P-Glycoprotein Efflux Activity in Steroid-Responsive versus Steroid-Resistant Autoimmune Hemolytic Anemia

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Abstract: Background: Multi-drug resistance 1 (MDR1) gene, encodes for P-glycoprotein may predict response to treatment of autoimmune hemolytic anemia includes agents that are substrates of P-glycoprotein. Objectives: The aim of this study was to evaluate the clinical relevance of P-glycoprotein efflux activity on lymphocytes to disease outcome in patients with autoimmune hemolytic anemia (AIHA).

Methods: The study was carried out on 52 subjects [ 37 patients; age ranged from 2.6 to 20 years, with a median 9.5 years (27 acute ,10 chronic AIHA and 15 controls; age ranged from 2.6 to 20 with a median 10 years). Three patients died (they were steroid resistant and excluded from the study). Patients were subjected to the following: History taking (including onset , course, duration of evidence of haemolysis and bleeding tendency, drugs, viral infections, treatment regimens and response), full clinical evaluation, complete blood picture, coombs’ test both direct and indirect. Functional assay of P-glycoprotein activity. Patients had been followed up for six months.

Results: According to the response to treatment, they were; 18 steroid responsive patients with sustained remission, 6 patients became chronic). Evan's syndrome was reported in 8 cases (32%), while 3 cases (11%) diagnosed to be SLE by serology and score. The efflux capability of its substrates increased in chronic cases of autoimmune hemolytic anemia than that in steroid responsive cases, and also higher than in controls, while not significantly different between responsive cases and controls, which means that the study found a correlation between chronicity and P-glycoprotein activity that makes resistance to steroid therapy. Conclusion: P-glycoprotein can efflux its substrate like steroid that used as the main line of treatment of autoimmune hemolytic anemia which leads to unresponsiveness and then chronicity. It could be used as a predictor of outcome.

Key Words: P-Glycoprotein, steroid-responsive, autoimmune hemolytic anemia.

1. Introduction

Autoimmune hemolytic anemia (AIHA) refers to disorders characterized by the presence of auto antibodies that bind to the patient’s own erythrocytes, leading to premature red cell destruction (1&2): Autoimmune hemolytic anemia is classified as either warm AIHA or cold AIHA, which includes cold agglutinin disease, and paroxysmal cold hemoglobinuria. These classifications are based on the characteristics of the auto antibodies involved in the pathogenesis of the disease. Each has a different underlying cause, management and prognosis, making classification important when treating a patient with AIHA(3,4). Warm autoimmune hemolytic anemia (wAIHA) accounts for about 50% to 70% of all cases of autoimmune hemolytic anemia and is characterized by the production of auto antibodies that target determinants on red blood cells (5).

It can be caused by a number of different auto antibodies, with IgG and IgM antibodies being the main causes. Depending on which is involved, the pathology will differ. As IgG is poor at activating complement but effectively binds the Fc receptor of phagocytic cells(6&7).

First line therapy for warm AIHA is usually with corticosteroids, such as prednisolone. Following this, other immunosuppressants are considered, such as rituximab, danazol, cyclophosphamide, azathioprine or cyclosporine. Beyond supportive measures, rituximab given either alone or in combination with chemotherapy may be helpful (3,8).

P-glycoprotein of membrane transporters, a product of the multiple drug resistance (MDR)-1 gene, is known to play a pivotal role in the acquisition of drug resistance to chemotherapy in malignancy. However, the relevance of MDR-1 and P-glycoprotein to resting and activated lymphocytes, which are the major target in the treatment of systemic autoimmune diseases, remains unclear. The use of both P-glycoprotein antagonists (e.g., cyclosporine) and inhibition of P-glycoprotein synthesis with intensive immunosuppressive therapy successfully reduces the efflux of corticosteroids (which are substrates of P-glycoprotein) from lymphocytes in vitro, suggesting that P-glycoprotein antagonists and P-glycoprotein synthesis inhibitors could be used to overcome drug-resistance in vivo and improve outcome (9).
Aim of the study
We aimed to evaluate the clinical relevance of P-glycoprotein efflux activity on lymphocytes to disease outcome in patients with autoimmune hemolytic anemia whether acute or chronic.

2. Subject and Methods
The present study was conducted on 52 subjects [37 patients; age ranged from 2.6 to 20 years, with a median 9.5 years (27 acute, 10 chronic AIHA and 15 controls age ranged from 2.6 to 20 with a median 10 years). Three patients died (they were steroid resistant) and excluded from the study. Patients suffering from AIHA were recruited from the admitted patients and from patients attending the Pediatric and Internal Medicine Hematology, clinic units, Menoufiya University Hospitals.

Studied groups were classified into:
Group I: Patients with recently diagnosed autoimmune hemolytic anemia; this group was treated with prednisone 2mg/kg./day for 4 weeks then tapered over 2 weeks; they were subdivided after 6 months follow up into:
Group IA: those patients who recovered from the disease.
Group IB: those patients who did not recover from the disease till 6 months from diagnosis (became chronic).

Group II: Patients with chronic autoimmune hemolytic anemia (> 6 months).

Group III: Normal age and sex matched subjects served as a control group.

Patients were subjected to the following:
History taking (including onset, course, duration of evidence of hemolysis and bleeding tendency, drugs, viral infections, treatment regimens and response). Full clinical evaluation. Complete blood picture using AC920 auto-counter. Coombs' test both direct and indirect. Functional assay of P-gp using facscaliba flow cytometry (BD, USA):

A. Reagents:

- Rhodamine 123 (Rh 123) obtained from (ICN Biomedicals Inc USA) had to be first dissolved in D.W. for preparation of stock at 5μg/ml. Before use, Rh123 was diluted at 1/250 (20 μl from the stock solution in 5 ml D.W.)
- Cyclosporine (Novarts, Neoral), 50 mg/ml in D.W. was prepared as stock solution. 2 μl of stock solution diluted with 8 μl D.W. (1:5) were used as a working solution.
- Ficoll-Hypaque from (Sigma Diagnostics – USA) was used for separation of Peripheral blood mononuclear cells.

B. Procedures:
1- Peripheral blood mononuclear cells (Pbmncs) were isolated from fresh blood using a Ficoll-Hypaque density gradient centrifugation.
2- After one wash with phosphate buffer saline (PBS) fresh mononuclear cells are adjusted at 2 x 10^6 cells /ml in PBS.
3- Three hundred μl of cell suspension are distributed in 6 test tubes:
   - Two tubes to evaluate cell autofluorecence: uptake control (UC) and efflux control (EC);
   - Two tubes to evaluate Rh123 uptake (UR) and efflux (ER);
   - Two tubes to evaluate cyclosporine effect on Rh123 uptake (U cyclo) and efflux (E cyclo).
4- Ten μl of PBS are added in tubes UC and EC. Three μl of cyclosporine working solution are added in tubes U Cyclo and E Cyclo. Then 25 μl of Rh123 working solution are added in tubes UR, ER, U Cyclo and E Cyclo. All tubes were incubated for 15-30 min at room temperature, avoiding light exposure.
5- Rh123 uptake is measured on green fluorescence detector (FL1). Data acquisition and data analysis were performed with cell Quest software (BD, USA). Mean fluorescence of Rh123 are evaluated for each cell incubation after acquisition of 10,000 viable cells.
Figure (2): Cyclosporine effect on Rh123 uptake and efflux in acute autoimmune hemolytic anemia.

Figure (3): Cyclosporine effect on Rh123 uptake and efflux in chronic autoimmune hemolytic anemia.

Statistical Analysis:
The data collected were tabulated and analyzed by SPSS statistical package version 11 on IBM compatible computer. Descriptive statistics was presented as mean & standard deviation (X±SD), and number and percentage (No & %) and analyzed by applying chi-square test. Student t-test was used for comparing two groups of normally distributed variables, Analysis of variance (F test) for comparing more than two groups. Mann-Whitney U test, Correlation co-efficient test (r-test) and regression analysis were also performed whenever appropriate. Results were considered of significance at P<0.05.

3. Results:
P-glycoprotein activity is significantly higher in chronic than in controls (p <0.001), and highly significantly higher in chronic than in acute (p <0.001) as shown in Table (1).

Table (1): Comparison of P-glycoprotein activity among the studied groups:

<table>
<thead>
<tr>
<th>Group</th>
<th>Range</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (n=24)</td>
<td>9.39% - 53.08%</td>
<td>22.5346% ± 11.2389%</td>
</tr>
<tr>
<td>II (n=10)</td>
<td>15.97% - 62.77%</td>
<td>43.5548% ± 12.5358%</td>
</tr>
<tr>
<td>III (n=15)</td>
<td>4.22% - 39.56%</td>
<td>16.3488% ± 10.5896%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>T-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I versus II</td>
<td>-4.025</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Group I versus III</td>
<td>1.096</td>
<td>0.08</td>
</tr>
<tr>
<td>Group II versus III</td>
<td>5.112</td>
<td>&lt; 0.001**</td>
</tr>
</tbody>
</table>
P-glycoprotein efflux activity and capacity was statistically significantly higher ($P<0.005$) in the acute cases that became chronic when compared with the acute cases that recovered ($P<0.005$) as shown in table (2).

**Table (2):** Comparison of P-glycoprotein activity between steroid responsive and steroid resistant subgroups.

<table>
<thead>
<tr>
<th></th>
<th>Group IA (steroid responsive)</th>
<th>Group IB (steroid resistant)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-glycoprotein efflux activity</td>
<td>$(19.0589% \pm 8.2379%)$</td>
<td>$(42.0045% \pm 5.986%)$</td>
<td>$P&lt;0.005**$</td>
</tr>
</tbody>
</table>

**Table (3):** Main predictors of disease outcome in autoimmune hemolytic anemia patients (after following up the recently diagnosed cases)

<table>
<thead>
<tr>
<th>Model</th>
<th>Standardized Coefficients</th>
<th>$T$-test</th>
<th>$P$-value</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-glycoprotein activity</td>
<td>0.685</td>
<td>3.452</td>
<td>$&lt;0.005**$</td>
<td>0.521</td>
</tr>
<tr>
<td>AGE (Years)</td>
<td>0.411</td>
<td>2.001</td>
<td>$&gt;0.05$</td>
<td>0.261</td>
</tr>
<tr>
<td>HB g/dl</td>
<td>-0.402</td>
<td>-1.650</td>
<td>$&gt;0.05$</td>
<td>0.203</td>
</tr>
<tr>
<td>WBCS</td>
<td>-0.054</td>
<td>-0.222</td>
<td>$&gt;0.05$</td>
<td>0.004</td>
</tr>
<tr>
<td>Platelets</td>
<td>0.008</td>
<td>0.034</td>
<td>$&gt;0.05$</td>
<td>0.000</td>
</tr>
</tbody>
</table>

When the variable has a beta coefficient $>0.5$ [as, P-glycoprotein activity (Beta coefficient = 0.685, $R^2=0.521$, $P<0.005$)] can be considered as a predictor factor.

4. Discussion

Multi-drug resistance 1 (MDR1) gene, encodes for P-glycoprotein found in a variety of normal tissues including peripheral blood lymphocytes (PBL), particularly T lymphocytes and natural killer (NK) cells. (10) Treatment of autoimmune hemolytic anemia includes agents that are substrates of P-glycoprotein (11). A high mortality was reported in this study; three steroid resistant acute autoimmune hemolytic anemia died (11%); they were excluded from the study. Meanwhile a high percentage of Evan’s syndrome was reported in this study; 8 cases (32%), while 3 cases (11%) diagnosed later to be SLE by serology and score.

A Korean study of 32 cases found thirteen patients (40.6%) were initially diagnosed with Evans’ syndrome. Nevertheless, 1 year after initiating treatment, 80% of the patients were still treatment-dependent and 14 of 25 patients (56.0%) were found to have SLE (12).

A reduced percentage of naturally occurring regulatory T cells and IL-10/IL-12 imbalance may play an essential role in the onset and/or maintenance of AIHA. Corticosteroids are the main drug used in the treatment of autoimmune hemolytic anemia especially in Egypt; (13) we often experience patients with systemic autoimmune diseases who are resistant to this treatment. Previous studies found that P-glycoprotein of membrane transporters, a product of the MDR1 gene, is found to be correlated between the expression level and disease activity in systemic autoimmune diseases (9).

Predictors of response to therapy as age or initial Hemoglobin were not correlated with response to steroids; while P-glycoprotein level was highly correlated with response to steroids. Our study found the efflux capability of its substrates increased in chronic cases of autoimmune hemolytic anemia than that in steroid responsive cases, and also higher in chronic cases than in controls, while not significantly different between responsive cases and controls, which means that the study found a correlation between chronicity and P-glycoprotein activity that makes resistance to steroid therapy. Drigo et al 2010, found that the increased activity of membrane transporters could be responsible for the selective response to different glucocorticoids, even if P-glycoprotein and MDR1 gene expression was not increased (14).

**Conclusion:**

P-glycoprotein can efflux its substrates, like steroid that used as the main line of treatment of autoimmune hemolytic anemia which leads to unresponsiveness and then chronicity and expect an increase in morbidity. P-glycoprotein could be used as a predictor of outcome from the start of diagnosis and so either use alternative line of therapy or use P-glycoprotein inhibitor with steroids.
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References