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ORIGINAL PAPER

Expression of multidrug resistance P-glycoprotein on lymphocytes from nephrotic children treated with cyclosporine A and ACE-inhibitor

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Abstract The aim of this work was to determine the expression of P-glycoprotein (P-gp) on peripheral lymphocytes (CD3) in children with steroid-dependent nephrotic syndrome (SDNS) during cyclosporine A (CyA) and ACE-inhibitor (ACE-I) treatment. The study group (I) consisted of 20 children with SDNS aged 5–18 years, with a subsequent proteinuria relapse at the time of prednisone dose reduction. All nephrotic syndrome (NS) children were examined three times: A—at proteinuria relapse, before CyA treatment; B—after 3 months; C—after 12 months of CyA administration. The control group (II) consisted of 20 healthy children. CD3/P-gp was measured using a flow cytometry assay. The serum CyA level was assessed by means of the immunofluorescence method. The expression of CD3/P-gp in NS relapse, prior to CyA+ACE-I administration, was much higher (median 9.15%, range 1.50–13.50%) when compared to healthy controls (median 1.20%, range 0.30–5.70%). The absolute number of CD3/P-gp in this examination was almost five times higher when compared to healthy controls ($p<0.01$). After 3 months of CyA+ACE-I therapy, the expression of CD3/P-gp decreased dramatically and was similar to the controls. Similar results were obtained after 12 months of treatment. A strong negative correlation was found between CD3/P-gp and serum CyA concentration in both examinations ($r=-0.624$, $p<0.01$; $r=-0.464$, $p<0.01$). We conclude that the results of our studies indicate that CyA+ACE-I in SDNS inhibits the expression of P-gp. CyA is an alternative therapy that may lead to the optimization of glucocorticoid (GC) doses, thus, reducing the risk that is associated with the treatment.

Keywords Children · Cyclosporine A · Nephrotic syndrome · P-glycoprotein

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Introduction

Nephrotic syndrome (NS) is a common chronic disorder, characterized by the alteration of permselectivity at the glomerular capillary wall, resulting in its inability to restrict the urinary loss of protein [1]. The pathogenesis of minimal change NS in children is still unclear. Recent knowledge indicates that antigen presentation to T-lymphocytes results in a polarized immune response, which may be type 1 (dominated by gamma-interferon and IL-2) or type 2 (IL-4, IL-10, IL-13). Type 1 cytokines predominate in cell-mediated immunity [1].

Nephrotic children are usually steroid-sensitive and respond to glucocorticoids (GC) quickly during the first episode of disease. But a majority of them relapse within the first 6 months of initial therapy. Almost 50–60% of them have frequent relapses or steroid-dependence. This group of children usually requires immunosuppressive treatment, such as cyclophosphamide or cyclosporine A (CyA). Among the multiple mechanisms of resistance to a variety of drugs, the overexpression of P-glycoprotein (P-gp) has emerged as the major molecule [2]. P-gp is a 170-kD product of the multidrug resistance 1 (MDR-1) gene. The MDR-1 gene belongs to the ATP-binding cassette (ABC) energy-dependent transporters [9]. P-gp

is expressed in most tissues and also on the surface of peripheral blood lymphocytes, which are the putative targets of pharmacotherapy in NS [4, 26].

In humans, P-gp is involved in xenobiotics efflux, protecting host tissue from toxic side effects. The over-expression of P-gp results in the reduction of the concentration of peptides, alcaloids, steroids, immunosuppressive drugs, and calcium channel blockers [22–24]. These are mainly substances whose molecular weights range from 300–2,000 Da [25]. Thus, P-gp is involved in both protecting cells from these drugs and in developing resistance to them.

Treatment resistance is observed not only in patients with malignancies [3, 8, 13], but also in those with systemic lupus erythematosus [6], inflammatory bowel disease [7], or other autoimmune diseases [17]. The expression of P-gp on lymphocytes is up-regulated by interleukin-2 (IL-2), a potent lymphocyte stimulus, which plays an important role in the pathogenesis of NS in children [18]. The increased expression of P-gp is mediated via activation by human Y-box-binding protein-1 (YB-1), which reduces the intracellular corticosteroid concentration [21]. It has been demonstrated that P-gp function can be circumvented by different compounds known as chemosensitizers, such as cyclosporine A or verapamin [8, 11].

Aim of this work

The aim of this work was to determine the expression of P-gp on peripheral lymphocytes (CD3) in nephrotic children during the CyA treatment.

Subjects and methods

The study group (I) consisted of 20 children with steroid-dependent nephrotic syndrome (SDNS) (12 male, 8 female), aged 5–18 years, with a subsequent proteinuria relapse (mean number of relapses 4.5±1.7) at the time of prednisone dose reduction. Apart from prednisone (0.5–1.0 mg per kg body weight on alternate days), CyA at an initial dose of 5–6 mg per kg body weight every day, max. 150 mg/24 h, and ACE inhibitor (ACE-I) 0.05–0.1 mg per kg body weight every day were applied. The serum concentration of CyA should not have exceeded 150 ng/ml during the first 6 months and 100 ng/ml in following months of treatment. All of the children were examined three times: A—at proteinuria relapse (protein/creatinine ratio 10.35) before CyA treatment; B—after 3 months; C—after 12 months of CyA administration. The prednisone dose following proteinuria regression (2–3 weeks) was reduced to (mean) 0.6±0.2 mg per kg body weight on alternate days, while the CyA dose was gradually decreased according to the serum concentration. All of the patients responded to CyA during the first weeks of treatment. None of the patients were steroid or cyclosporine A resistant. The control group (II) consisted of 20 healthy children (12 male, 8 female) aged 6–18 years.

Blood and urinary samples were collected from the children with NS treated with CyA, who were hospitalized every 6 weeks to assess the serum concentration of CyA. Control samples were collected from outpatient healthy children.

We measured P-gp expression on peripheral blood lymphocytes T (CD3) of patients with NS using a flow cytometry assay. P-gp expression was quantified by the percentage of P-gp-positive cells and the absolute number of cells per μl. Double (FITC/PE) monoclonal antibodies for immunophenotyping analysis were purchased from Becton Dickinson (BD). All of the other reagents were obtained from Coulter. Flow cytometric analysis was performed by Coulter Epics XL in a whole blood procedure.

The serum CyA level was assessed by means of the immunofluorescence method in polarized light, using monoclonal antibodies. The serum albumin level was measured on a Hitachi analyzer; a nephelometric method was used to assess the albumin level in the urine and the Jaffe method was used to determine the creatinine level.

Data analysis was performed using the computer program Statistica 6.0. Since no features of a normal distribution were found in the groups examined with the Shapiro-Wilk test, statistical analysis was performed using the non-parametric Mann-Whitney U test or the Wilcoxon test. Spearman correlation coefficients were calculated for statistically significant correlations. A p value of less than 0.05 was considered to be statistically significant.

The study was approved by the ethics committee of the Medical Academy in Białystok, Poland in accordance with the Declaration of Helsinki.

Results

The age and sex of the examined children did not differ from the healthy controls ($p>0.05$). In NS children (I), during proteinuria relapse (A), before CyA institution, the serum albumin and creatinine levels were reduced and the protein/creatinine ratio increased as compared to the control group. During CyA therapy (in examinations B and C), these parameters were close to those observed in healthy children (II) ($p>0.05$). The serum CyA level decreased during treatment proportionally to dose reduction (Table 1).

The total number of lymphocytes and of the CD3 lymphocytes in children with a relapse during GC therapy were not different from those of the controls (Table 2). During the CyA treatment and GC dose reduction, the

Table 1 Biochemical parameters examined in nephrotic syndrome (NS) patients (I) before and during treatment (IA-C) and in the control group (Controls). *p*-value comparison of the results in both groups: *$p<0.05$, **$p<0.01$; CyA—cyclosporine A, ACE-I—ACE-inhibitor, GC—glucocorticoids

Group	Serum, median (min.–max.)			Urine protein/ creatinine ratio (g/g)	CyA dose (mg/kg b.w. per 24 h)
	Albumin (g/dl)	Creatinine (mg/dl)	Concentration of CyA (ng/ml)		
IA—during proteinuria relapse and GC treatment	3.3 (1.4–4.1)**	0.4 (0.3–0.4)*	-	10.35 ** (4.5–16.3)	-
IB—after 3 months of CyA+ACE-I+GC	4.1 (3.7–4.5)	0.6 (0.3–0.9)	118.1 (36.7–215.3)	0.25 (0.0–0.7)	4.62 (1.66–5.35)
IC—after 12 months of CyA +ACE-I+GC	4.2 (3.7–5.0)	0.7 (0.4–0.9)	68.45 (39.4–171.1)	0.2 (0.0–0.4)	2.7 (1.47–3.60)
Controls	4.3 (3.7–4.9)	0.7 (0.4–0.9)	-	0.18 (0.0–0.3)	-

number of lymphocytes increased and still did not differ from control group.

The expression of P-gp in CD3 lymphocytes (CD3/P-gp) in NS relapse and during GC therapy, but prior to CyA+ ACE-I administration, was much higher (median 9.15%, range 1.50–13.50%) when compared to healthy controls (median 1.20%, range 0.30–5.70%) (Fig. 1). The absolute number of CD3/P-gp in this examination was almost five times higher when compared to healthy controls ($p<0.01$). The absolute number of P-gp in cells other than CD3 cells was also higher than in the control group ($p<0.01$).

After 3 months of CyA+ACE-I therapy and reduced prednisone dose (0.5 mg per kg body weight), the expression of CD3/P-gp decreased dramatically and was similar to the values of healthy controls. The expression of total P-gp was a little higher than in the controls, but the difference was not statistically significant ($p>0.05$). The values of P-gp expression in the examination performed after 6 months of continued treatment (data not shown) were similar to examination B, so we decided to check the expression of P-gp after 12 months, when the dose of CyA was reduced. We found out that the CD3/P-gp expression was almost the same as in the examination performed after 3 months, only the total P-gp expression was a little higher, but the difference was not statistically significant ($p>0.05$).

When analyzing the correlation between CD3/P-gp and serum CyA concentration, a strong negative correlation was found in both examinations (Fig. 2). The correlation was stronger in group IB (during the treatment with higher CyA doses) ($r=-0.624$, $p<0.01$) than in IC (after reduction of the CyA dose) ($r=-0.464$, $p<0.01$). The correlation between the total P-gp and the serum CyA concentration was a little weaker.

The CD3/P-gp and total P-gp expression was also negatively correlated with CyA dose, but the correlation was not statistically significant.

Discussion

Although most patients with minimal change nephropathy respond to high-dose GCs, in the course of the diseases,

Table 2 Characteristics of the examined parameters in children with NS (median and ranges) (I) before treatment (A), after 3–4 weeks of treatment (B), and in remission (C), and in the control group (II). a.n.—absolute number; *p* comparison with healthy controls: *$p<0.05$, **$p<0.01$; median (range)

Group		*n*	Total lymphocytes (%)	Total lymphocytes/μl×10^3 (a.n.)	CD3 (%)	CD3/μl a.n.
Controls		20	30.8 (11.8–47.6)	2.180 (1.140–3.960)	65.7 (41.9–81.9)	1347 (756.0–2130.0)
I	A	20	29.6 (12.3–45.6)	1.765 (0.510–4.100)	60.7 (41.8–77.7)	1110.9 (234.0–2660)
	B	20	32.55 (9.7–53.4)	2.035 (0.370–4.660)	66.4 (38.1–86.0)	1236.6 (163.3–2970.4)
	C	20	33.95 (9.7–45.0)	1.950 (0.370–3.960)	63.2 (30.5–80.0)	1153.4 (169.8–1916.6)
Group		*n*	CD3/P-gp (%)	CD3/P-gp (molecules/cell)	Total P-gp (%)	Total P-gp (molecules/cell)
Controls		20	1.20 (0.30–5.70)	18.75 (2.49–75.5)	3.6 (1.8–13.2)	72.5 (14.0–184.0)
I	A	20	9.15 (1.50–13.50)**	93.74 (11.0–229.7)**	16.06 (3.5–33.5)**	228.45 (19.0–908.0)**
	B	20	2.15 (0.8– 7.8)	22.88 (6.01–168.3)	4.60 (1.8–91.0)	71.5 (14.0–324.0)
	C	20	2.05 (0.60–6.40)	22.07 (4.32–96.2)	5.40 (1.2–11.4)	76.5 (9.3–176.0)

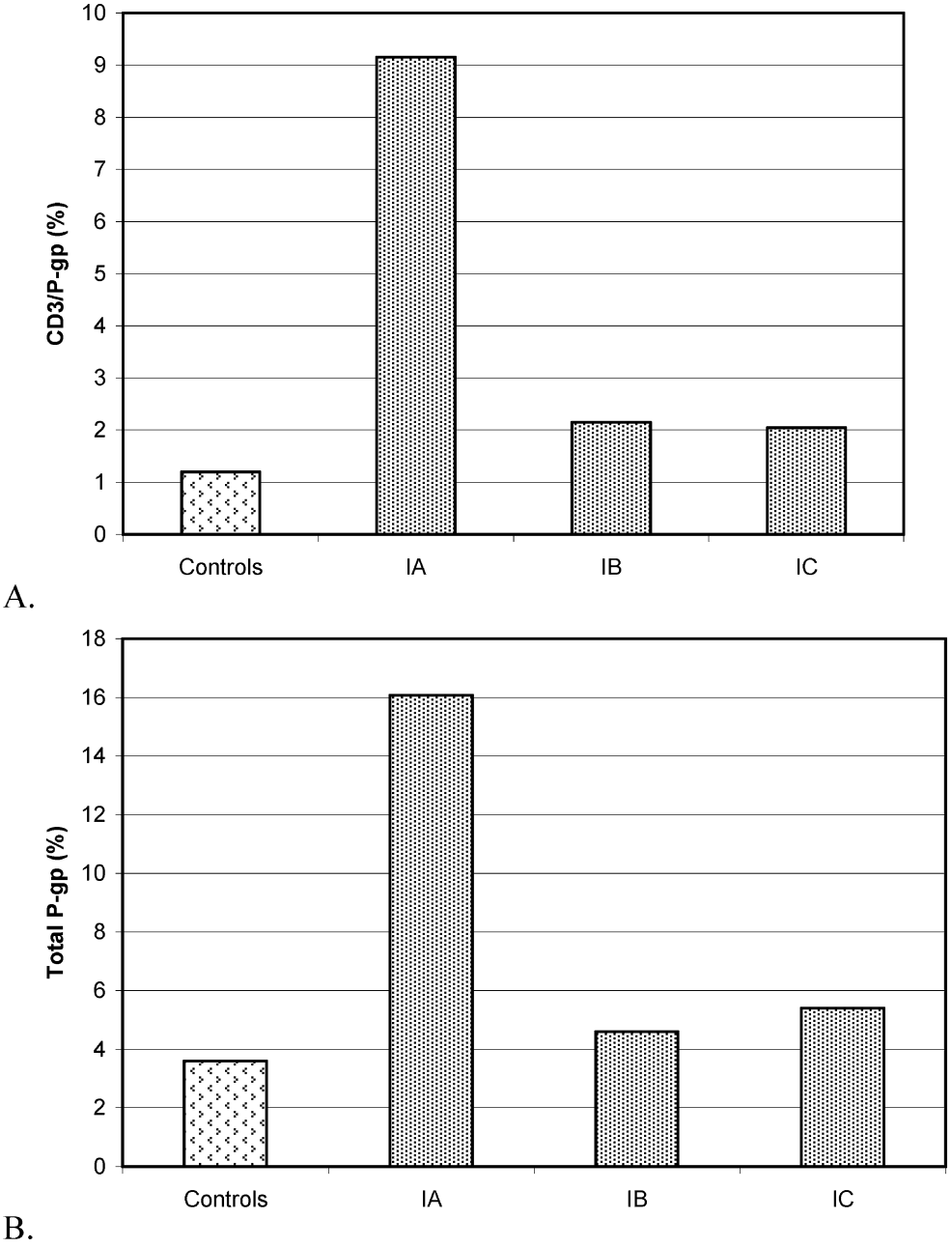

Fig. 1a, b Expression of CD3/P-gp (**a**) and total P-gp (**b**) in children with NS treated with CyA and ACE-I in comparison to healthy controls

some of them show poor response or steroid dependency. In this clinical situation, immunosuppressive agents like cyclophosphamide or cyclosporine A are essential for the control of disease activity. Clinical observation of children with SDNS treated with CyA indicates that, in most patients, complete remission of proteinuria is observed within several weeks of treatment.

The main immunosuppressive effect of CyA is the inhibition of T-cell proliferation by a reduction of IL-2 secretion. However, CyA also plays another role. Tsujimura et al. [21], in experimental studies, found that IL-2 upregulated the P-gp expression on lymphocytes via activation by human Y-box-binding protein-1 (YB-1), an MDR-1 transcription factor. He also noticed that the concentration of intracellular corticosteroids was reduced.

The clinical studies in patients with systemic lupus erythematosus (SLE) showed that increased IL-2 usually decreased below a threshold level following GC therapy [10, 20]. However, the authors noticed that, in poor responders, it remained at a high level.

In our previous studies, we found that, in children with NS, in relapse of NS, the P-gp expression was increased, but mainly in children with subsequent relapses, which was probably caused by the GC treatment [27]. In children with a first episode of NS, the initial expression of P-gp was similar to healthy controls and it increased significantly

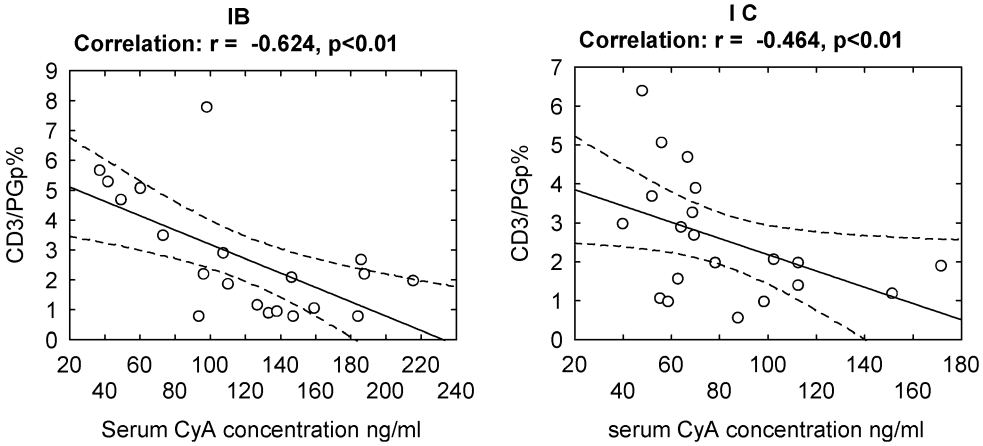

Fig. 2 Correlation between CD3/P-gp and serum CyA concentration in children treated with CyA and ACE-I in examinations IB and IC

during GC treatment. We demonstrated that the GC therapy rather than IL-2 influenced the expression of P-gp on lymphocytes.

In the present study, we demonstrate the influence of CyA and ACE-I treatment on CD3/P-gp expression in children with SDNS, in whom the initial P-gp expression was very high. The effort to reduce the dose of GC caused immediate relapse of proteinuria. Therapy with CyA and ACE-I caused marked reductions in P-gp expression on lymphocytes in almost all of the children after 3 months of therapy. Further CyA and ACE-I treatment did not influence the P-gp expression significantly. The final study after 12 months of treatment showed that the P-gp expression was similar to the values of the control group. We found a strong negative correlation between the CD3/P-gp and serum CyA concentration in all of the children treated with CyA; however, the correlation was stronger during the treatment with higher CyA doses. In our analysis, we also took into account the possible role of ACE-I in the modification of P-gp expression. The data from the literature show that the mechanism of action of this drug is different and ACE-inhibitors are neither P-gp substrates nor inhibitors [12]. Also, the fact that all of the examined children received the same dose of ACE-I during the whole therapy, in contrast to the dose of CyA, which was reduced after 6 months of treatment, may confirm that it is CyA which is responsible for changing the P-gp expression. Another argument is the presence of strong negative correlation between the serum CyA concentration and the P-gp expression.

Clinical observation and the results of our examination suggest that the disappearance of P-gp expression causes a recovery of steroid responsiveness and, in most of the children, leads to successful subsequent treatment with oral GC.

P-gp is widely expressed in many tissues and is often overexpressed in tumors, making them resistant to drug therapy [19]. The drug may modulate the expression and function of P-gp. Parasrampuria et al. [15], in experimental studies, showed that lymphocytes have functional P-gp, which is inhibited by various drugs. They found out that the strongest inhibitor of P-gp-mediated efflux of Rh123 is the CyA, rather than tacrolimus, quinidine, and the weakest is verapamil. Cy-A at a micromolar concentration appeared to exert a protective effect on lymphocytes, which is why they can be used for studying the effect of drugs on P-gp function and expression. Lymphocytes can be used for studying both the induction and inhibition effects of drugs on transporters, but there are some limitations. The expression of P-gp in lymphocytes is low and the measurement requires the use of a flow cytometer, which is a very sensitive instrument for appropriate analysis. That is the reason why we choose this method in our studies.

Cyclosporine and tacrolimus are substrates and potent inhibitors of the multidrug transporter, P-gp, in vitro. There are only a few studies relating the assessment of the P-gp expression in patients treated with CyA. Most of them concern transplanted people. Parasrampuria et al. [16] investigated the effect of chronic therapy with immunosuppressive drugs on the expression and function of P-gp in T lymphocytes and suggested that the calcineurin inhibitors might be modulating the expression and function of the transporters in lymphocytes.

The function of P-gp can be circumvented by a variety of compounds known as chemosensitizers, such as CyA and Verapamil [5, 8, 11]. The efforts to reverse multidrug resistance with these drugs in neoplastic processes have been disheartening for side effects. The required doses were too high and the side effects outweighed the potential benefits [2, 14]. The results of our study indicate that medium doses of CyA (5–6 mg per kg body weight) have been used successfully for SDNS in children. Three-month therapy with these doses of CyA are enough to decrease the high CD3/P-gp expression in children with SDNS treated with prednisone alone before.

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