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Identification of novel indicators of cyclosporine A nephrotoxicity in a CD-1 mouse model

Séin O'Connell, Craig Slattery, Michael P. Ryan, Tara McMorrow *

UCD School of Biomolecular and Biomedical Research, UCD Conway Institute, University College Dublin, Ireland

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ABSTRACT

The calcineurin inhibitor cyclosporine A (CsA) is a widely used immunosuppressive agent. However, nephrotoxicity is a serious side effect observed in patients which limits clinical use of CsA. CsA nephrotoxicity is associated with tubulointerstitial injury progressing to nephropathy. This is typically diagnosed by invasive renal biopsy and is often only detected when the disease process is well advanced. Therefore identification of novel, early indicators of CsA nephrotoxicity could be clinically advantageous. This study aimed to establish a murine model of CsA nephrotoxicity and to identify urinary proteins that may indicate the onset of CsA-induced nephropathy using 2-D gel electrophoresis. CsA nephrotoxicity was induced in CD-1 mice by daily CsA administration for 4 weeks. By week 4, elevated serum creatinine and proteinuria were observed after CsA treatment indicating significant renal dysfunction. Decreased cadherin-1, increased α-smooth muscle actin and fibroblast specific protein 1 in kidney tissue indicated disruption of normal tubular architecture. Alterations in podocin and uromodulin were also observed which may indicate damage to other segments of the nephron. Proteomic analysis of urine identified a number of differentially regulated proteins that may be involved in early CsA nephropathy including cadherin 1, superoxide dismutase and vinculin. These findings suggest novel mechanisms of CsA nephrotoxicity and identify novel potential markers of the disease.

Introduction

The use of calcineurin inhibitors (CNIs), cyclosporine A (CsA) and tacrolimus has revolutionised solid organ transplantation over the last 30 years (Gaston, 2009). However, clinical use of CsA is associated with both acute and chronic nephrotoxicity which is a major limiting factor in its use. While alternative therapeutics have been sought, CNIs remain our most effective and widely used immunosuppressants (Gaston, 2009). While acute CsA nephrotoxicity is managed clinically through careful monitoring of renal function and appropriate regimen adjustment, the balance between preventing immunologic allograft loss and the management of chronic CNI nephrotoxicity (particularly CsA nephropathy) is still a major issue in renal transplantation (Bestard et al., 2005). Early diagnosis of nephropathy can greatly improve patient prognosis. However the initial stages of CsA nephropathy are largely asymptomatic making early diagnosis difficult. Therefore identification of novel, early disease indicators is currently a major research focus.

Current diagnostic techniques employed to detect CsA nephropathy are inadequate. The primary method is estimation of glomerular filtration rate (eGFR) (Cockcroft and Gault, 1976). However this technique is limited since eGFR varies greatly both between patients, and over time within a patient (Kwong et al., 2010). eGFR is the net result of the complex interaction of multiple factors including age, blood pressure and other diseases. Many of these factors are variable and so compensation can often occur, leading to stabilisation of eGFR, effectively masking early renal functional decline. These factors mean that eGFR can be a very insensitive indicator of renal damage. Determination of serum creatinine and blood urea nitrogen (BUN) are also used to estimate renal function although these tests can be insensitive and have poor diagnostic value (Dieterle et al., 2010). Measurement of albumin and/or protein in the urine to detect renal damage may be more sensitive than the determination of eGFR on an individual basis, especially in early disease states. However biopsy studies have clearly shown that intra-renal pathology often occurs well in advance of microalbuminuria (Rastaldi et al., 2002). Furthermore, the relationship between proteinuria and CsA nephropathy is complex (Li and Yang, 2009) limiting its power as an early indicator of CsA nephrotoxicity.

CsA nephropathy is characterised by tubulointerstitial fibrosis (TIF), tubular vacuolisation, glomerulosclerosis, and arteriolopathy (Hara et al., 2009). Of these, TIF is thought to be the primary mechanism driving the progression of CsA nephropathy (Bobadilla and Gamba, 2007). TIF is characterised by the gradual loss of tubular epithelial cells, and progressive accumulation of fibroblasts and myofibroblasts (α-smooth muscle actin (α-SMA) and fibroblast specific protein-1 (FSP-1) positive cells). The accumulation of myofibroblasts results in excessive

* Corresponding author at: School of Biomolecular and Biomedical Science, Conway Institute of Biomolecular & Biomedical Research, University College Dublin, Dublin 4, Ireland. Fax: +353 1 7166456.
E-mail address: tara.mcmorrow@ucd.ie (T. McMorrow).

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production and deposition of extracellular matrix (ECM) in the tubulointerstitium (Lan, 2003). Previous studies from this research group have demonstrated the direct toxic effects of CsA on renal tubular epithelial cells *in vitro* (Kiely et al., 2003; McMorrow et al., 2005; Slattery et al., 2005; Martin-Martin et al., 2010). It is clear that some of the downstream pathogenic effects of CsA are mediated by transforming growth factor beta 1 (TGF-β1) and it's downstream effector connective tissue growth factor (CTGF) , (Grotendorst, 1997; Gupta et al., 2000; Shihab et al., 2003; Slattery et al., 2006). Conversely, the TGF-β1 antagonist bone morphogenetic protein 7 (BMP-7) is downregulated in rat models of CsA nephrotoxicity (Tuglular et al., 2004). However the precise mechanism of CsA-induced nephrotoxicity remains to be fully elucidated.

The aims of this study were to establish an *in vivo* mouse model of CsA nephropathy and evaluate a number of putative nephrotoxicity markers. These markers included TGF-β, CTGF and BMP-7 which have been proposed in published studies, across a number of other models of nephrotoxicity, as being significantly involved in disease progression and may be suitable as indicators of toxicity or therapeutic targets (El Chaar et al., 2007; Dudas et al., 2009; Phanish et al., 2010). TGF-β, CTGF and BMP-7 were therefore evaluated in a CsA nephrotoxicity model, and a high-throughput proteomic screening methodology was utilised to identify novel, early indicators of CsA nephropathy. 2-DE has been widely used to identify potential urinary markers of disease in a range of settings including hepatocellular carcinoma (Jia et al., 2010) and juvenile idiopathic arthritis (Rosenkranz et al., 2010). Identification of discriminating urinary proteins in the current model may lead to novel markers of CsA nephrotoxicity and may also help to further elucidate the mechanisms underlying CsA nephrotoxicity.

Methods

Animal treatment

Male CD-1 mice weighing 25–35 g (6–8 week) were housed in the UCD biomedical facility according to ethical and legal guidelines in a temperature and light controlled environment. All experiments were approved by the UCD Animal Research Ethics Committee (P04-07). Government approval (B100/3539) was also granted by the Irish Department of Health under section 11 of the Cruelty to Animals Act. For the duration of the experiment mice were maintained on a low sodium diet (Harlan, UK Ltd.). CsA (Sigma-Aldrich, Cat no. C1832) was made up as a 1 mg/ml stock solution in olive oil (Sigma-Aldrich). CsA was administered by intraperitoneal injection (15 mg/kg/day), daily for 1 week or 4 weeks, as indicated. Control mice received 1 ml/kg of vehicle (olive oil) by intraperitoneal injection, daily for 1 week or 4 weeks, as indicated. These doses and time points were based upon data from other relevant *in vivo* studies (Thomas et al., 1998; Clarke and Ryan, 1999; Yang et al., 2002; Ling et al., 2003). Using this protocol ensured that by 4 weeks of CsA treatment significant nephropathy had developed. The 1 week CsA group was utilised to examine early toxic effects of CsA before overt histological alterations had manifested (Ling et al., 2003; Chaaya et al., 2011). Mice had free access to food and water throughout the experiments.

At the end of each treatment period mice were housed in group metabolism cages for 24 h for urine collection which was then frozen at −80 °C. After this 24 h period mice were euthanised and blood samples obtained by cardiac puncture. Kidneys were collected for analysis of histology, gene and protein expression. Half a kidney was used for RNA isolation (Trizol method, T9424 Sigma-Alrdich) and half for protein isolation (RIPA buffer method Sigma-Aldrich, R0278). Half of the other kidney was snap frozen in liquid N_2 and the other half was fixed in neutral buffered formalin. Each treatment group i.e. 1 week control; 1 week CsA; 4 week control and 4 week CsA contained 6 mice. Urine was collected from each group of 6 mice and processed for analysis as one pooled sample for each group.

Renal function and histology

Renal function was assessed by determination of serum creatinine and urinary protein (proteinuria). Serum creatinine was measured using the Quantichrom™ Creatinine Assay Kit (Cat no. DICT-500, Bioassay systems assay kit), according to the manufacturer's protocol. This colorimetric assay is based on the improved Jaffe method. Proteinuria was measured using the Bradford Assay for assessing total protein quantities in a biological sample (Bradford, 1976). Urine samples were normalised for urinary output. Half a kidney was fixed in neutral buffered formalin, paraffin embedded and sectioned at 5 μm. After de-waxing, gross renal histology was examined using standard haematoxylin and eosin (H&E) staining (Sigma-Aldrich). Collagen staining of sections was performed using Masson's Trichrome stain (Sigma-Aldrich). Sections were stained using an automated slide stainer (Leica autostainer XL).

Quantitative polymerase chain reaction (PCR). Total RNA was isolated using the trizol method from half a kidney stored at −80 °C in RNAlater™ (Ambion Cat no. AM7020) according to the manufacturer's protocol. One microgram of total RNA was used to synthesise cDNA. A Real-Time PCR TaqMan assay was used to quantify the relative expression levels of genes of interest and has been described previously (Feighery et al., 2008). Briefly, cDNA was amplified on the ABI 7900HT Sequence Detection System at default thermal cycling conditions: 2 min @ 50 °C, 10 min @ 95 °C for enzyme activation and then 40 cycles of 15 s @ 95 °C for denaturation and 1 min @ 60 °C for annealing and extension. Results were analysed using the delta Ct method of analysis. Primer sequences for murine TGF-β1 were designed in the Conway Institute genomics core facility and synthesised by Applied Biosystems. Primer specificity was assessed by nBLAST in the NCBI database.

Name: Mouse TGF-β1 NM_011577.tx-529 Forward
Sequence: AATTCCTGGCGTTACCTTGGT
Name: Mouse TGF-β1 NM_011577.tx-600 Reverse
Sequence: GACGTCAAAAGACAGCCACTCA

Commercially available gene expression assays were used for mouse CTGF (Mm00515790_g1), Cadherin 1 (Mm00486906_m1), α-SMA (Mm00426835_g1) and FSP-1 (Mm00803372_g1) all from Applied Biosystems.

Quantitative enzyme-linked immunosorbant assay (ELISA). A TGF-β1 ELISA was used to determine the effect CsA had on urinary TGF-β1 protein levels. This was done according to the manufacturing company's (Cat no. DB100B, R&D systems) protocol. The specificity and sensitivity of the assay was assessed using 5 ng of TGF-β1 as a positive control and sterile water as a negative control.

Western blot analysis. Total kidney protein was isolated using the RIPA buffer method (Sigma-Aldrich, R0278) from renal homogenates according to the manufacturer's protocol. The SDS-PAGE procedure used was that of Laemmli (1970). Expression levels of renal proteins following CsA treatment was determined by Western blot and has been described previously (McMorrow et al., 2005; Slattery et al., 2005; Feighery et al., 2008). Proteins of interest were detected using the following antibodies according to the manufacturer's protocol, mouse monoclonal anti-α-SMA antibody (A2547 Sigma-Aldrich), mouse monoclonal anti-Cadherin 1 (CDH-1) antibody (61081, BD biosciences) or rabbit anti-CTGF polyclonal antibody (A gift from Dr. John Crean, UCD Conway Institute). In urinary western blots colloidal coomassie blue stain (Sigma-Aldrich, B8522) which stains all proteins within a polyacrylamide gel was used as a loading control to ensure equal loading of proteins.