have been previously published. To our knowledge, GR expression in PBMC by flow cytometry in children with SRNS has not been widely assessed before. Wasilewska et al. studied the expression of GR in mononuclear cells in 23 children with SSNS by Coulter flow cytometry. They reported that in children with SSNS, lymphocyte CD3 and monocyte CD14 expression were not different from healthy controls before the start of GC therapy [23]. In SSNS, T lymphocyte (CD3+) expression of GR was significantly lower in the late steroid responders (LR) than in the control group, whereas it was similar in the early steroid responders (ER) and control groups. GR expression was also decreased in the LR group compared with the ER group [13]. Szilagyi et al. observed lower expression of GR in PBMC, tested by Western blotting, in SRNS than in SSNS and controls [19]. Bagdasarova et al., assessing GR expression in monocytes by immunoassay, reported a lower level of GR in SRNS than in SSNS patients [12]. Haack et al. investigated GRs in the PBMC of 28 pediatric patients with NS by testing the density and binding affinity of the receptors using a dexamethasone binding assay. They found no significant differences between

Table 2 Comparison of the percentages of expression of glucocorticoid receptors in lymphocytes (CD3/GR) and monocytes (CD14/GR)*

Group	Number		Median %	IQR	*P*	*P***
I	30	CD3/GR	56.3	51.6–67.9	0.06	<0.0001
II	21		17.6	13.5–18.4	<0.0001	
Controls	20		51.7	43.6–60.6		
I	30	CD14/GR	41.5	38.9–46.2	0.07	<0.0001
II	21		17.3	11.6–19.4	0.002	
Controls	20		35.5	15.2–51.4		

*Mann–Whitney test

P—comparison of I or II and controls, *P*—comparison of I and II

SSNS and SRNS patients and between these and healthy controls, although a few patient values fell outside the range of controls [14]. The variations in these studies may be due to the timing of sampling, use of heterogeneous material or different analytical techniques for the assessment of GR.

Our results showed that before starting GC therapy, lymphocyte and monocyte GR expression was significantly lower in SRNS patients in comparison to healthy controls. Low GR expression in these patients is likely one of the factors responsible for their poor response to steroids, but not to the pathogenesis of NS. This hypothesis was strengthened by the finding of significantly lower lymphocyte and monocyte GR expression in SRNS in comparison to SSNS children. Thus, the nephrotic state is not the cause of the lower GR expression observed in SRNS because both study groups had active disease at the time of sampling and none of the patients had an attack of nephrotic relapse before. It is well known that steroid therapy is associated with down-regulation of GR expression in PBMC in patients with NS [23] and renal transplant patients [24]. The mechanism of GR down-regulation may be explained by a possible movement of GR to the cell nucleus after binding to steroids [25]. However, in the present study, since the blood samples had been obtained before therapy, decreased GR expression in SRNS cannot be attributed to steroid therapy.

The causes of steroid resistance in NS are variable and the underlying mechanisms may be complicated. At a molecular level, resistance to the anti-inflammatory effects of glucocorticoids can be induced by several mechanisms, and these may differ between different patients [26]. It is widely accepted that GC resistance may be caused by GR gene mutations [26, 27]. Reduced number of GRs, altered affinity of GRs for their ligands, reduced ability of GRs to bind to DNA, or increased expression of inflammatory transcription factors, such as AP-1, that compete for DNA binding have been described as possible mechanisms [26].

We conclude that patients with initial SRNS show decreased GR expression in PBMC before starting therapy and that this low expression may be one of the pathophysiological mechanisms of steroid resistance in these children. The results of the current work may be limited by the small numbers of patients studied; further studies on larger numbers of patients are recommended.

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