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Differential alteration in peripheral T-regulatory and T-effector cells with change in P-glycoprotein expression in Childhood Nephrotic Syndrome: A longitudinal study

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ABSTRACT

Introduction: Childhood Idiopathic Nephrotic Syndrome (INS) responds to glucocorticoid therapy, however, 60–80% of patients relapse and some of them become steroid non responsive. INS may occur because of T cell dysfunction, abnormal cytokines and podocytopathies which reverse on steroid treatment. The reason of relapses could be imbalances in T cells phenotypes and respective cytokines. Herein, we hypothesize that relapses in INS may occur due to imbalance in T-regulatory and T-effector cell with their respective cytokines and overexpression of P-gp on lymphocytes.

Methods: The frequency of peripheral blood $CD4^+CD25^+FoxP3^+$ Treg, $CD4^+IFN\text{-}\gamma^+$ Th1 and $CD4^+IL\text{-}4^+$ Th2 lymphocytes and their respective cytokines and P-gp expression on peripheral blood lymphocytes (PBLs) were analyzed in INS patients at baseline ($n = 26$), during remission ($n = 24$) and at relapse ($n = 15$).

Results: Compared to baseline, the frequency of Tregs was significantly increased at remission and decreased during relapse. In contrast, the frequency of Th1 and Th2 lymphocytes was significantly decreased during remission and increased at the time of relapse. Similarly, expression of P-gp was significantly high at baseline and at the time of relapse as compared to remission. Levels of cytokines IL-10 and TGF-β in the supernatant of stimulated PBMCs was increased during remission and decreased during relapse. In contrast, levels of IFN-γ and IL-4 were decreased during remission and increased at the time of relapse.

Conclusions: Steroid therapy in INS induces decreased P-gp expression on PBLs along with increased frequency and cytokine response of T-regulatory cells, and reduced frequency and respective cytokine response of Th1 and Th2 cells during remission. However, reversal in the frequency and respective cytokines of T-regs, Th1 and Th2, and P-gp expression on PBLs occurs during relapses on follow-up.

1. Introduction

Minimal change disease (MCD) is one of the most common glomerular diseases causing Idiopathic Nephrotic Syndrome (INS) in children. MCD may be associated with T-cell dysfunction [1], increase in cytokines level [2–4] and podocyte injury which may be reversed after steroid therapy [5,6]. The exact cause of this podocytopathy in MCD remains unknown. Podocytopathy in MCD may be idiopathic, genetic; associated with NPHS2 mutation or reactive associated with malignancy or other immunologic stimuli. Non genetic idiopathic and reactive forms of MCD are usually steroid responsive and cell mediated immunity has been invoked as etiologic factor for this form of MCD [1,5]. However the predominant role of different T cell phenotypes; regulatory T cells (Tregs), and effector T cells (Teff) T helper cells (Th1) and T helper cells (Th2) remain poorly understood [7]. The predominance of Th2 phenotype was reported in one study while another study did not report any skewing of Th1/Th2 and suggested a role of Tregs [8]. Araya et al have shown that suppressor function of Tregs cell was deficient in INS that could lead to persistence of pathogenic cytokines released by Teff cells resulting into proteinuria [9]. A case of INS going in to remission following influenza B infection without the need of steroids has been reported. Authors have demonstrated increased Tregs population during remission in the case [10]. Recently, we have observed greater ratio of Tregs/Effector (Teff)

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cells in remission in INS patients and its reversal during treatment resistant state [11].

Approximately 60–80% of steroid responsive INS patients experience relapses of proteinuria on follow up despite initial clinical response to steroids and some of them become steroid non responsive [12]. The treatment of relapsing Nephrotic Syndrome with multiple courses of steroids, cytotoxic agents and calcineurin inhibitors remains a challenging clinical scenario [13]. The reason of poor steroid response could be change in histological pattern from MCD, a condition with podocyte injury without podocytopenia, to FSGS podocyte injury with podocytopenia [14], or changes in pharmacological intervention with overexpression of permeable glycoprotein (P-gp) on lymphocytes which has emerged as one of the major molecule causing poor response to many drugs, peptides, alkaloids, steroids, immunosuppressive drugs, and calcium channel blockers [15]. P-gp is a 170-kD product of the multidrug resistance 1 (MDR-1) gene. Our previous study indicated that homozygous mutant of MDR-1 gene influences steroid responsiveness in NS patients [16]. The MDR-1 gene belongs to the ATP-binding cassette (ABC) energy-dependent transporters [17]. In humans, P-gp is involved in xenobiotics efflux, protecting host tissue from toxic side effects. The overexpression of P-gp causes efflux of steroid from inside of cells to the outside. This limits the concentration of steroids within the cells and their site of action that results in poor response and steroid resistance [18]. P-gp is expressed on the surface of peripheral blood lymphocytes (PBLs), which are the putative targets of pharmacotherapy in INS [19] and the expression of P-gp is regulated by certain cytokines at transcription level which plays role in pathogenesis of INS [20].

Corticosteroids and calcineurin inhibitors are the drugs used for the treatment of NS. It is possible that podocyte itself could be the target of steroid or calcineurin inhibitors in INS as receptors of these drugs has been seen on podocytes. The effect of corticosteroids on lymphocytes and cell mediated immunity in INS is well established and the effect of steroid therapy on PBLs and phenotypic changes in patients during the course of the therapy is easier to monitor than any changes on podocytes. Corticosteroids are the substrate of P-gp and calcineurin inhibitors are substrate as well as inhibitor of P-gp expressed on lymphocytes [19]. The many poor steroid responsive INS patients respond well to calcineurin inhibitors. There is paucity of data on this novel biomarker during the course of the disease. It may help in better monitoring of the disease status. Therefore, we aimed this study to evaluate the alterations in frequency of different peripheral blood T cell phenotypes; Tregs, Th1, Th2 cells, and P-gp expression on PBLs in INS patients receiving steroids at baseline, on achieving remission and at time of relapse.

2. Patients and methods

We longitudinally studied the frequency of CD4+CD25+FoxP3+ Treg, CD4+IFN-γ+ Th1 and CD4+IL-4+ Th2 lymphocytes from whole blood and P-gp expression at baseline ($n = 26$) (before initiating steroid therapy); during remission after stopping steroid for at least 4 weeks (SSNS, $n = 24$); and at the time of relapse before starting immunosuppression ($n = 15$) during follow up. All patients were subjected for the analysis of cytokine production from stimulated PBMCs at baseline, during remission and at the time of relapse. Ten healthy children as controls were also subjected for the different T cell phenotypes, cytokine production and P-gp expression experiments.

Children of less than 2 years and greater than 16 years; and those with a family history of NS were excluded from the study. Definitions of INS, remission, and relapse were based on established criteria according to the International Study for Kidney Diseases in Children [21]. NS in children was defined as proteinuria of 40 mg/m^2/h or, ratio of 2 for spot urine protein (milligram)/creatinine (milligram) in the first morning urine sample with hypo-albuminemia (serum albumin <2.5 g/dL) and presence of edema. All children were treated with prednisolone of 60 mg/m^2 daily for 6 weeks followed by 40 mg/m^2 alternate day for the next 6 weeks. Samples from patients who achieved remission were taken after 4 weeks of stopping steroid treatment. Remission of NS was defined by urinary protein excretion <4 mg/m^2/h or urine dipstix nil/trace for three consecutive days. Relapse was defined as urinary protein excretion >40 mg/m^2/h or urine dipstix ++ or more for three consecutive days. In cases of infection associated relapse, blood sample were not collected if there was any clinical evidence of infection in the patients. In 3 patients with presumed upper respiratory tract viral infection (URTI) associated relapse, blood sample for analysis was collected after 2 weeks of resolution of infection when proteinuria was persisting before staring steroid. Steroid resistance was defined as unresponsiveness of 60 mg/m^2 body surface area per day for 4 weeks of prednisolone therapy. Patients enrolled in this study did not have any (i) underlying secondary causes, they were negative for hepatitis B surface antigen seropositivity, anti hepatitis C virus seropositivity and human immunodeficiency virus seropositivity and had normal serum complement (C3 and C4) levels. An informed consent was obtained from a parent or guardian of both patients and controls when participant <15 years and from the participant when age was >15 years as per institute guidelines. This study was performed in accordance with declaration of Helsinki and approved by the Institute Ethics Committee, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.

2.1. T cell phenotype analysis from whole blood

Whole blood was diluted 1:1 with RPMI 1640 (Sigma Aldrich, St. Louis) culture media and stimulated with phorbol 12-myristate 13-acetate (20 ng/ml; Sigma Aldrich, St. Louis) and ionomycin (1 μg/ml; Sigma Aldrich, St. Louis) for 5 h. Monensin (2 μM; BD Biosciences, San Diego, CA) was also added for the final 2 h of activation as a protein transport inhibitor. For surface staining, FITC conjugated mouse anti-human CD4 and PerCP-Cy5.5 conjugated mouse anti-human CD69 were added for staining of activated CD4 cells. After surface staining, RBCs were lysed with BD FACS lysing solution. Cells were washed, fixed and then permeabilized with Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's instruction for intracellular cytokine staining with Alexa Fluor 647- conjugated mouse anti-human IFN-γ for Th1 and Allophycocyanin (APC)-conjugated mouse anti-human IL-4 for Th2 cells. Isotype-matched antibodies were used as controls in each experiment. At least 10,000 lymphocytes were acquired on BD FACS Calibur (Becton Dickinson, Mount View, CA, USA) for each sample and analyzed with FlowJo software (Ashland, OR, USA).

For T regulatory cells, whole blood was incubated with a cocktail of 2 mAb directed to CD4 (FITC) and CD25 (PerCP-Cy 5.5). RBCs were lysed with BD FACS lysing solution. For intracellular staining of FoxP3, cells were subsequently fixed and permeabilized with BD Human FoxP3 Buffer Set according to the manufacturer's guidelines before Alexa Fluor 647-conjugated Mouse Anti-Human FoxP3 was added. Isotype-matched antibodies were used as controls. All the antibodies were purchased from BD biosciences (BD Pharmingen, Sanjose, California, USA). A minimum 50,000 events in lymphocyte counts were acquired on a FACSCalibur (Becton Dickinson, CA, USA) flow cytometry and analyzed with FlowJo software (Ashland, OR, USA). All fluorescein conjugated monoclonal antibodies were purchased from BD Pharmingen CA, USA.

2.2. Analysis of In vitro production of cytokines

The mitogen stimulated PBMCs were cultured and supernatant was analyzed for various cytokines as has been described