Differential Expression of Plasma Proteins in Cyclosporine A-Induced Rat Acute Nephrotoxicity

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Received September 26, 2008; Accepted November 25, 2008; Online Publication, March 7, 2009

Cyclosporine A (CsA) is an effective and widely used immunosuppressive agent. Nevertheless, its intense nephrotoxicity restricts clinical application. In this study, Wistar rats were randomly divided into a control group, a low-dose group and a high-dose group. The rats in the low-dose group and those in the high-dose group were given CsA at a daily dose of 5 mg per kg and 100 mg per kg by gavage for 7 days respectively, while the control group was given distilled water of equal volume. The extent of renal impairment was evaluated by renal function assay, urease and pathological observation. The results showed that the rats in the low-dose group did not suffer remarkable renal impairment, while the rats in the high-dose group did. Two-dimensional gel electrophoresis (2DE) was utilized to resolve the plasma protein profile. Six spots showing stable and significantly different expression were identified by MALDI-TOF-MS and ESI-TOF-MS/MS respectively, the two markedly different expression were identified by MALDI-TOF-MS. The results showed that the rats in the low-dose group and those in the high-dose group were significantly different from the control group. The protein profile of plasma showed changes in key proteins such as α-1-acid glycoprotein (AAG), clusterin (CLUS), and haptoglobin (Hp). The protein profile of plasma showed changes in key proteins such as α-1-acid glycoprotein (AAG), clusterin (CLUS), and haptoglobin (Hp).

Key words: cyclosporine A; acute nephrotoxicity; rat plasma; two-dimensional gel electrophoresis

Organ and bone marrow are successfully transplanted nowadays, but rejection reactions occur when a foreign organ is attacked by the body’s immune system. Many immunosuppressive drugs are used to prevent rejection reactions.1) Cyclosporine A (CsA), a fungal peptide, is an important immunosuppressive agent widely used in organ transplantations, its use has resulted in increased survival rates of both patients and grafts since its introduction in the market in the early 80’s.2) However, CsA treatment is associated with some serious adverse effects, most notably nephrotoxicity.3,4) CsA nephrotoxicity is a reality, it leads to premature renal transplant dysfunction and deterioration,5) which can be divided into acute and chronic effects. Acute nephrotoxicity leads to renal blood flow, reduced glomerular filtration rates, and increased renal cascular resistance. It has been found to be mediated by increased endothelin activity, increased levels of angiotensin II, and reductions in the synthesis and secretion of nitric oxide from the endothelium.6,7) These adverse effects restrict cyclosporine’s clinical applications.

Nowadays, routine therapeutic drug monitoring (TDM) of the concentration of CsA in the blood is essential, but the pharmacokinetics of CsA exhibit high intra- and interpatient variation, and hence it is difficult to define the therapeutic margin and there are serious consequences when the compound is an overdose or underdose.8) Some groups have reported that cyclosporine trough blood levels (C0), on which the cyclosporine dose is usually based, correlate poorly with acute rejection and cyclosporine nephrotoxicity after renal transplantation.9,10) The limitations of the routine methods are clear to see, so there is great need for alternatives.

Proteomics is an area rapidly changing our approach to drug development. Potentially it might contribute to the field of drug toxicology by identifying new markers of toxicity or by providing new insights into complex mechanisms of toxicity.11,12) In support of this, several reports using two-dimensional gel proteomic technologies have documented novel mechanistic pathways and new biomarkers of toxicity. Specifically, several studies have been performed on rat renal tissue in an attempt to gain new mechanistic insight. For example, a proteomic analysis of kidney homogenates from CsA-treated rats defined the calcium-binding protein calbindin-D (28 K) as a novel marker of CsA renal toxicity.12) Studies using the aminoglycoside antibiotic gentamicin reported changes in a variety of proteins responsible for stress response, glucose metabolism, and lipid biosynthesis.13,14) However, serum or plasma toxicity markers would be more attractive to monitor patient health than other tissues since blood samples can readily be obtained with minimal risk to the patient. Several studies have been published on analyzing changes in blood samples. For example, proteomic patterns in serum have been successfully used to identify ovarian cancer.15) Kennedy et al. found that some proteins in urine and serum samples had the potential to be non-

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Abbreviations: AAG, α-1-acid glycoprotein; CLUS, clusterin; CsA, cyclosporine A; Hp, haptoglobin; TDM, therapeutic drug monitoring; 2-DE, two-dimensional gel electrophoresis
invasive markers for gentamicin toxicity. In addition, several investigations into differential expression of proteins in rat plasma exposed to benzene, dioxin, and nicotine have been reported separately. To date, however, surprisingly few proteomic studies have been published analyzing protein changes in the blood following a CsA toxic insult.

Biological fluids, such as plasma/serum and urine, are easily available samples for analysis in the clinical chemistry. Variations of the proteins in plasma have been associated with different physiological and pathological conditions; 2-DE allows evaluation at both the qualitative and the quantitative level. In this study, we chose to examine the acute nephrotoxicity of CsA in greater detail by monitoring protein changes in the plasma of treated rats using 2D gel proteomics technology. Those proteins which showed differences were identified by MALDI-TOF-MS and CapLC/ESI/Q-TOF MS/MS.

Materials and Methods

Chemicals. CsA (Hangzhou Zhongmei Huadong Pharmaceutical, Hangzhou, China) was commercially obtained. IPG Drystrips, a ProteoExtract™ Albumin/IgG Removal Kit, Urea, Bio-Lyte, pH 4–7, acrylamide and N,N-methylene bisacrylamide were purchased from Bio-Rad (Hercules, CA, USA). Phenylmethanesulfonyl fluoride (PMSF), CHAPS, TEMED, DTT, and iodoacetamide were from Merck (Darmstadt, Germany). BCA™ Protein Assay Kits were from Pierce (Rockford, USA). SDS and glycin were from Promega (Madison, USA). Low molecular weight markers for proteins were from Shanghai Dongfeng Biotechnology, (Shanghai, China). All chemicals used in 2-DE, MALDI-TOF, and CapLC/ESI/Q-TOF analysis were of electrophoresis or analysis grade.

Animals and treatment. Eighteen male wistar rats weighting 200 ± 20 g were purchased from the Department of Laboratory Animal Science at the Peking University Health Science Center, and were allowed to acclimatize for 3 d. Six rats were housed in one cage and had free access to water and food. They were maintained under standard conditions: 12 h light-dark cycle, 22–24°C, and 50–60% humidity. In the present study, all experiments were performed under the guidelines of the Experimental Laboratory Animal Committee of Peking University Health Science at the Peking University Health Science Center, and were in strict accordance with the Animal Welfare Act (D.C., USA) and with the institutional guidelines. The rats in the low-dose and high-dose groups were selected from randomly divided rats at the Peking University Health Science Center and were in strict accordance with the manufacturer’s guidelines. The acetone precipitation method was carried out for the protein concentration and the desalting step. Five volumes of ice-cold acetone were added to each sample and the samples were kept overnight. They were centrifuged at 11,180 g, 4°C for 30 min, the supernatants were removed, and the pellets were air dried. Each pellet was suspended in rehydration solution (8 mol/l urea, 4% CHAPS) and an aliquot taken to determine the protein concentration using the BCA™ Protein Assay Kit. The remains were stored at −80°C until protein analysis.

2-DE. IPG Drystrips (17 cm, pH 4–7) were used for isoelectric focusing (IEF). Proteins (150 μg and 15.5 mg) were diluted with a 300 μl solution of (8 mol/l urea, 4% CHAPS, 0.2% Bio-Lyte (pH 4–7), 50 mmol/l DTT, and a trace of bromophenol), and the Drystrips were rehydrated for 1 h without current and another 12 h with a current of 50 V applied. IEF was then carried out using the Protean IEF System (Bio-Rad, Hercules, CA), a voltage ramping as follows: 250 V for 30 min; 1,000 V for 1 h; 10,000 V for 5 h, and 10,000 V, up to a total of 60,000 Vh. After IEF, the individual strip was emersed in solution A (375 mmol/l Tris–HCl buffer (pH 8.8), 6 mol/l urea, 20% glycerol, 2% SDS, 2% DTT) for 15 min and then in solution B (same as A, except that DTT was replaced with 2.5% iodoacetamide) for 15 min. The strips were then removed from solution B, and protein transfer from the first to the second dimension (12.5% SDS-polyacrylamide gels) was achieved by running a constant current of 10 mA for 1 h. Electrophoresis was carried out applying a constant voltage of 100 V using the Protean II xi cell (Bio-Rad, Hercules, CA) until the bromophenol arrived at the bottom of the gels.

Image analysis. Analytical gels were silver-stained, and micro-preparative gels were stained with CBB-R250. First, the proteins were visualized by the silver staining method, as follows: the gels were fixed in 45% methanol/10% acetic acid v/v for 15 min, then sensitized by soaking in Farmer’s Reducer for 2 min, rinsed 3 times for 10 min in deionised water, suspended for 15 min in 0.1% silver nitrate, rinsed for 30 min in deionised water and 2.5% sodium carbonate, then developed in 2.5% sodium carbonate with 0.1% formaldehyde until the desired intensity of staining was achieved. Finally the development was stopped using a solution containing 10% acetic acid, and the gels were stored in deionised water.

For the second staining method, the gel was stained in CBB-R250 (0.1 g/100 ml) solution (V$_{HCl}$/V$_{CHOH}$:V$_{H2O}$ = 9:9:2) for 2 h. It was washed and submerged into methanol-acetic acid solution (V$_{HCl}$/V$_{CHOH}$:V$_{H2O}$ = 8:1:1) to destain it.

In order to obtain a credible result, samples from three randomly selected rats from each group were taken to perform 2-DE. Computer analysis of the 2-DE image was carried out using PDQuest Software 7.1.0 (Bio-Rad, Hercules, CA). The separated 2-DE gels obtained for different CsA doses were analyzed, and spots separated and differentially expressed in gels were selected for identification by MALDI-TOF-MS and CapLC/ESI/Q-TOF MS/MS.

Protein identification.

Peptide mass fingerprinting by MALDI-TOF-MS. In-gel digestion of proteins from the CBB R250-stained gels was performed as follows: Bands were washed twice with 200 μl 50% ACN/50% H$_2$O for 30 min, and destained with 200 μl of 100 mmol/l NH$_4$HCO$_3$/30% ACN for 30 min, 200 μl of 50% ACN/50 mmol/l NH$_4$HCO$_3$ for 30 min, washed once with 200 μl of 100% ACN for 10 min, and dried. Approximately 10 μl of 20 ng/μl trypsin was added to the dried residue, and the sample was incubated at 37°C for 16 h. Peptides were subsequently extracted twice with 55 μl of 60% ACN/5% trifluoroacetic acid for 15 min, and dried to 10 μl. One μl of the digest extract sample was mixed with 1 μl CFA (10 mg of CFA dissolved in 1 ml ACN/0.1% TFA = 1:1 solution). One μl of these mixed solutions was evaporated on the metal MALDI-TOF target at room temperature. All analyses were carried out using an AXIMA-CFR plus MALDI-TOF Mass spectrometer (Shimadzu, Kyoto, Japan) with an internal mass calibration.
2.5 nm pore size, Waters, Milford, USA) and fractionated by a capillary LC system (Waters, Milford, USA), which connected to an Q-TOF Ultima Global mass spectrometer equipped with an electrospray ion source (Waters, Milford, USA). Data was processed using MassLynx 4.0 and GLOBAL SERVER software.

**Database search.** The resulting peptides mass fingerprints and MS/MS spectra were searched against the NCBInr database using the MASCOT search program (www.matrixscience.com).

### Results and Discussion

**Pathology**

During administration, the weights of the rats in the control and low-dose groups showed a similar change, while the high-dose group lost weight significantly. After 7 d of administration, a decrease of 16.1% in weight on average was found in the high-dose group, probably due to a reduction in food intake. The control group and the low dose CsA-treated group gained weight (on average the increases of 13.1% and 15.8% respectively). An assessment of renal impairment results is shown in Table 2. It can be seen that there were no significant differences between the control and low-dose groups, but, serum BUN and urine NAG/Cr in the high-dose group increased significantly compared with the control. Additionally, although serum Cr of the high-dose group was higher than the control group, this was not statistically significant. Microscopic observations of PAS-stained rat kidney slices are shown in Fig. 1. Rat kidney in the control group showed no morphological abnormality (Fig. 1A). In the low-dose group, a loss of brush borders of the proximal tubular epithelial cells was found (Fig. 1B), while in the high-dose group, an evident loss of brush borders and clear vacuolization (Fig. 1C) with numerous empty vacuoles clustered in the proximal tubules were found in all rats. Microvacuolization was the only characteristic histological abnormality observed in the high-dose group. This was not observed in the other groups. Microvacuolization, observed here, has been described as a characteristic of acute CsA-induced nephrotoxicity in rats and rabbits. These results indicate that nephrotoxicity appears to increase with dose of CsA in rats, and remarkable acute nephrotoxicity model was established for the high-dose group.

**Protein analysis results**

A high abundance of proteins such as albumin and IgG in the plasma might be expected to obscure the identification/quantification of low-abundant proteins, leading to unreliable results on proteomic analysis. However, a sample preparation method to deplete the albumin/IgG-content of protein pellet was investigated. Figure 2 shows a comparison of the results of 2D electrophoresis analysis before and after pretreatment of

#### Table 1. A Non-Linear Gradient Elution Program of Capillary LC System

<table>
<thead>
<tr>
<th>Time</th>
<th>Eluent A</th>
<th>Eluent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1–3.5 min (injection)</td>
<td>97%</td>
<td>3%</td>
</tr>
<tr>
<td>3.5–40 min</td>
<td>95%–60%</td>
<td>5%–40%</td>
</tr>
<tr>
<td>40–60 min</td>
<td>60%–40%</td>
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<td>60–65 min</td>
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<tr>
<td>65–70 min</td>
<td>10%–95%</td>
<td>90%–5%</td>
</tr>
<tr>
<td>70–90 min</td>
<td>95%</td>
<td>5%</td>
</tr>
</tbody>
</table>

#### Table 2. Renal Function and Urine NAG Assays Results after 7 d of Administration (x ± s, n = 6)

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Cr (μmol/l)</th>
<th>Serum BUN (mmol/l)</th>
<th>Cr clearance (ml/min)</th>
<th>Urine NAG/Cr (IU/g Cr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80.2 ± 5.0</td>
<td>5.8 ± 1.4</td>
<td>0.42 ± 0.12</td>
<td>29.8 ± 4.4</td>
</tr>
<tr>
<td>Low-dose</td>
<td>77.6 ± 6.5</td>
<td>5.5 ± 1.1</td>
<td>0.45 ± 0.08</td>
<td>37.7 ± 15.1</td>
</tr>
<tr>
<td>High-dose</td>
<td>97.8 ± 31.0</td>
<td>11.5 ± 3.6*</td>
<td>0.20 ± 0.10*</td>
<td>62.8 ± 28.1*</td>
</tr>
</tbody>
</table>

*Compared with control group: p < 0.01 by Student’s t-test.

Fig. 1. Typical Images of PAS-Stained Rat Kidney Slices from the Control Group (A), the Low-Dose Group (B), and the High-Dose Group (C) at the Original Magnification (×200).
rat plasma. In general, the strong spots representing albumin, and heavy and light IgG chains are weaker (Fig. 2B) than the starting material (Fig. 2A), and a few spots are darker. No significant differences were observed between untreated plasma (Fig. 2A) and treated samples (Fig. 2B), although the latter were concentrated. Some spots disappeared in the treated sample, which means that protein loss during the procedure probably took place. Although affinity-based methods are effective in removing albumin and IgG, non-specific protein associations might result in the binding and removal of other proteins.25) Based on these data, pretreatment of plasma was not carried out in this study.

In Fig. 3, the 2-DE patterns of rat plasma proteins of the control group are compared with the low-dose group and the high-dose group. By PDQuest software analysis, it was found that there were no significant changes in protein 2-DE patterns between control group and the low-dose group. The match rate of these two maps reached 80% ± 3%, and different expression appeared in the high-dose group. The match rate with the control group was only 65% ± 5%. In particular, three areas marked in Fig. 3, displayed evident and stable differences between the high-dose group and the other groups. Down-regulated (disappearing in the high-dose group) and up-regulated (appearing only in the high-dose group) spots were detected. The up- and down-regulated spots correspond to the newly-induced spots and the spots that disappeared due to high-dose CsA induce, respectively. Selection of differential proteins was based on whether spots appeared stably or disappeared in all the plasma samples of the high-dose group as compared with the control and low-dose groups. The differential protein spots are shown in Figs. 4 and 5. Spots 1, 2, 3, and 4 were up-regulated in areas I and II (Fig. 4). The four spots were present in all the rats of the high-dose group, but were not present or hard to identify in the control and low-dose groups, but, two down-regulated spots, 5 and 6, appeared in area III (Fig. 5), which did not exist in all the rats of the high-dose group. Magnified 2-DE maps of three rats in each group are also shown in Figs. 4 and 5.

Identification of proteins

The differentially isolated proteins were identified by mass spectrometry analysis, as shown in Table 3. Spots 1, 2, 3, and 4 showed up-regulation, and spots 5 and 6 showed down-regulation of expression by high-dose CsA. Spots 2 and 4 were identified by MALDI-TOF-MS analysis as α-1-acid glycoprotein (AAG) and albumin segments respectively, and spot 3 was a protein mixture of AAG and clusterin (CLUS). We deduce that spot 4 originates from the degradation of albumin. Since we could not obtain the masses of spots 1, 5, and 6 by MALDI-TOF-MS analysis, CapLC/ESI/Q-TOF MS/MS analysis was carried out to identify these spots. We could not obtain the mass of spot 1, since the protein was not sufficient in amount for mass determination. Spots 5 and 6 were identified as the same haptoglobin protein (Hp). The two spots were the same protein, but appeared with slightly shifted isoelectric points (pIs). This was caused by the proteins’ post-translational modifications, resulting in a progressive change in pI.18,26)

AAG was newly induced by high-dose CsA-treatment. It is well known that A1AG is one of the major acute phase proteins (APP) in humans, rats, and other species, and it is also considered a natural anti-inflammatory and immunomodulatory agent. Like most acute phase proteins, its serum concentration increases in response to systemic tissue injury, inflammation, or infection, and these changes in serum protein concentrations have been correlated with increases in hepatic synthesis.27) But now it is well established that the acute phase response might take place in extra-hepatic cell types, and might be regulated by inflammatory mediators, as observed in hepatocytes.27) A growing body of evidence indicates that acute phase response might take place in extra-hepatic cell types, notably epithelial cells, and might be regulated by inflammatory mediators, as observed in hepatocytes.27) A growing body of evidence indicates that acute phase response might take place in extra-hepatic cell types, notably epithelial cells, and might be regulated by inflammatory mediators, as observed in hepatocytes.27) A growing body of evidence indicates that acute phase response might take place in extra-hepatic cell types, notably epithelial cells, and might be regulated by inflammatory mediators, as observed in hepatocytes.27)
indicate that plasma AAG can be synthesized and secreted by renal cells and other extra-hepatic cells as well as in hepatic synthesis when the acute phase response occurs. Therefore, expression of AAG in CsA-treated rats might contribute to maintain homeostasis by reducing the tissue damage associated with the inflammatory process. However, it must be pointed out that AAG appears to be a marker for renal toxicity, but is unsuitable for patients with systemic injury, such as those having had transplant surgery, since its concentration increases in response to systemic tissue injury.

Clusterin (CLUS), which was also newly induced in rats treated with high-dose CsA, is a heterodimeric glycoprotein induced after renal tubular cell injury. Although there are a few reported studies on CsA nephrotoxicity inducing CLUS, a great number of publications have reported the function of CLUS in the physiology and pathology of the kidney. The basal expression of CLUS in the kidney is minimal and confined to rare distal tubular cells, but it is markedly induced in a variety of tubular injury states. Numerous functions have been proposed for CLUS, including complement inhibition, lipid transport, apoptosis, membrane protection, and maintenance of normal cell interactions. Maintenance of cell-cell and cell-substratum contacts appears to be particularly relevant to acute renal injury, given the potent ability of CLUS to promote renal epithelial cell interactions. After nephrotoxicity, CLUS plays a protective or reparative role by enhancing cell interactions, and scavenging necrotic cell debris and toxic denatured macromolecules. Darby et al. reported vascular expression of the clusterin gene in the kidney during the development of cyclosporine-induced nephrotoxicity. Girton et al. found dose-dependent and time-dependent reduction of gentamicin-induced proximal tubular cell cytotoxicity by CLUS. Marked induction of CLUS mRNA or CLUS in renal epithelial cells was seen after cisplatin nephrotoxicity and puromycin aminonucleoside nephrosis.

Fig. 3. 2-DE Maps of Rat Plasma. A, control group; B, low-dose group; C, high-dose group. IPG, 7 cm, pH 4–7. Sample loading, 1.5 mg. CBB-R250 staining.

Fig. 4. Spots, 1, 2, 3, and 4 Expressed in the High-Dose Group Up-Regulated after CsA Treatment. The Magnified 2-D images of these spots in rats, 1, 2, and 3 of each group are shown in the right lane.
Hence it can be assumed that expression of CLUS in rat plasma is concomitant with CsA nephrotoxicity.

Hp is a $\alpha_2$-sialoglycoprotein with hemoglobin (Hb)-binding capacity. Expression of haptoglobin (Hp) was markedly decreased in the high-dose rat plasma. It is well-known that the concentration of Hp in serum decreases after intravascular hemolysis, whether immune, infectious, hereditary, or mechanical. The amplitude of this decrease depends largely on the initial serum Hp concentration and hemolysis. Serum Hp concentrations also decrease in malnutrition and chronic liver disease. Although renal toxicity is a notable adverse effect of CsA, CsA treatment is also associated with hepatotoxicity and a high incidence of hypertension, which might progress to microangiopathic hemolysis. These adverse effects might lead to a decrease in the expression of Hp in the plasma.

**Concluding Remarks**

We examined plasma protein alterations caused by CsA administration a high dose (100 mg/kg/d) and a low dose (5 mg/kg/d) using 2-DE. The extent of renal impairment was evaluated through renal function and pathologic observation. Serum BNU and urine NAG/Cr of the rats in the high-dose group increased significantly, while Cr clearance decreased markedly and a loss of brush borders and clear vacuolization was also observed. Differentially expressed proteins were characterized by MALDI-TOF-MS and CapLC/ESI/Q-TOF MS/MS. Two up-regulated proteins were identified, one as AAG, a protein involved in stimulation of the immune system by CsA acute nephrotoxicity, and the other CLUS, a protein that plays a protective role by scavenging cellular debris and toxic denatured macromolecules. A down-regulated protein was identified as Hp, which is known to be associated with liver impairment and microangiopathic hemolysis. Known occurrences follow the toxicity of CsA. Further studies are necessary to determine the quantitative relationships between doses of CsA and the expression levels of these proteins (especially for CLUS). These identified proteins might be used to evaluate CsA acute nephrotoxicity and, to help in further understanding of the mechanism of CsA-induced nephrotoxicity.

**Acknowledgments**

This study was supported by the National Program for Key Basic Research Projects (no. 2004CB519802) of China.

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