Tacrolimus Improves the Proteinuria Remission in Patients with Refractory IgA Nephropathy

Qingxian Zhang, Su-fang Shi, Li Zhu, Ji-cheng Lv, Li-jun Liu, Yu-qing Chen, Hong Zhang, Hai-yan Wang

Renal Division, Department of Medicine, Peking University First Hospital, Peking University Institute of Nephrology, Key Laboratory of Renal Disease, Ministry of Health of China, Key Laboratory of Chronic Kidney Disease Prevention and Treatment (Peking University), Ministry of Education, Beijing, PR China

Abstract

Background: Tacrolimus has been reported to be effective in refractory nephrotic syndrome, such as focal segmental glomerulosclerosis and membranous nephropathy. Some IgA nephropathy (IgAN) patients with massive proteinuria showed resistance to steroids and/or cytotoxic immunosuppressants based on the supportive therapy with renin-angiotensin system blockade. The efficacy and safety of tacrolimus in such refractory IgAN patients are extremely ambiguous, and the mechanism of tacrolimus improving proteinuria remission needs to be investigated. Methods: 14 refractory IgAN patients were enrolled. The patients received tacrolimus (0.05–0.1 mg/kg/day) and prednisone (0.5 mg/kg/day) for at least 6 months. Synaptopodin and calcineurin expression were detected in renal tissues of patients who received re-biopsy. A puromycin aminonucleoside (PAN)-induced human podocyte injury model was applied to investigate the possible role of tacrolimus in proteinuria remission. Results: Of the 14 patients enrolled, 3 were withdrawn because serum creatinine increased over 30% baseline. In 11 patients treated with tacrolimus over 6 months, 9 showed complete or partial remission and 7 achieved remission within 1 month. In renal tissues, the expression of calcineurin increased while synaptopodin decreased and recovered partially after tacrolimus therapy. In an in vitro study, F-actin disrupted in human podocytes after stimulation of PAN, while calcineurin increased and synaptopodin decreased. After co-treatment with tacrolimus the reorganization of F-actin and the expression of calcineurin and synaptopodin recovered. Conclusions: Tacrolimus showed a rapid proteinuria remission in refractory IgAN patients. The possible mechanism of tacrolimus to proteinuria remission might be podocyte cytoskeleton stabilization through inhibition of calcineurin expression.

Introduction

Primary IgA nephropathy (IgAN) is the most common form of idiopathic glomerulonephritis throughout the world and the main cause of end-stage renal disease.

Qingxian Zhang and Su-fang Shi contributed equally to this work.
IgAN patients usually present with different degrees of proteinuria, which was thought to be an important predictive risk factor for renal dysfunction [1]. Renin-angiotensin system (RAS) blockade is a crucial therapeutic strategy for proteinuria [2, 3], but RAS blockade alone may be ineffective for some patients with massive proteinuria. Studies have shown that IgAN patients who were resistant to RAS blockade alone could obtain benefit from steroids and/or immunosuppressive agents [4–6]. However, there are still some patients with massive proteinuria showing resistance to steroids and/or cytotoxic immunosuppressants and are more likely to progress to end-stage renal disease [7]. Therefore, a new and effective strategy for such patients needs to be explored.

Studies have shown that tacrolimus, a new and potent kind of calcineurin inhibitor, is effective in nephrotic syndrome (NS), especially in refractory NS both in children and adult patients [8, 9]. In our previous clinical practice, we found that tacrolimus could decrease proteinuria in IgAN patients who showed resistance to steroids and/or immunosuppressants with RAS blockade. However, the efficacy and safety of tacrolimus in IgAN patients with refractory proteinuria as well as the potential mechanism need further investigation.

Tacrolimus can reduce proteinuria by suppressing the immune response through downregulating the transcription of various genes in T cells. However, for patients with refractory NS who had already been treated with a full dose of immunosuppressive agents, non-immunological mechanisms may take part in the action of proteinuria remission. A recent study showed that calcineurin inhibitors may have an anti-proteinuric action through an effect on the stability of the podocyte cytoskeleton [10, 11]. It was definite that puromycin aminonucleoside (PAN) could induce podocyte cytoskeleton disruption [12], so a PAN-injured human podocyte model was performed in this study to investigate whether tacrolimus could decrease proteinuria by stabilizing the podocyte cytoskeleton.

**Methods**

**Patients**

In this observational study, 14 renal biopsy-proven primary IgAN patients with refractory proteinuria who were registered in the Peking University First Hospital IgAN Database (http://www.renal-online.org) from January 2007 to December 2008 were enrolled. Refractory proteinuria was defined as a persistent nephrotic range of proteinuria (>3.5 g/day) after RAS blockade therapy together with steroids and/or immunosuppressant therapy for at least 6 months. RAS blockades indicated any exposure to either angiotensin-converting enzyme inhibitors or angiotensin-receptor blockers, or both. We convert angiotensin-converting enzyme inhibitor and angiotensin-receptor blocker units dispensed to defined daily doses using WHO classifications (http://www.whocc.no/atcd/). After at least 3 months of RAS blockade therapy during which patients’ blood pressure was well controlled, steroids and/or immunosuppressive therapy were administered to patients who were still in the nephrotic range of proteinuria. The immunosuppressants refer to cyclophosphamide and others including mycophenolate mofetil and rituximab. Patients with an estimated glomerular filtration rate (eGFR; estimated by using the MDRD equation for Chinese population [13]) <30 ml/min-1.73 m² were excluded. The study protocol was reviewed and approved by the Ethics Committee of Peking University First Hospital and written informed consents were provided by all participants.

**Indications for Tacrolimus Therapy**

After immunosuppressants were dropped for at least 1 month, tacrolimus was started at 0.05–0.1 mg/kg/day in two divided doses over 12-hour intervals for 6–12 months. The dose was adjusted according to a target trough level of 5–10 ng/ml. Oral prednisone was started at 0.5 mg/kg/day and maintained for 8 weeks; the dose was tapered by 5 mg every 2 weeks to 2.5 mg/day and maintained for 4 weeks. Patients on RAS blockade therapy were maintained on the same dose. Other antihypertensive drugs were added if needed to achieve an adequate blood pressure control of <125/75 mm Hg.

Follow-up was twice a week for the first 4 weeks, then monthly. After initiation of tacrolimus treatment the whole-blood tacrolimus trough level was detected after 2 weeks and then monthly until stable levels of FK-506 to 5–10 ng/ml were achieved. Patients’ blood pressure and any complaints of adverse events possibly associated with tacrolimus were obtained at each visit. Urine proteinuria, serum creatinine, eGFR, glucose, albumin, and complete blood counts were measured at each visit during the observation period.

**Outcome and Definitions**

The primary outcome was the complete or partial remission of proteinuria. Complete remission was defined as a decrease in proteinuria to a level ≤1.0 g/day, and partial remission as a 50% decrease in proteinuria and a level <3.5 g/day but >1.0 g/day. Resistance to tacrolimus therapy was defined as persistence of nephrotic proteinuria (>3.5 g/day) after 6 months of tacrolimus treatment. The time required for complete or partial remission was defined as the time from the start of therapy to the day on which complete or partial remission was observed. The safety profile of tacrolimus was assessed by the increase of serum creatinine less than 30% of baseline.

**Renal Biopsy and Pathology**

Renal specimens were evaluated using direct immunofluorescence, light and electron microscopy. In order to estimate the potential nephrotoxicity of tacrolimus and changes in severity of histological involvement, repeat renal biopsy was performed in 4 patients after informed consent. Features of typical nephrotoxicity induced by calcineurin inhibitors were carefully identified, including arteriolopathy, striped interstitial fibrosis, tubular vacuolization and microcalcification of tubular epithelial cells [14].
The degree of glomerular sclerosis, interstitial fibrosis, tubular atrophy and hyalinosis as well as the histological stage of the disease were compared between the biopsy specimens of each patient by the Oxford Classification of IgAN and Haas grading [15, 16].

**Immunohistochemistry**

After deparaffinization and rehydration, the paraffin-embedded biopsy specimens were incubated with mouse anti-synaptopodin (1:20; Meridian Life Science, Inc.) or rabbit anti-calcineurin (1:50; Millipore) antibody at 4°C overnight, followed by incubation with a Dako EnVision® system (ready to use; Dako). Four cases of peritumoral normal kidney tissue were used as controls. The immunostaining slides were analyzed using Image-Pro® Plus version 6.0. The average optical density of each captured glomerulus (original magnification ×400) was calculated by dividing the integral optical density by the glomerular capillary loop total area.

**Human Podocyte Culture**

The conditionally immortalized human podocytes (gift from Prof. Moin A. Saleem, Children’s Renal Unit and Academic Renal Unit, University of Bristol, UK) were grown in RPMI 1640 containing 10% FBS, penicillin (100 U/ml), streptomycin (100 mg/ml), and ITS (insulin, transferrin, sodium selenite; Sigma) at 33°C and 5% CO2/95% air to promote cell propagation [17]. After moving to 37°C, cells were transplanted to a 6-well plate or Lab-Tek™ chamber slide. Cells were divided into three groups, including a normal control group (Control), a PAN (Sigma, USA) injury group (10 µg/ml PAN was given, PAN), and a tacrolimus- (Sigma, USA) treated group (5 µg/ml tacrolimus was added, PAN+T). Cells in 6-well culture plates were collected for protein extraction. Cells on Lab-Tek™ chamber slides were fixed in 4% paraformaldehyde.

**Western Blotting**

Human podocytes were lysed and the protein samples were subjected to SDS-PAGE and then electrophoretically onto Hybond PVDF membrane. Membranes were incubated with rabbit anti-calcineurin (1:500; Millipore), mouse anti-synaptopodin (1:300; Meridian Life Science, Inc.) and GAPDH (1:1,000; Sigma) antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were detected by ECL™ chemiluminescent detection reagent (PerkinElmer Life Sciences), and then quantified.

**Immunofluorescence**

Paraformaldehyde-fixed cells were permeabilized with 0.2% Triton X-100. For cytoskeletal staining, cells were incubated with 0.5 µg/ml tetramethylrhodamine B isothiocyanate-phalloidin (Sigma). For nephrin staining, cells were stained with rabbit anti-nephrin antibody (1:50), followed by treatment with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. Cells were observed and imaged by confocal microscopy (FluoView™ FV1000; Olympus).

**Statistical Analysis**

Normally distributed variables were expressed as mean ± SD and compared using Student’s t test, one-way ANOVA. \( p < 0.05 \) was considered statistically significant. All statistical analyses were performed with SPSS statistical software (version 16.0).

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**Results**

**Clinical and Pathological Baseline Data of Enrolled Patients**

The baseline characteristics of the 14 enrolled refractory IgAN patients are listed in table 1. There were 6 male patients and mean age was 28.6 ± 9.1 years. The level of urinary protein excretion of 9/14 patients was >3.5 g/day at the time of renal biopsy, but only 2 patients were diagnosed as having NS, and the pathological features of the 2 NS patients were not minimal-change disease. All patients were treated with RAS blockade, and the mean defined daily dose was 1.7. After at least 3 months of RAS blockade therapy, blood pressure had been well controlled. A total of 9/14 patients were then treated with steroids and cyclophosphamide for 6–8 months, 3/14 patients were treated with steroids alone for 6–8 months, 1 patient was treated with mycophenolate mofetil, and 1 patient was treated with two courses of rituximab. Six months later the proteinuria levels of all 14 patients were consistently >3.5 g/day and diagnosed as refractory IgAN.

**Response to Tacrolimus Therapy**

Three out of 14 patients withdrew after tacrolimus treatment for only 1 month because their serum creatinine had increased over 30% versus baseline. In the remaining 11/14 patients who were treated for more than 6 months, 9/11 presented with proteinuria remission, including complete remission in 8/11 and partial remission in 1/11. The time to achieve remission was less than 1 month in 7/9 patients (table 2). There were 2/11 patients who were resistant to tacrolimus therapy.

Of the 11 patients who were treated more than 6 months, the level of urinary protein excretion decreased significantly 1 month after initiation of tacrolimus therapy (5.22 ± 1.63 vs. 2.27 ± 1.26, \( p < 0.001 \)) and maintained until the end of the tacrolimus therapy (5.22 ± 1.63 vs. 1.56 ± 1.71, \( p < 0.001 \)), the level of serum albumin increased significantly (38.73 ± 4.39 vs. 43.20 ± 3.49, \( p = 0.016 \)) and the serum creatinine did not change (103.45 ± 53.49 vs. 128.55 ± 84.18, \( p = 0.41 \)) at the end of tacrolimus therapy (table 2). The mean tacrolimus trough level was 6.07 ± 2.34 ng/ml.

Four patients had a repeat renal biopsy after 6 months of tacrolimus therapy. No typical signs of nephrotoxicity could be found. The proliferative lesions – mesangial and endocapillary – were improved, while the chronic tubular and interstitial lesions did not change according to the Oxford Classification of IgAN (tables 1, 2).
Table 1. Clinical baseline data before tacrolimus treatment

<table>
<thead>
<tr>
<th>No.</th>
<th>Gender</th>
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<th>Clinical manifestation at the time of renal biopsy</th>
<th>Pathology</th>
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<td>eGFR ml/min 1.73 m²</td>
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Scr = Serum creatinine; eGFR = estimated glomerular filtration rate; Salb = serum albumin; MAP = mean arterial pressure; RAS blockades = renin-angiotensin system blockades; DDD = defined daily dose; CTX = cyclophosphamide; MMF = mycophenolate mofetil.

M₀ = Mesangial hypercellularity score <0.5; M₁ = mesangial hypercellularity score >0.5; E₀ = non-endocapillary hypercellularity present; E₁ = endocapillary hypercellularity present; S₀ = non-segmental glomerulosclerosis present; S₁ = segmental glomerulosclerosis present; T₀ = tubular atrophy/interstitial fibrosis range from 0 to 25%; T₁ = tubular atrophy/interstitial fibrosis range from 26 to 50%.

* As patient 4 is not yet 18 but only 14 years of age, eGFR is demonstrated as creatinine clearance rate.

Table 2. Overview of tacrolimus treatment

<table>
<thead>
<tr>
<th>No.</th>
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<th>After tacrolimus treatment</th>
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<th>Maintaining dosage mg/day</th>
<th>Duration of therapy months</th>
<th>Mean blood trough level ng/ml</th>
<th>Therapeutic effect at the end of therapy</th>
<th>Time required for remission months</th>
<th>Pathology of re-biopsy</th>
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compl. = Complete; part. = partial.
Adverse Effects during Therapy

Three patients got gastrointestinal symptoms including abdomen pain and nausea. These symptoms improved about 1 week later, and there was no need for a reduction of tacrolimus. One patient suffered from colpitis mycotica after tacrolimus therapy. Hypertension and hyperglycemia were not reported in any of the 14 patients.

During tacrolimus treatment, 5/14 patients showed a serum creatinine increase over 30% from baseline, including 3/5 patients after 1 month and 2/5 patients after 6 months. All of these 5 patients had severe renal function injury before tacrolimus treatment (eGFR ranged from 30.1 to 48.3 ml/min·1.73 m²). The mean tacrolimus trough level of the 5 patients was 4.9 ± 0.7 ng/ml.
Relapse after Tacrolimus and Steroid Discontinuation
Remission was persistent after the steroid was withdrawn. In 4/9 (44.4%) patients who had shown remission, relapses after cessation of tacrolimus were observed within 6 months. The time to relapse ranged from 2 to 5 months. Two 2 patients received a second trial of only tacrolimus therapy and both achieved complete remission within 1 month.

Expression of Synaptopodin and Calcineurin in Renal Tissues before and after Tacrolimus Treatment
Calcineurin expressed weakly in the glomeruli, while the synaptopodin expressed strongly and was orderly distributed along the basement membrane in control renal tissues (fig. 1A-a, A-d). Calcineurin was upregulated and synaptopodin was decreased segmentally in podocytes before tacrolimus therapy (fig. 1A-b, A-e), which were recovered partially in a repeated biopsy (fig. 1A-c, A-f). A semiquantitative analysis showed that the expression of calcineurin decreased significantly and the synaptopodin expression recovered partially in patients with complete remission (fig. 1B). The semiquantitative analyses before and after tacrolimus therapy were not compared in patient 11 because there were only two glomeruli in the repeated renal tissue.

Podocyte Cytoskeleton Could Be Stabilized by Tacrolimus in the PAN-Injured Model
In the control group, F-actin of the cultured podocytes was distributed regularly and extended into the foot processes. The cell-cell junctions were shown clearly (fig. 2a). The podocyte cytoskeleton was reorganized after the stimulation of PAN in a time- and dose-dependent manner (fig. 2b–d), and the expression of nephrin also decreased (fig. 2h). After co-treatment with 5 μg/ml of tacrolimus, F-actin showed clearly as in the control group, the cell-cell junctions could be found again (fig. 2e), and the expression of nephrin recovered partially (fig. 2i).
Tacrolimus Reversed the PAN-Induced Calcineurin and Synaptopodin Expression

PAN caused a significant increase of calcineurin and decrease of synaptopodin in a dose-dependent manner (fig. 3a). Tacrolimus alone did not affect the expression of calcineurin or synaptopodin and even a large dose of tacrolimus was added (20 μg/ml) (fig. 3b). PAN (10 μg/ml, 48 h) induced a significant decrease of synaptopodin and increase of calcineurin (fig. 3c). However, after co-treatment with tacrolimus (5 μg/ml), the expression changes of calcineurin and synaptopodin recovered (fig. 3c; p < 0.05).

Discussion

In IgAN patients, proteinuria is regarded as one of the most important predictive factors of renal prognosis and RAS blockade is considered the most important strategy for proteinuria [1]. In patients with proteinuria still >1 g/day after a full dose of RAS blockade, steroids and/or immunosuppressive agents are suggested [4, 5]. However, some patients still present with massive proteinuria after steroids and/or immunosuppressive agents combined with RAS blockade therapy. It is very important to find an effective therapeutic strategy for IgAN patients with refractory proteinuria.
Tacrolimus has been used as a useful therapeutic agent in various glomerular diseases, especially refractory NS [8, 9, 18]. However, there is a lack of studies on the effectiveness of tacrolimus in IgAN. In the present study, we observed the efficacy and safety of a combination of tacrolimus and a moderate dose of steroids in a group of 14 IgAN patients who were well defined as having refractory IgAN. Our results are important as they present the first clinical observation of refractory IgAN patients treated with tacrolimus with a rapid proteinuria remission. Li et al. [19] and Roberti and Vyas [20] reported tacrolimus could reduce proteinuria both in adults and children with steroid-resistant nephrotic syndrome rapidly, which was consistent with our results.

It has been proven that calcineurin inhibitors can suppress the immune response by downregulating the transcription of various genes in T cells. In the current study, all the enrolled patients had been fully treated with steroids and/or immunosuppressive agents, so it was difficult to explain the quick remission of proteinuria by the immunosuppressive processes of tacrolimus. We suspect that tacrolimus might reduce proteinuria through non-immunological mechanisms. It is well known that podocytes are a key component of the glomerular filtration barrier to prevent proteinuria [21, 22]. Podocyte injury may be a candidate of the pathogenesis of proteinuria in kidney disease, including IgAN [23, 24]. It was reported in an animal model that the podocyte cytoskeleton could be stabilized by the calcineurin inhibitor, cyclosporine, and then induce proteinuria remission [10]. In our study, we found that calcineurin was upregulated in most of the refractory IgAN patients and the expression of synaptopodin was decreased and disappeared segmentally. This result indicated that podocyte cytoskeleton injury was present and was possibly the reason for the massive proteinuria excretion in refractory IgAN. After tacrolimus therapy, calcineurin and synaptopodin expression were recovered partially in complete remission patients. We assumed that tacrolimus may cause the recovery of synaptopodin by inhibiting the expression of calcineurin, so a PAN-induced podocyte cytoskeleton injury model was performed. Consistently, an in vitro study showed that tacrolimus could also decrease the upregulation of calcineurin expression in PAN-stimulated human podocytes. Moreover, cytoskeleton disruption recovered after co-treatment with tacrolimus. The histological and in vitro results indicated that tacrolimus may have an effect on podocyte cytoskeleton protection through the inhibition of calcineurin. As we know, calcineurin inhibitors have an inhibitory effect on NFAT activation in T cells, and it was reported that NFAT activation also occurred in podocytes which could cause proteinuria and FSGS-like changes [25]. Whether NFAT is activated in refractory IgAN patients and whether tacrolimus could inhibit the NFAT activation in podocytes needs further investigation.

In four re-biopsy specimens, no significant drug-associated nephrotoxicity could be found, while the proliferative lesions were improved and the chronic lesions did not aggravate, which indicates that tacrolimus was safe in these patients. Adverse events were rare. No patients experienced new-onset or worsening of hypertension or diabetes mellitus. During the time of tacrolimus therapy, kidney function remained stable in most of the patients who experienced complete or partial remission. Only those patients with severe renal function injury before initiation of tacrolimus showed an increase in serum creatinine. It has been reported that renal toxicity has been associated with tacrolimus dose and trough levels [14]. However, in this study, the trough levels of patients who experienced a serum creatinine elevation were all <6 ng/ml. It was difficult to confirm the possible reason for the serum creatinine increase in our patients, as we do not have histological evidence to verify the existence of drug-associated nephrotoxicity. Given the potential harmful effects of tacrolimus with respect to serum creatinine, more attention should be paid to the treatment of IgAN patients with severe renal dysfunction. In this study, relapse was observed in 4/9 (44.4%) patients after tacrolimus was discontinued, which was consistent with the result in resistant NS [19]. The reason for the high relapse incidence of tacrolimus was not clear, which should be a concern in future studies.

This study was an observational study and was limited by its small sample size. To draw a conclusion for clinical practice, a randomized controlled study with long-term follow-up is necessary. In addition, the PAN-injured podocyte model in the current study was not directly associated with IgAN.

In summary, our results showed that tacrolimus could improve proteinuria remission in refractory IgAN rapidly and effectively. The possible mechanism of tacrolimus in proteinuria remission might be podocyte cytoskeleton stabilization through inhibition of calcineurin.
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Disclosure Statement

The authors have no conflicts of interest to disclose.

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