Glucocorticoid receptors, in vitro steroid sensitivity, and cytokine secretion in idiopathic nephrotic syndrome

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Background. Glucocorticoids (GC) represent the mainstay of treatment of idiopathic nephrotic syndrome (INS) and might be involved in the pathogenesis of the disease. We evaluated basal secretion of cortisol, number and affinity of glucocorticoid receptors, dexamethasone (Dex)-mediated inhibition of concanavalin-A (Con-A)-stimulated peripheral blood mononuclear cell (PBMC) proliferation, and cytokine secretion in patients with INS.

Methods. Blood and saliva were obtained from 20 INS patients in relapse and 11 control patients. Cortisol concentrations were measured by radioimmunoassay. PBMC were isolated for binding and in vitro GC sensitivity assays. Cytokines were measured in supernatants of PBMC culture by enzyme-linked immunosorbent assay (ELISA).

Results. Salivary cortisol concentrations were similar in INS patients and control patients. Density and affinity of GC receptors were similar in steroid-sensitive (SS) patients and control, whereas in steroid-resistant (SR) patients they were variable. Lymphocyte proliferation after Con-A stimulation was inhibited by Dex in a dose-dependent manner in control and SS patients. Control and all clinically SS patients were steroid-sensitive by in vitro test, but control patients significantly presented more suppression of PBMC proliferation compared with SS patients. Basal- and Con-A–stimulated interleukin (IL)-6, IL-10, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α levels were similar in control and INS patients, and all cytokines but IL-10 were significantly inhibited by Dex 10−6 mol/L. In SR patients, cytokine secretion remained elevated after treatment with high doses of Dex.

Conclusion. Abnormalities of number and affinity of the GC receptor and altered secretion of cytokines may be involved in tissue sensitivity to GC in INS patients.

Key words: glucocorticoid receptor number and affinity, Th1/Th2 cytokines, in vitro steroid sensitivity, idiopathic nephrotic syndrome.

Steroid responsiveness is the major determinant of prognosis in idiopathic nephrotic syndrome (INS). Approximately 85% to 90% of patients with INS respond to glucocorticoid (GC) therapy and have complete remission of proteinuria; 10% to 15% have partial or no response to corticosteroid treatment [1]. Children with steroid-sensitive (SS) INS have a favorable long-term outcome, even after multiple relapses, whereas 50% of steroid-resistant (SR) patients progress to end-stage renal failure within 1 to 4 years [2]. There are no clinical predictors of the response to corticosteroids in INS.

The etiology of INS remains obscure. Cellular immune disturbances, particularly T-lymphocyte abnormalities, have been previously implicated in the pathogenesis of the disease [2]. A suppressed immune response, the absence of renal histologic alterations on light microscopy, and no significant immune deposits in glomeruli have suggested that INS is a T-cell disorder associated with a functional renal compromise [3]. Proteinuria in INS is thought to be caused by the effect of circulating cytokines on glomerular permeability. Thus, several authors have looked for a cytokine that could render the glomerular basement membrane more permeable to proteins [2, 3].

Glucocorticoids act by binding to a cytoplasmic GC receptor, which then translocates to the nucleus to act as a transcription factor [4]. Previous studies have demonstrated diminished GC receptor number and binding affinity in peripheral blood mononuclear cells (PBMC) from rheumatoid arthritis [8] and asthma SR patients [9]. In INS patients, there are few GC binding studies and they are still controversial [10, 11]. Other factors besides GC receptors might mediate the variable response to GC, such as cytokines [11]. However, there is no consensus concerning the production of several cytokines in patients with INS [12–15].

In the present study, we evaluated the basal secretion of plasma and salivary cortisol, the number and affinity of GC receptors, and the pattern of cytokine [tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin
(IL)-6, IL-10] secretion in control patients and in children with INS. In order to classify patients with INS as SR or SS by the in vitro test, we evaluated dexamethasone (Dex)-mediated inhibition of concanavalin-A (Con-A)–stimulated PBMC proliferation. In addition, to evaluate the role of cytokines in the mechanism of corticosteroid resistance, we studied the effect of different doses of Dex on cytokine secretion by Con-A–stimulated PBMC in culture.

METHODS

Subjects

This prospective study was approved by the institutional review board for human research and informed consent was obtained from all subjects and their parents. We studied 11 healthy subjects (6 males, 5 females) ranging in age from 1.2 to 16.2 years, with no signs of acute or chronic illness and who were taking no drugs. In addition, we also studied 20 patients with INS (9 males, 11 females) aged 2 to 15.9 years, followed-up by the Division of Pediatric Nephrology-Faculty of Medicine of Ribeirão Preto, University of São Paulo. The diagnostic criteria for INS were based on the International Study of Kidney Disease in Children [16]. At the time of the study, all patients had active disease characterized by massive proteinuria (>40 mg/m²/hr or urinary protein/creatinine ratio >1), with normal serum creatinine levels and who were not using steroids. Ten patients were off corticosteroid therapy for 4 to 40 months (median 16 months). Ten patients were evaluated during the initial course of INS and only received corticosteroids after blood and saliva collection for this study. According to the clinical response to treatment, patients who had complete remission of proteinuria with corticosteroid therapy were classified as SS (N = 17). Patients who persisted with proteinuria after daily prednisone therapy (60 mg/m²/day) for 4–6 weeks, followed by high-dose intravenous pulse methylprednisolone (30 mg/kg/dose, maximum 1000 mg) on alternate days for 3–6 doses were considered SR (N = 3). Renal biopsy was performed in the SR patients and revealed focal segmental glomerulosclerosis in all.

Plasma and salivary cortisol measurement

Blood and saliva samples were obtained from healthy donors and INS patients between 8:00 and 9:00 a.m. Serum and salivary cortisol concentrations were measured by radioimmunoassay, as previously described [17–19].

Cell preparation

PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque (Histopaque; Sigma Chemical Co., St. Louis, MO, USA), washed three times in Hank’s buffered saline solution (HBSS), and resuspended in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) containing 2 mmol/L HEPES buffer (Sigma Chemical Co.), 10% fetal calf serum, 100 IU/mL penicillin, 100 g/mL streptomycin, and 10 mg/mL gentamicin.

Binding assay

PBMC Dex-binding assay was performed as previously described [20]. Cells were suspended in RPMI media and adjusted to 2 × 10⁶ cells per tube in duplicate and incubated with six concentrations (1.56–50 nmol/L) of Dex (1, 2, 4, 6, 7, ³H) (Dexamethasone; Amersham Life Science, Buckinghamshire, UK) at 37°C in the presence or absence of a 1000-fold molar excess of unlabeled Dex (Dexamethasone; Sigma Chemical Co.) for 1 hour. After incubation, the cells were washed three times to separate bound- from free-steroid with 1.5 mL cold phosphate-buffered saline (PBS) and centrifuged at 400g for 10 minutes. After the third wash, the pellets were suspended in 100 μL of RPMI, transferred to vials, and the radioactivity was counted in a β counter. Specific binding was calculated by subtracting nonspecific binding from total binding. Receptor assay data were analyzed by the method of Scatchard using computerized linear regression analysis. The binding capacity (B_max) was expressed as fmol of dexamethasone bound per mg of protein, and the dissociation constant (K_d), inversely proportional to ligand affinity, was expressed in nmol/L.

Proliferation and in vitro corticosteroid sensitivity assay

To perform the in vitro steroid sensitivity assay we measured the inhibitory effect of Dex on Con-A–stimulated PBMC proliferation. PBMC (2 × 10⁶ cells per well) were plated onto 96-well flat-bottomed plates (Nunc, Naperville, IL, USA) in triplicate and cultured at 37°C in the presence of 5% CO₂. Con-A at the dose of 50 μg/mL was used to stimulate the cells in the presence or absence of different doses (10⁻⁸, 10⁻⁶, 10⁻⁴ mol/L) of Dex. After 48 hours of culture, the cells were pulsed with 1 μCi/well tritiated thymidine (³H-thymidine, Amersham, Pharmacia Biotech, UK) for 18 hours before collecting the supernatants for cytokine quantification. The cells were harvested with a multiple automated sample harvester and radioactivity was counted in a liquid scintillation β counter (Beckman Coulter, Fullerton, CA, USA).

Percent inhibition of proliferation by steroid was calculated using the following formula: \(\frac{1 – (x – n/y – n)}{100}\), where \(x = \) counts in Dex and Con-A; \(n = \) counts in RPMI alone; \(y = \) counts in Con-A alone. The IC₅₀ was defined as the concentration of Dex that caused a 50% inhibition of cell proliferation. Subjects with an IC₅₀ > 10⁻⁶ mol/L were considered SR. Percent inhibition of cytokine secretion was calculated by the formula above, where \(x = \) cytokine secretion in Dex and Con-A; \(n = \) cytokine secretion in RPMI alone; \(y = \) cytokine secretion in Con-A alone. By using these calculations, the variations for
Con-A–induced PBMC proliferation, or cytokine secretion occurring between and within individuals at different times, were compensated.

Cytokine quantification

The supernatants collected during cell culture were stored at −70°C for measurement of IL-6, IL-10, TNF-α, and IFN-γ levels by ELISA. The antibodies used for coating 96-well plates were 18891D (anti-IFN-γ, PharMingen, San Diego, CA, USA), JES3-19F1 (anti-IL-10, PharMingen), MAB206 (anti-IL-6, R&D Systems, Minneapolis, MN, USA), and MAB610 (anti-TNF-α, R&D Systems). Second-step biotinylated detection monoclonal antibodies were 19751N (anti-IFN-γ, PharMingen), JES3-12G8 (anti-IL-10, PharMingen), BAF206 (anti-IL-6, R&D Systems), and BAF210 (anti-TNF-α, R&D Systems). The minimum detection limits for IFN-γ, IL-10, IL-6, and TNF-α were 19751N (anti-IFN-γ, R&D Systems). The linearity of the Scatchard plots indicates a single class of binding site affinity.

Statistical analysis

All results are expressed as median with 95% CI. Data were compared using Friedman followed by Dunn test and Mann-Whitney test, when appropriate, with the level of significance set at P < 0.05. Due to the small sample size, the SR patients were analyzed individually.

RESULTS

Plasma and salivary cortisol measurement

Plasma cortisol levels were lower (P = 0.01) in SS patients (5.4 μg/dL; 95% CI: 4.6–8.3 μg/dL) compared to normal control patients (8.5 μg/dL; 95% CI: 7.6–12.2 μg/dL). However, both groups presented similar salivary cortisol concentrations (705 ng/dL; 95% CI: 525–1333 ng/dL vs. 710 ng/dL; 95% CI: 600–1216 ng/dL, respectively). Plasma and salivary cortisol levels in patients SR1, SR2, and SR3 were 7.2, 7.8, 2.0 μg/dL, and 1600, 430, 360 ng/dL, respectively.

Binding assay

The linearity of the Scatchard plots indicates a single class of binding site affinity. The number of binding sites of GC receptors (Bmax) in PBMC and their apparent dissociation constant (Kd) were similar in the SS group (Bmax = 8.3 fmol/mg of protein; 95% CI: 7.2–12.4 fmol/mg of protein and Kd = 5.5 nmol/L; 95% CI: 3.8–8 nmol/L) and control patients (Bmax = 8.2 fmol/mg of protein; 95% CI: 6.4–11.6 fmol/mg of protein and Kd = 3.7 nmol/L; 95% CI: 2.8–5.8 nmol/L). Patient SR2 presented higher Bmax and Kd (25.5 fmol/mg of protein and 39.8 nmol/L, respectively) in comparison to the control and SS groups (Fig. 1). Bmax and Kd in patients SR1 (6.9 fmol/mg of protein and 4.2 nmol/L) and SR3 (12.9 fmol/mg of protein and 6.7 nmol/L) were similar to the values of control and SS patients.

Proliferation assays and sensitive or resistant phenotypes

Basal lymphocyte proliferation was stimulated by Con-A in the control group (359 cpm; 95% CI: 249–915 cpm vs. 43,530 cpm; 95% CI: 31,790–58,700 cpm, P = 0.0001) and SS group (1165 cpm; 95% CI: 790–1584 cpm vs. 39,180 cpm; 95% CI: 35,320–56,830 cpm, P < 0.0001). Different doses of Dex (10−8, 10−6, 10−4 mol/L) inhibited lymphocyte proliferation in a dose-dependent manner in the control group (23,130 cpm, 95% CI: 12,810–35,620 cpm; 11,890 cpm, 95% CI: 4496–16,420 cpm, 10,680 cpm, 95% CI: 3263–12,200 cpm; P < 0.0001) and SS group (22,440 cpm, 95% CI: 17,410–47,850 cpm; 3366 cpm, 95% CI: 2666–7092 cpm; 3816 cpm, 95% CI: 3377–6851 cpm; P < 0.0001). The steroid-resistant INS patients also presented lymphocyte proliferation after Con-A stimulation (SR1, 2009 vs. 60,847 cpm; SR2, 318 vs. 15,899 cpm; SR3, 369 vs. 30,428 cpm). Lymphocyte proliferation after different doses of Dex (10−8, 10−6, 10−4 mol/L) was 42,405, 9709, and 18,382 cpm; 11,462, 8578, and 8339 cpm; 29,167, 1584, and 1068 cpm (1165 cpm; 95% CI: 790–1584 cpm vs. 39,180 cpm; 95% CI: 35,320–56,830 cpm, P < 0.0001). Different doses of Dex (10−8, 10−6, 10−4 mol/L) inhibited lymphocyte proliferation in a dose-dependent manner in the control group (23,130 cpm, 95% CI: 12,810–35,620 cpm; 11,890 cpm, 95% CI: 4496–16,420 cpm, 10,680 cpm, 95% CI: 3263–12,200 cpm; P < 0.0001) and SS group (22,440 cpm, 95% CI: 17,410–47,850 cpm; 3366 cpm, 95% CI: 2666–7092 cpm; 3816 cpm, 95% CI: 3377–6851 cpm; P < 0.0001). The steroid-resistant INS patients also presented lymphocyte proliferation after Con-A stimulation (SR1, 2009 vs. 60,847 cpm; SR2, 318 vs. 15,899 cpm; SR3, 369 vs. 30,428 cpm). Lymphocyte proliferation after different doses of Dex (10−8, 10−6, 10−4 mol/L) was 42,405, 9709, and 18,382 cpm; 11,462, 8578, and 8339 cpm; 29,167, 1584, and 1068 cpm (1165 cpm; 95% CI: 790–1584 cpm vs. 39,180 cpm; 95% CI: 35,320–56,830 cpm, P < 0.0001).
of Dex (10−8, 10−6, and 10−4 mol/L) in healthy control patients, steroid-sensitive (SS) patients (○), and steroid-resistant (SR) patients: SR1 (□), SR2 (△), SR3 (○). Boxes indicate the 95% CI in control patients.

between the control (58%; 95% CI: 39–70) and SS (33%; 95% CI: 21–43) groups (P = 0.02).

Cytokines

Cytokine levels were measured in the supernatant collected from fresh cultured PBMC in the basal condition and after stimulation with Con-A alone or Con-A plus different doses of Dex in control, SS, and SR patients. There was no difference in basal production of IL-6, IL-10, IFN-γ, or TNF-α between the control and SS groups. Con-A–stimulated PBMC increased IFN-γ, TNF-α, IL-10, and IL-6 secretion significantly compared with the basal production in the control (P < 0.005) and SS (P < 0.0001) groups. The percentage of inhibition of TNF-α, IFN-γ, IL-6, and IL-10 secretion after different doses of Dex in the control, SS, and SR patients are shown in Figure 3. The secretion of IFN-γ, TNF-α, and IL-6 was significantly inhibited by Dex 10−6 mol/L in the control (P < 0.01) and SS (P < 0.001) groups. IL-10 secretion was significantly inhibited by Dex 10−6 mol/L in the SS group (P < 0.001). However, in the control group, a higher dose of Dex (10−4 mol/L) was required to significantly inhibit IL-10 secretion (P < 0.001). In SR2 and SR3 patients, the levels of cytokines increased after Con-A stimulation, but remained elevated even after a high dose of Dex (10−4 mol/L).

DISCUSSION

Glucocorticoids are crucial mediators of the endocrine-immune interaction and play an important role in modulating cellular and humoral immune responses [21, 22]. An imbalance on the immune-hypothalamic-pituitary-adrenal (HPA) axis may be involved in inflammatory diseases, such as asthma [9], rheumatoid arthritis [8], and INS. Thus, we hypothesized that GC might be important not only to the treatment, but also to the pathogenesis of INS. In the present study, we demonstrated that plasma cortisol levels were decreased in INS patients compared to normal control patients, which may be explained by the lower concentration of plasma proteins during the active phase of the disease. Indeed, salivary cortisol concentrations, which are unaffected by the concentration of plasma proteins and reflect the biologically active serum unbound cortisol levels [23], were similar in INS patients and normal control patients, suggesting that endogenous cortisol secretion is preserved in INS.

We also evaluated the number of binding sites and affinity of GC receptors in patients with INS and healthy control patients. There was no difference in the number and affinity of GC receptors in PBMC of SS patients compared to control patients. Among SR patients, the number and affinity of GC receptors were variable. A former study [10] including patients with different causes of nephrotic syndrome demonstrated that the number of GC receptors was increased in SS patients and decreased in SR patients. In contrast, Haack et al [11] showed no change in the density and binding affinity of GC receptors in SR INS patients, which were off steroid therapy for a median time of 5 months. The use of corticosteroids has been shown to influence the expression of GC receptors, causing down-regulation of the receptors in cells [24, 25]. Taken together, these data suggest that GC receptors might mediate glucocorticoid resistance in some patients with INS. It is also important to point out that binding assays evaluate the GC receptor α, which is the classic ligand-binding protein for corticosteroid. Therefore, we cannot rule out an overexpression of GC receptor β, a dominant negative inhibitor of GC receptor α, inducing steroid insensitivity in INS, as well established for asthmatic patients [26, 27].

In an attempt to identify predictive factors of the response to GC in INS, we evaluated the pattern of Dex-mediated inhibition of Con-A–stimulated PBMC proliferation. Using the IC50 to classify patients by in vitro study, our data showed that all clinically SS patients presented more than 50% inhibition of PBMC proliferation with Dex 10−6 mol/L. However, there was a significant difference in the percentage of suppression in vitro between the control and SS groups using a low dose of Dex (10−8 mol/L), indicating a diminished sensitivity to corticosteroids even in SS patients. In addition, patients SR2 and SR3 presented both in vivo and in vitro resistance to GC, whereas patient SR1 responded to Dex in vitro but had a poor clinical response to GC in vivo. This raises questions about compliance to therapy or the presence of mutations in genes that encode proteins of the slit diaphragm of the glomerular basement membrane, such as podocin and nephrin, as recently described [28, 29]. Although we have studied a limited number of SR patients.
Indeed, it has been shown that lymphocytes and mono-
cells, natural killer cells, or monocytes/macrophages, but also in other cells such as suppressor/cytotoxic
cells, but also in other cells such as suppressor/cytotoxic
logic abnormality in INS may not lie only in T-helper

A shift in the balance of Th1 and Th2 cytokine response has been related to the development of autoimmune dis-
cases or atopic reactions [30, 31, 32]. Moreover, abnormal cytokine release has been studied to investigate the primary insult in INS [12–15]. Studies evaluating serum cytokines have demonstrated a shift to Th1 pattern in patients with focal segmental glomerulosclerosis unrespon-
sive to steroids [13]. In addition, children with INS during

To our knowledge there is no data in the literature
evaluating the effect of dose-response of Dex in PBMC-
stimulated cytokine production in INS. In our study,

The authors suggest failure of endogenous glucocorticoid
resistance after prolonged corticosteroid adminis-
tration [36].
CONCLUSION
Our data showed that INS patients have a preserved endogenous cortisol secretion, but they present a spectrum of glucocorticoid sensitivity, ranging from diminished sensitivity in SS patients to resistance in SR patients, compared with control patients. Although SR nephrotic syndrome represents a heterogeneous group of kidney diseases, the present data also suggest that the clinical response to corticosteroid therapy of patients with INS can be predicted accurately using an in vitro test of GC suppression of Con-A–induced cell proliferation. Finally, abnormalities of number and affinity of the GC receptor and altered secretion of cytokines may be involved in tissue sensitivity to corticosteroids in INS patients.

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