Endoplasmic Reticulum Stress Predicts Clinical Response to Cyclosporine Treatment in Primary Membranous Nephropathy

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Key Words
Primary membranous nephropathy \cdot Cyclosporine \cdot Calcineurin \cdot Glucose regulated protein 78 \cdot Endoplasmic reticulum stress

Abstract
Background: Little is known about the endoplasmic reticulum stress (ERS) marker glucose regulated protein 78 (GRP78) and calcineurin in the kidney in primary membranous nephropathy (PMN) and if they could predict post-cyclosporine treatment outcome. Methods: This is a retrospective study using a dataset of biopsy-confirmed PMN from Peking Union Medical College Hospital from 1996 to 2014. Seventy-six adult patients treated with cyclosporine as primary immunosuppression for at least 6 months were studied. Immunohistochemistry was used to detect GRP78 and calcineurin in the kidney. Serum calcineurin was assayed by ELISA. Patients were grouped into no-remission (NR, n = 17), partial remission (PR, n = 39), or complete remission (CR, n = 20) at the end of 6 months of treatment. Results: There was no difference of initial dose of cyclosporine among NR, PR, and CR groups. Kidney calcineurin expression in PMN was significantly increased compared to that in controls (p < 0.0083). The glomerular GRP78 in NR PMN was higher than that in control, CR and PR patients (p < 0.0083). Kidney calcineurin expression and GRP78 expression was positively correlated. However, there were no differences in either serum calcineurin levels or kidney calcineurin expressions among NR, PR or CR groups. There was a negative correlation between serum calcineurin activity and whole kidney calcineurin expression (p = 0.034) or glomerular calcineurin expression (p = 0.007). Neither kidney calcineurin nor GRP78 expression was correlated with proteinuria. Conclusions: ERS marker GRP78 in the glomeruli but not serum or kidney calcineurin expression could be a useful marker in PMN to negatively predict the response to cyclosporine treatment at the sixth month.

Introduction

Primary membranous nephropathy (PMN) is a leading cause of nephrotic syndrome in adults. Although 5–30% of untreated PMN patients achieve spontaneous remission in 5 years, approximately 50% of patients with PMN develop progressive disease leading to end-stage renal disease requiring dialysis or renal transplantation in 5–10 years [1, 2]. KDIGO [3] recommends that cyclosporine or tacrolimus be used for a period of at least 6 months.
Calcineurin is a Ca\(^{2+}\)/calmodulin-dependent serine/threonine protein phosphatase and serves as a target of calcineurin inhibitors (CNIs), cyclosporine and tacrolimus [5]. CNIs induce complete or partial remission (PR) in proteinuria was seen in approximately 2/3 of the patients [2], it would be ideal that potential non responders should be selected to avoid such therapy and given other options. We were thus interested to find new biomarkers to predict clinical response of PMN patients to cyclosporine therapy prior to treatment, to prevent unnecessary cyclosporine exposure and avoid potentially severe side effects in non-responders.

Endoplasmic reticulum (ER) is an organelle for protein folding, assembling and maturation. ER function is vulnerable to various pathophysiological effects such as hypoxia, Ca\(^{2+}\) depletion, viral infections and oxidative stress. These effects lead to the activation of the unfolded protein response (UPR), a protective response to accumulation of misfolded proteins in the ER, namely, ER stress (ERS) [8]. Glucose regulated protein 78 (GRP78), also known as ‘immunoglobulin heavy chain-binding protein’ (BiP), a member of the heat-shock protein-70 family, is involved in the folding and assembly of proteins in the ER [9]. As a major ER chaperone, GRP78 is anti-apoptotic and protects the host cell against ERS-induced cell death [10, 11]. Evidence shows that increased proteinuria can induce ERS in renal proximal tubule cells [12–14] and podocytes [15]. Our previous in vitro study has also shown increased expression of calcineurin in podocytes during palmitate-induced ERS, thus linking these 2 markers [16].

It has also been suggested that ERS is a mediator of cyclosporine nephrotoxicity [17, 18]. From a clinical perspective, no study had been done about status of ERS or calcineurin in the kidney of PMN. In this study, we tried to explore if serum calcineurin, renal calcineurin, or renal GRP78 expression, as a marker of ERS, was correlated with patients’ clinical responses to cyclosporine treatment.

**Methods**

**Patients and Data Collection**

Seventy-six patients at Peking Union Medical College Hospital (from January 1996 to June 2014), who had newly diagnosed, biopsy-proven PMN and were treated with cyclosporine as primary immunosuppression for at least 6 months, were retrospectively reviewed. Kidney biopsies were evaluated by light microscopy, electron microscopy and immunofluorescence microscopy. Secondary causes were carefully excluded to confirm PMN. Subjects with previous cyclophosphamide or other immunosuppressive agent treatment were excluded. Kidney samples (away from the tumor) from 5 renal carcinoma patients in the Division of Urology were used as healthy controls in staining. Serum from IgA nephropathy (IgAN, n = 12), lupus nephritis (LN, n = 12), minimal change disease (MCD, n = 12), and PMN (n = 42) without any immunosuppressant exposure during the past 6 months before serum collection were assayed for serum calcineurin activity. Serum from 14 healthy subjects during their regular physical checkup were used as controls in the serum calcineurin assay. Kidney biopsy tissues of all PMN patients (n = 76) were used for calcineurin and GRP78 immunohistochemistry (IHC) staining.

**Grouping**

PMN patients were divided into 3 groups at the end of 6 months of cyclosporine treatment based on their clinical response. Complete remission (CR) was defined as urinary protein <0.30 g/day and serum albumin >35 g/l; PR was defined as urinary protein excretion between 0.3 and 3.5 g/day, with a decrease in proteinuria by of at least 50% from the initial value and <3.5 g/day; accompanied by an improvement or normalization of the serum albumin concentration and stable serum creatinine (Scr) [3]. Patients who did not meet the definitions of CR or PR were assigned to no-remission (NR).

**Kidney IHC of Calcineurin and GRP78**

Kidney tissues were fixed with 10% formaldehyde solution, embedded in paraffin and cut into 2-μm thick sections. After deparaffinization, hydration and antigen retrieval, the tissue samples were incubated with antibody at a dilution of 1:2,000 against calcineurin (Abcam, ab71149, UK) at 4 °C overnight or a dilution of 1:100 of an antibody against GRP78 (Santa Cruz, sc-1050, USA) at 37 °C for 1 h. In staining of calcineurin, antigen retrieval was performed by heat-induced retrieval by keeping slides at a temperature of 120 °C for 1 h. In staining of calcineurin, antigen retrieval was performed by heat-induced retrieval by keeping slides at a temperature of 120 °C for 1 h. In staining of calcineurin, antigen retrieval was performed by heat-induced retrieval by keeping slides at a temperature of 120 °C for 1 h. Slides were then exposed to color development by diaminobenzidine. Images were semi-quantitative.
titatively analyzed by Image-Pro Plus 6 software. At least 5 images were captured at random in each slide of kidney tissue for quantification. Calcineurin and GRP78 expression were assessed by the integrated optical density (OD) of the color object above a baseline threshold.

Serum Calcineurin Activity Assay

Serum calcineurin activity was measured by a colorimetric assay kit (calcineurin phosphatase assay kit, Enzo Life Sciences, BML-AK804, USA). This method is based on the principle that calcineurin can dephosphorylate the RII phosphopeptide substrate. The detection of released free-phosphate is based on the classic malachite green assay. The standard curve was made by serial dilution of phosphate standard and its corresponding OD 620 nm value on a microplate reader. The OD values were converted to the amount of released free-phosphate using a standard curve formula, which indirectly indicates calcineurin activity.

Statistical Analysis

All data analyses were performed using SPSS version 17.0. For continuous measurements, if they were normally distributed, they were expressed as mean ± SD and one-way analysis of variance was used within group comparisons. If continuous data were not normally distributed, they were presented by median (IQR), and non-parametric test Mann–Whitney U test was applied within group comparisons. In order to control the type I error, Bonferroni’s correction was made for all possible comparisons, according to the number between any 2 subgroups being compared among the total groups, \( p < 0.0083 \) (0.05/6) was considered statistically significant for serum calcineurin, renal calcineurin expression, and renal GRP78 expression comparisons between control and sub-groups in PMN, and \( p < 0.005 \) (0.05/10) was considered statistically significant for comparisons of serum calcineurin activity among control, IgAN, MCD, LN and PMN sub-groups.

For normally distributed data, Pearson’s correlation analysis was used. For non-normally distributed data, Spearman correlation analysis was applied. \( p < 0.05 \) was considered significant unless otherwise indicated.

Results

Baseline Characteristics of Patients Enrolled in This Study

Baseline data for the 76 patients were listed in table 1. There was no difference in the initial cyclosporine dose among the 3 subgroups. Proteinuria in the NR group was significantly higher than those in the CR or PR groups \( (p < 0.001) \). Correspondingly, the serum albumin level was significantly lower in the NR group than those in the CR or PR group \( (p < 0.001) \). Scr level was also significantly higher in the NR group than in the CR or PR group \( (p = 0.022) \). A total of 64 patients were treated with prednisone at the same time, and the mean daily dose was 0.48 mg/kg/day, with no difference among these 3 subgroups \( (p = 0.169) \).

After 6 months of cyclosporine therapy, there were 20, 39, and 17 patients respectively in CR, PR and NR groups. The overall complete and PR rate was about 77%. The proteinuria, serum albumin level, Scr level, the cyclosporine and prednisone doses were listed in table 2. Patients in NR and PR groups maintained higher cyclosporine dosage as compared to the CR group \( (p = 0.009) \).

Calcineurin and GRP78 Expression in the Kidney and Their Correlation

IHC demonstrated that calcineurin was mainly expressed in podocytes and distal tubules (fig. 1a–d). Calcineurin staining was increased in PMN patients compared to HC before cyclosporine therapy in the glomerular and tubular compartments. Although no significant differences were observed among CR, PR, and NR subgroups in either the glomerular or tubular compartment (fig. 1e, f and g), there was a clear trend that non-responders to

| Table 1. Baseline characteristics of 76 adult patients with IMN treated with cyclosporine |
|----------------------------------------|------------------|------------------|------------------|------------------|
|                                       | CR (n = 20)      | PR (n = 39)      | NR (n = 17)      | p value         |
| Age, years                            | 46.4±16.5 (20)   | 47.9±15.8 (39)   | 53.0±14.4 (17)   | 0.411           |
| Gender, male/female                   | 10/10            | 26/13            | 15/2             | 0.047           |
| SBP, mm Hg                            | 126.4±16.5 (19)  | 125.5±17.2 (33)  | 134.4±20.7 (12)  | 0.263           |
| DBP, mm Hg                            | 76.1±9.5 (19)    | 75.6±11.4 (33)   | 81.8±9.8 (15)    | 0.159           |
| Proteinuria, g/day                    | 3.6±3.4 (20)     | 5.6±3.4* (39)    | 10.3±6.2* (17)   | <0.001          |
| Serum albumin, g/l                    | 28.8±4.3 (20)    | 24.8±5.0* (39)   | 22.0±4.7* (17)   | <0.001          |
| Scr, μmol/l                           | 69.3±13.1 (20)   | 74.7±18.5 (39)   | 84.8±16.6* (17)  | 0.022           |
| CsA dose, mg/kg/day                   | 2.9±1.1 (20)     | 2.7±0.8 (39)     | 3.0±1.0 (17)     | 0.49            |
| Prednisone dose, mg/kg/day            | 0.55±0.15 (18)   | 0.46±0.20 (33)   | 0.45±0.21 (13)   | 0.169           |

n = The number of patients studied. SBP = Systolic blood pressure; DBP = diastolic blood pressure; CsA = cyclosporine A. * Compared to CR or PR group; ^ compared to CR group.
cyclosporine had a higher renal calcineurin expression than responders.

IHC demonstrated that GRP78 was mainly expressed in the cytoplasm of podocytes and distal tubules (fig. 2a–d). The GRP78 in the whole kidney was significantly higher in NR group than in the CR or PR subgroup (fig. 2e). The glomerular GRP78 in the PMN NR group was higher than that of healthy control, CR or PR subgroup (fig. 2f). The tubulointerstitial GRP78 in the NR group of PMN was also higher than that in the CR or PR subgroup (fig. 2g).

There was a significant positive correlation between calcineurin and GRP78 in overall expression (p = 0.025; fig. 3a), in the glomerular compartment (p < 0.001; fig. 3b) and in the tubular interstitial compartment (p = 0.025; fig. 3c). There was no correlation between whole kidney, glomerular or tubular interstitial calcineurin or GRP78 expression with baseline proteinuria (fig. 3d–i). There was no correlation between whole kidney, glomerular or tubular interstitial calcineurin or GRP78 expression with baseline serum albumin level or Scr level, either (data not shown).

Table 2. Clinical profile of 76 adult patients with IMN treated with cyclosporine after 6 months

<table>
<thead>
<tr>
<th></th>
<th>CR (n = 20)</th>
<th>PR (n = 39)</th>
<th>NR (n = 17)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria, g/day</td>
<td>0.14±0.10</td>
<td>0.82±0.85*</td>
<td>5.11±4.24*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum albumin, g/l</td>
<td>42.0±3.8</td>
<td>40.0±6.0</td>
<td>30.0±9.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Scr, μmol/l</td>
<td>79.0±33.1</td>
<td>82.0±23.0</td>
<td>88.0±32.1</td>
<td>0.359</td>
</tr>
<tr>
<td>CsA dose, mg/kg/day</td>
<td>1.69±0.91</td>
<td>2.44±0.81*</td>
<td>2.31±0.98*</td>
<td>0.009</td>
</tr>
<tr>
<td>Prednisone dose, mg/kg/day</td>
<td>0.31±0.20</td>
<td>0.31±0.14</td>
<td>0.41±0.06</td>
<td>0.392</td>
</tr>
</tbody>
</table>

n = The number of patients studied. CsA = Cyclosporine A.
* Compared to CR or PR group; # compared to CR group.
Levels of Calcineurin Activity in Serum and Its Correlation with Renal Calcineurin Expression

Serum calcineurin activity in PMN patients (n = 42) was significantly higher than HC (n = 14), but there were no differences among CR, PR and NR subgroups (fig. 4a). In order to verify whether high serum calcineurin expression is unique in PMN, sera from IgAN, LN, and MCD patients (n = 12 in each group) were analyzed. The characteristics of those patients are listed in table 3. PMN, LN and MCD patients had higher calcineurin activity than HC and IgAN patients (fig. 4b). PMN patients also had significantly higher calcineurin activity than LN and MCD patients. A total of 37 PMN patients in this cohort had both renal tissues evaluated by IHC for calcineurin and serum assayed for its activity. There was a negative correlation between serum calcineurin activity with the whole renal calcineurin expression (p = 0.034; fig. 4c). There was a negative correlation between serum calcineurin activity with the glomerular calcineurin expression (p = 0.007; fig. 4d). There was no correlation between serum calcineurin activity with the tubulointerstitial calcineurin expression (fig. 4e). There was also no correlation between serum calcineurin activity and baseline proteinuria in PMN patients (fig. 4f).

Discussion

PMN is the most common nephrotic syndrome in adults in western country and China. In a meta-analysis, Xie et al. [19] included 17 randomized controlled trials encompassing 696 Chinese PMN patients, who had been treated with different immunosuppressive agents, where 7 trials involving 282 patients in comparison CNIs with alkylating agents, found that CNIs led to a higher effect on CR and PR rate with fewer side effects in the Chinese population. While studies have demonstrated efficacy of CNIs, safety concerns include multiple side effects, including the risk of nephrotoxicity with long-term exposure [20]. This makes it very attractive to find a biomarker predictive of the clinical response to CNIs [21].

ERS is defined by an accumulation of unfolded or misfolded proteins in the ER, both physiologically and pathologically. In response to this accumulation of unfolded/misfolded proteins, cells adapt to the stressful conditions through a UPR [22]. The UPR involves transient attenuation of new protein synthesis, induction of ER chaperones, such as GRP78, and activation of ER-associated degradation to eliminate immature proteins [23].
Accumulating evidence indicates that ERS contributes to glomerular and tubular injury in kidney disease. These findings emphasize the importance of ERS as a not yet unidentified pathway in its pathogenesis and the potential possibility of reno-protective strategies targeting ERS [24, 25]. Surprisingly, studies specific for GRP78 expression in glomerular disease are lacking. There is some evidence showing that GRP78 protein levels are increased in PMN compared with MCD in biopsied human kidney samples [23]. This raised another question: how does GRP78 expression change in the renal histology of PMN patients, and can this be a useful marker to predict patients’ clinical outcomes? 

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ERS in PMN
responses to cyclosporine treatment? If it were, we could predictively shift those patients who will not respond to cyclosporine treatment to other treatments and avoid unnecessary potential CNI nephrotoxicity [26].

CNIs exert their immunosuppressive effect by blocking T-cell-mediated responses through the inhibition of calcineurin-dependent nuclear factor of activated T-cells signaling [27]. CNIs have also been suggested to be cytoprotective in podocytes through the inhibition of synaptopodin degradation and stabilization of the podocyte actin cytoskeleton [28]. Cyclosporine has also been shown to induce stiffening of the actin network in tubular interstitial calcineurin expression (e). The correlation analysis between serum calcineurin activity and glomerular calcineurin expression (d). The correlation analysis between serum calcineurin activity and proteinuria in PMN patients (f). * p < 0.0083 vs. HC; # p < 0.005 vs. IgA; § p < 0.005 vs. LN; & p < 0.005 vs. MCD.

Table 3. Clinical characteristics of patients from IgAN, MCD, LN, and PMN with serum calcineurin activity assayed

<table>
<thead>
<tr>
<th></th>
<th>IgA (n = 12)</th>
<th>MCD (n = 12)</th>
<th>LN (n = 12)</th>
<th>IMN (n = 42)</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>Age, years</td>
<td>37.8±14.3</td>
<td>28.8±14</td>
<td>34.3±14</td>
<td>47.9±16.4</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>10/2</td>
<td>10/2</td>
<td>3/9</td>
<td>29/13</td>
<td>0.008</td>
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<tr>
<td>Serum albumin, g/l</td>
<td>36.4±6.4</td>
<td>20.6±5.7</td>
<td>25.2±9.5</td>
<td>24.8±4.9</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>Scr, μmol/l</td>
<td>177.9±116.7</td>
<td>104.4±83.3</td>
<td>128.2±110.3</td>
<td>77.0±18.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Proteinuria, g/24 h</td>
<td>3.8±3.4</td>
<td>11.3±10.1</td>
<td>9.7±8.9</td>
<td>6.8±4.0</td>
<td>0.016</td>
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* Compared to the IgAN group; # compared to the MCD group.
Ers in PMN

The authors do not have any competing interests.

Acknowledgments

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Disclosure Statement

The authors do not have any competing interests.
References


In the article by Tao J, et al., entitled 'Endoplasmic reticulum stress predicts clinical response to cyclosporine treatment in primary membranous nephropathy' [Am J Nephrol 2016;43:348–356, DOI: 10.1159/000446293], the following 2 errors occurred in table 2: it should be 0.82 (0.85) instead of 0.82±0.85 and similarly 5.11 (4.24) instead of 5.11±4.24.

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