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LABORATORY INVESTIGATION

Comparison of acute rapamycin nephrotoxicity with cyclosporine and FK506

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Comparison of acute rapamycin nephrotoxicity with cyclosporine and FK506. Acute cyclosporine (CsA) nephrotoxicity is characterized by a reduction of glomerular filtration rate (GFR), hypomagnesemia and tubular injury. The mechanisms of CsA's immunosuppressive action and presumably its nephrotoxicity are mediated through inhibition of the renal phosphatase, calcineurin. FK506 (FK), which has a different chemical structure and binding immunophilin, also inhibits calcineurin. We compared the renal effects of these drugs to those of rapamycin (RAPA), which although similar in structure and intracellular binding to FK, does not work by changing calcineurin activity. Rats were given CsA (15 mg/kg/s.c.), FK (6 mg/kg/p.o.), RAPA (3 mg/kg/p.o.) or vehicle (V) for two weeks on a low salt diet. CsA and FK strikingly decreased urinary excretion of nitric oxide, renal blood flow and GFR, whereas RAPA did not. In contrast, all these three drugs caused significant hypomagnesemia associated with inappropriately high fractional excretion of magnesium, suggesting renal magnesium wasting. In addition, with all three drugs there were lesions in the rat kidneys consisting of tubular collapse, vacuolization and nephrocalcinosis. We thus showed that only the calcineurin inhibitors produced glomerular dysfunction in an acute experimental model of nephrotoxicity. The mechanism of hypomagnesemia and tubular injury induced by all three immunosuppressive drugs is unclear but may be independent of calcineurin. The mechanism of renal vasoconstriction on the other hand may be related to inhibition of calcineurin.

Cyclosporine A (CsA) has played a large role in the improvement in solid organ transplant graft survival rates. Thus indications for the drug are being extended to the treatment of autoimmune and primary renal diseases [1]. However, the nephrotoxicity of CsA continues to be a major problem [2, 3]. Acute CsA nephrotoxicity is characterized by a decrease in glomerular filtration rate (GFR) and renal blood flow (RBF), hypomagnesemia and tubular injury [4]. The mechanism and mediators of these hemodynamic and structural changes are yet to be defined, and possible links between functional changes and structural damage remain to be elucidated. Hypomagnesemia has been implicated in the pathophysiology of neurotoxicity and essential hypertension [5]. Hypomagnesemia is common in transplant patients receiving CsA and has been proposed as an important factor in the pathogenesis of CsA nephrotoxicity [6]. FK506 (tacrolimus, FK) and rapamycin (sirolimus, RAPA) have also been used clinically to treat transplant rejection [7, 8]; in addition, they have been useful agents in helping to define the mechanisms of immunosuppression and nephrotoxicity with this class of drugs. Despite structural differences, CsA and FK inhibit T-lymphocyte activation by a similar mechanism, namely, through inhibition of the renal phosphatase calcineurin, while CsA and FK bind to different families of intracellular binding proteins immunophilins [9]. RAPA has a distinct mechanism of action, despite being structurally homologous to FK and binding to the same immunophilin. RAPA does not inhibit calcineurin [10]. Recently, Lea et al reported that CsA and FK reduced Na^+,K^+-ATPase activity in medullary thick ascending limb (mTAL) through inhibition of calcineurin with the same potency as the drugs inhibit IL-2 transcription in T cells, while RAPA did not [11]. Na^+,K^+-ATPase in the kidney might be linked to active magnesium transport in tubules. Preliminary data indicate that RAPA shows considerably less nephrotoxicity than CsA and FK [12, 13]. The purpose of this study is to compare the effects of these structurally and pharmacologically different immunosuppressants, on renal tubular and glomerular dysfunction to gain insights into whether CsA nephrotoxicity is mediated through inhibition of renal phosphatase calcineurin or if nephrotoxicity can be dissociated from the immunosuppressive actions of these drugs.

Methods

Animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) weighing 225 to 250 g were housed in individual cages in a temperature- and light-controlled environment. They received a low salt diet (0.05% sodium; Teklad Premier, Madison, WI, USA) and were allowed free access to tap water. They were weighed and examined daily.

Drugs

CsA, provided by Sandoz Research (East Hanover, NJ, USA), was diluted in olive oil and subcutaneously given to animals at 15 mg/kg/day. FK (Fujisawa Pharmaceutical Co., Ltd, Osaka, Japan) was provided in a solid dispersion vehicle formulation, dissolved in distilled water and orally given to animals at 5 mg/kg/day. RAPA, obtained from Wyeth-Ayerst Research (Princeton, NJ,

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USA), was dissolved using a dilution of Tween 80 (10%), N-N-dimethylacetamide (20%), and polyethylene glycol 400 (70%) and orally given to animals at 3 mg/kg/day. The control animals were received the same formulation of vehicles of those three drugs.

Design of experiments

After one week on low salt diet with *ad libitum* tap water, weight-matched pairs of rats were randomly assigned to receive CsA 15 mg/kg, FK 6 mg/kg, RAPA 3 mg/kg or an identical volume of vehicles (VH) for 14 days. The doses of these drugs were chosen from published data reporting the same potency for immunosuppression [14–16]. The numbers of pairs for each experimental groups were determined by the number of designated measurements (8 rats for each measurement of GFR and RBF or perfused kidney, respectively). The vehicle, FK and RAPA rats were pair fed to the amount of food consumed the day before by the CsA rats. On day 14 tail blood pressure (BP) was measured with a plethysmography (Natsume Seisakusyo, Tokyo, Japan) and the rats were placed in metabolic cages (Nalge Company, Rochester, NY, USA) for urine collection. The urine was collected into a plastic tube cooled with ice. After 24 hours the animals were anesthetized with intraperitoneal ketamine and a blood sample was drawn. At this point, as previously designated for each rat, GFR and RBF were measured by ^{14}C-inulin clearance and a blood flow probe, or one kidney was collected for histology after *in vivo* perfusion.

Inulin clearance and RBF

After anesthesia with intraperitoneal ketamine (50 to 100 mg/kg), a polyethylene catheter (PE-50) was inserted into the left carotid artery and connected to a digital blood pressure analyzer (World Precision Instruments, Inc., Sarasota, FL, USA) for continuous measurement of the mean arterial blood pressure and heart rate. The animal's cavity was opened with a midline incision and the left renal artery was carefully exposed, and a transit-time blood flow probe (Transonic Systems, Inc., Ithaca, NY, USA; model 1RB) was placed around the vessel. The flow probe was connected to a small animal, digital transit-time blood flow meter (model T106, Transonic Systems, Inc.). Other polyethylene tubes (PE-50) were placed in the jugular vein for blood collection and in the femoral vein for infusion of solutions. A polyethylene tube (PE-90) was placed and sutured in the bladder. A heating lamp was positioned above the animal to maintain the body temperature. A loading dose of 0.25 μCi ^{14}C-inulin (NEN Research Products, Wilmington, DE, USA) in 6 ml 1% $NaHCO_3$ was given followed by a maintenance infusion of 2.5 μCi ^{14}C-inulin in 10 ml 1% $NaHCO_3$ at a rate of 52 μl/min using a pump (Harvard Bioscience, South Natic, MA, USA). After a 30-minute equilibration time, urine was collected over four periods of at least 20 minutes each in preweighed vials. A blood sample (0.30 ml) was drawn at the midpoint of each urine collection and replaced with an equal volume of 1% $NaHCO_3$. Fifty microliters of urine from these samples were placed in a scintillation vial to measure ^{14}C radioactivity with a Beckman LS 3801 liquid scintillation counter (Beckman Instruments Inc., Palo Alto, CA, USA). The blood sample was centrifuged and a 100 μl aliquot of the plasma was placed into a scintillation vial for measurement of ^{14}C radioactivity by liquid scintillation. Inulin clearances values expressed as ml/min/100 g body wt represent the mean of the four clearance periods. After the end of the inulin clearance the animal was sacrificed and the left kidney was weighed. GFR was represented for both kidneys and calculated per 100 grams of body wt by the standard formula.

Histology

After anesthesia a ventral midline incision was made exposing the abdominal aorta, posterior vena cava and renal vessels. The aorta and vena cava, just above the bifurcation to the iliac and the mesenteric arteria were tied. A ligature was loosely tied around the aorta and vena cava above the left renal artery. An 18-gauge needle was introduced in the abdominal aorta pointing toward the kidneys. An infusion of 20 ml of saline with 500 U of heparin was started simultaneously as the ligature above the left renal artery was tied and a hole was made in the left renal vein. After the saline flush, six minutes of perfusion with 1% glutaraldehyde in a modified 3/4 Tyrode buffer was done by using a pump. The pressure in the system was kept stable at 140 to 160 mm Hg and the flow at 10 to 12 ml/min. After perfusion fixation the left kidney was emersed in the same fixative for 24 hours, following which it was washed in cold Tyrode solution for 24 hours. Multiple transverse sections of the kidney were processed and embedded in paraffin. Two to four micron-thick sections were stained with periodic acid-Schiff stain and examined by light microscopy by an observer (DCH) masked to treatment groups. The histological findings were subdivided into two categories: tubular injury and nephrocalcinosis. Finding ascribed to tubular injury included cellular and intercellular vacuolization; tubular collapse (unassociated with interstitial fibrosis or tubular basement membrane thickening) and tubular distention. The extent of changes in cortical tubules was graded according to the following criteria: Score 0, no tubular injury; Score 0.5, $< 5\%$ of tubules injured; Score 1, 5 to 20% of tubules injured; Score 1.5, 21 to 35% of tubules injured; Score 2, 36 to 50% of tubules injured; Score 2.5, 51 to 65% of tubules injured; Score 3, $> 65\%$ of tubules injured. Nephrocalcinosis was defined as the presence of calcium crystals within the tubular lumen and was primarily found at the corticomedullary junction. Nephrocalcinosis was semiquantitatively assessed using the following scoring system: Score 0 no nephrocalcinosis present; Score 0.5, $< 5\%$ of tubules in corticomedullary junction; Score 1, 5 to 10% of tubules in corticomedullary junction; Score 1.5, 11 to 15% of tubules in corticomedullary junction; Score 2, 16 to 20% of tubules in corticomedullary junction; Score 2.5, 21 to 25% of tubules in corticomedullary junction; Score 3, $> 25\%$ of tubules in corticomedullary junction.

Magnesium supplementation

After one week on low salt diet with *ad libitum* tap water, additional weight-matched pairs of rats were allowed to drink tap water with 2% $MgCl_2$ added and were randomly assigned to receive CsA 15 mg/kg ($N = 8$) or an identical volume of VH ($N = 8$) for 14 days. On day 14 BP was measured with a plethysmography and the rats were placed in metabolic cages for urine collection. After 24 hours the animals were anesthetized with intraperitoneal ketamine and a blood sample was drawn and GFR was measured.

Analytical methods

Urinary and plasma sodium and potassium were measured by flame photometry (Instrumentation Laboratories, Lexington, MA, USA). Urinary and plasma osmolality were measured by

freezing point (Osmette A; Precision Systems Inc., Natick, MA, USA). Urinary and plasma creatinine, urinary and plasma magnesium, plasma calcium, urinary alanine aminopeptidase (AAP) and N-acetyl β-D-glucosaminidase (NAG) were measured by a Cobas autoanalyzer (Roche Diagnostics, Hoffmann-La Roche, Nutley, NJ, USA). Urinary excretion of AAP and NAG were standardized by urinary creatinine and expressed in IU/g creatinine. Serum and urine radioactivity were measured by a Beckman LS 3801 liquid scintillation counter (Beckman Instruments Inc., Palo Alto, CA, USA). The fractional excretion of sodium (FE_{Na}), fractional excretion of potassium (FE_K) and fractional excretion of magnesium (FE_{Mg}) were calculated by standard formulas.

Whole blood trough level

Blood was collected in plastic syringes, transferred to metal-free tubes containing EDTA and was stored at −20°C until determined by a double sandwich ELISA assay that employed mouse monoclonal anti-FK 506 antibody (Fujisawa Pharmaceutical Co., Osaka, Japan) or a monoclonal radioimmunoassay for CsA (Incstar Co., Stillwater, MN, USA) [17].

Urinary nitrate (NO_3)/nitrite (NO_2) assay

Urinary excretion of the stable nitric oxide (NO) metabolites, NO_2 and NO_3 were determined. Urinary NO_2 was assayed by the Griess reaction. Urinary NO_3 was estimated by the reduction to NO_2 using nitrate reductase from Aspergillus species (Boehringer Mannheim, Indianapolis, IN, USA) [18]. The NO_3 reduction was 95 to 100% effective. Urinary NO_3 excretion was considered the difference between total NO_2 (after reduction) minus initial NO_2 values. The results were expressed in μ mol/24 hours of urine.

Statistical analysis

Results are presented as mean ± SEM, and all statistical analyses were calculated with SYSTAT for Macintosh v. 5.2 (SYSTAT Inc., Chicago, IL, USA). Comparisons between groups were done by ANOVA (Kruskal-Wallis test followed by Tukey test). The level of statistical significance was chosen as $P < 0.05$.

Results

Physiologic studies

Weight gain was progressive in the three treatment groups of VH, CsA and FK with no significant difference throughout this experiment, while despite a pair feeding, animals treated with RAPA failed to gain as much weight as those of the VH rats with the difference achieving statistical significance ($P < 0.01$) at day 14 (Table 1). Hypertension was not observed in any group. CsA whole blood trough level in CsA-treated animals was 3250 ± 200 ng/ml and FK whole blood trough level in FK-treated animals was 9.8 ± 2.5 ng/ml at day 14. RAPA levels were unavailable.

Renal function

CsA and FK strikingly decreased urinary excretion of nitric oxide, RBF and GFR ($P < 0.01$ vs. VH), whereas RAPA did not (Table 1). In contrast, all these three drugs caused significant hypomagnesemia associated with inappropriately high fractional excretion of magnesium (FE_{Mg}), suggesting renal magnesium wasting ($P < 0.01$ vs. VH). The fractional excretion of sodium (FE_{Na}) was very low in all groups reflecting the low sodium diet, and there was no significant difference among the groups (Table 2). Plasma calcium was normal in all groups (VH, CsA, FK, RAPA: 9.8 ± 0.2, 9.6 ± 0.1, 9.8 ± 0.1, 9.5 ± 0.2 mg/dl, respectively). Plasma phosphorus and potassium were also normal in all groups (data not shown). CsA, FK and RAPA caused a significant increase in the urinary volume ($P < 0.05$ vs. VH) and decreases in the urinary osmolality (U_{Osm}) and free water reabsorption ($P < 0.01$ and 0.05 vs. VH, respectively), suggesting that urinary concentrating ability was clearly impaired. The urinary excretion of AAP was significantly higher in these three immunosuppressive agents groups ($P < 0.05$ vs. VH). There were no changes in the urinary excretion of NAG in all groups.

Table 1. Change in body weight, GFR, RBF and blood pressure in the different groups at day 14

	VH	CsA	FK	RAPA
Body wt change *g*	43 ± 6	30 ± 7	35 ± 5	9 ± 5[b]
GFR *ml/min/100 g*	1.1 ± 0.1	0.2 ± 0.1[b]	0.2 ± 0.1[b]	1.0 ± 0.1
RBF *ml/min/100 g*	3.3 ± 0.2	1.9 ± 0.2[b]	2.0 ± 0.2[b]	3.1 ± 0.3
BP *mm Hg*	145 ± 5	139 ± 6	140 ± 5	141 ± 6
UNO μ mol/24 hr	15.2 ± 1.2	9.5 ± 0.6[b]	10.6 ± 0.8[b]	19.4 ± 1.7

Data are mean ± SEM of eight rats. Abbreviations are: VH, vehicle control; Body wt, body weight; GFR, inulin clearance (both kidneys); RBF, renal blood flow (left kidney); BP, systolic blood pressure; UNO, urinary excretion of NO_2/NO_3.
[a] $P < 0.05$ vs. VH
[b] $P < 0.01$ vs. VH

When individual animals in all groups were compared by linear regression, a significant correlation was demonstrated between the FE_{Mg} and U_{Osm} ($N = 64$, $r = -0.68$, $P < 0.001$). However, there was no correlation between the FE_{Mg} and GFR or RBF.

Magnesium supplementation

CsA whole blood trough level in $MgCl_2$ supplemented rats treated with CsA was 3270 ± 240 ng/ml and was similar to that of $MgCl_2$ non-supplemented rats treated with CsA (3250 ± 200 ng/ml). Magnesium supplementation successfully prevented hypomagnesemia in CsA-treated animals (plasma magnesium 2.1 ± 0.1 vs. 2.0 ± 0.1 mg/dl in VH treated animals, NS). There was no increase in the urinary excretion of AAP (46 ± 7 IU/g Cr in CsA vs. 41 ± 4 IU/g Cr in VH, NS) as well as no decrease in urinary osmolality between CsA- and VH-treated animals (2600 ± 240 mOsm/kg vs. 2500 ± 180 mOsm/kg, respectively, NS) when magnesium was supplemented. On the other hand the decrease in GFR induced by CsA was not modified by magnesium supplementation (0.2 ± 0.1 ml/min/100 g in CsA vs. 1.0 ± 0.1 ml/min/100 g in VH, $P < 0.01$).

Histologic changes

Table 3 summarized the histologic data of all groups. Tubular injury was present in rats on CsA, FK and RAPA and consisted of tubular dilation, tubular atrophy, tubular cast formation and thickening of the tubular basement membrane (Fig. 1). The injury score of these groups was significantly higher compared to the VH control. Tubular injury was supported by observing a significantly elevated urinary AAP excretion (proximal tubular brush border

Table 2. Renal functional parameters in the different groups at day 14

	VH	CsA	FK	RAPA
UV *ml/24 hr*	11.3 ± 1.5	18.1 ± 1.7[a]	17.4 ± 1.5[a]	19.7 ± 2.1[b]
U_{Osm} *mOsm/kg*	2200 ± 250	1050 ± 180[b]	980 ± 200[b]	850 ± 150[b]
TcW *ml/day*	58 ± 5	39 ± 4[a]	42 ± 5[a]	40 ± 4[a]
Plasma Na *mEq/liter*	142 ± 2	143 ± 3	144 ± 3	143 ± 3
FE_{Na} %	0.05 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
Plasma Mg *mg/dl*	1.9 ± 0.03	1.1 ± 0.04[b]	1.0 ± 0.04[b]	1.1 ± 0.04[b]
FE_{Mg} %	5.5 ± 0.5	13.7 ± 0.9[b]	12.5 ± 1.3[b]	15.3 ± 1.1[b]
Urine AAP *IU/g Cr*	41 ± 5	85 ± 12[b]	78 ± 10[a]	75 ± 8[a]
Urine NAG *IU/g Cr*	27 ± 1	25 ± 3	28 ± 2	24 ± 2

Data are mean ± SEM of eight rats. Abbreviations are: VH, vehicle control; UV, urinary volume; U_{Osm}, urinary osmolality; TcW, free water reabsorption; FE_{Na}, fractional excretion of sodium; FE_{Mg}, fractional excretion of magnesium; AAP, alanine aminopeptidase; NAG, N-acetyl β-D-glucosaminidase.

[a] $P < 0.05$ vs. VH

[b] $P < 0.01$ vs. VH

Table 3. Semiquantitative histologic injury score (0 to 3+) in the different groups at day 14

	VH	CsA	FK	RAPA
Tubular injury	0.1 ± 0.1	1.5 ± 0.3[b]	1.7 ± 0.4[b]	1.4 ± 0.3[a]
Nephrocalcinosis	0 ± 0	1.1 ± 0.2[a]	1.5 ± 0.3[b]	1.3 ± 0.4[a]

Data are mean ± SEM of eight rats. Abbreviation is VH, vehicle control.

[a] $P < 0.05$ vs. VH

[b] $P < 0.01$ vs. VH

membrane marker enzyme, see above), renal magnesium wasting and impaired urinary concentrating ability. Nephrocalcinosis was also prominent at the corticomedullary junction in the animals treated with these three drugs (Fig. 2).

When individual animals in all groups were compared by linear regression, a significant correlation could be demonstrated between the injury score and U_{Osm}, FE_{Mg} or urinary AAP ($N = 32$, $r = -0.65$, $P < 0.01$; $r = 0.75$, $P < 0.001$; $r = 0.70$, $P < 0.001$, respectively).

Discussion

In this study we have compared the acute renal effects of CsA, FK and RAPA, which have different structures and inhibitory action on the renal phosphatase calcineurin. This was intended to provide indirect insight into the issue of whether acute CsA nephrotoxicity is mediated through inhibition of calcineurin. Confirming previous studies in our laboratory, rats given CsA or FK on low salt diet decreased their RBF and GFR [19, 20]. Also these two drugs caused significant hypomagnesemia with inappropriately high FE_{Mg}, suggesting renal magnesium wasting, and lesions in the rat kidneys consisting of partial tubular collapse, vacuolization in proximal tubules and nephrocalcinosis. FK has a different chemical structure and binding immunophilin from CsA but does potently inhibit the phosphatase activity of calcineurin similar to CsA. RAPA despite being structurally similar to FK and binding to the same immunophilin, does not inhibit calcineurin activity or calcineurin-dependent signaling cascades [10]. Our major findings were that while RAPA does not acutely decrease RBF nor GFR; it does produce profound hypomagnesemia with high FE_{Mg} and the same parenchymal kidney lesions as those caused by CsA and FK.

Preliminary experimental data indicate that RAPA shows considerably less nephrotoxicity than CsA and FK presumably due to a different target enzyme for its immunosuppressive effects and by inference its renal actions [12, 13, 21]. Our study supports this hypothesis since CsA and FK strikingly decrease urinary excretion of nitric oxide, RBF and GFR ($P < 0.01$ vs. VH), whereas RAPA does not. Recently, nitric oxide (NO) synthase has been found to be a substrate of calcineurin *in vitro* [22]; its inhibition may, in part, help to explain the renal dysfunction induced by CsA and FK. It has been demonstrated that chronic CsA administration impairs NO production by rat kidney arteries [23, 24]. Experimental evidence suggests an important role of the decrease of vasorelaxant mediators in the pathogenesis of the renal vasoconstriction initiating CsA nephrotoxicity [25]. Among these substances, NO is one of the most prominent [26]. It is produced in many tissues by a family of NO synthases. A constitutive Ca^{2+}/calmodulin-dependent form (cNO synthase) is blocked by the binding of CsA to calmodulin via the formation of the complex CsA/cyclophilin [27]. More recently, several groups have found that acute CsA nephrotoxicity was enhanced by simultaneous NO blockade with an analogue of L-arginine, whereas significant improvement in renal dysfunction was achieved by administration of L-arginine in rats [28–30]. Therefore our data suggest that glomerular dysfunction induced by CsA may be mediated at least partly through inhibition of calcineurin and NO biosynthesis.

Lea, Sands and McMahon reported that the inhibition of Na^+,K^+-ATPase activity in mTAL of the kidney by CsA and FK involves calcineurin, while RAPA does not inhibit either Na^+,K^+-ATPase or calcineurin [11]. It has been suggested that Na^+,K^+-ATPase might have an important role in reabsorption of magnesium in the mTAL via secondary active transport [31]. Thus, it could be hypothesized that CsA reduces Na^+,K^+-ATPase activity by inhibiting calcineurin which induces hypomagnesemia and other nephrotoxic effects. In this study, CsA treatment in rats caused a striking increase in the urinary excretion of AAP. This brush-border enzyme is very sensitive to small renal insults and its release occurs in early stages of proximal tubular epithelial cell nephrotoxicity. The lower urinary osmolality and high FE_{Mg} in CsA-treated animals suggest a CsA-mediated physiological defect in the mTAL. Magnesium supplementation prevented both the CsA induced tubular abnormalities in our present study. These results suggest a role of hypomagnesemia in the pathophysiology of CsA nephrotoxicity. Magnesium is an essential cation, involved in many enzymatic reactions, as a cofactor to adenosine triphosphatase [32]. It is critical in energy-requiring metabolic processes, as well as protein synthesis and anaerobic phosphorylation. Serum Mg concentration is maintained within a narrow range by the kidney and small intestine. In addition hypomagnesemia has been implicated in the pathophysiology of neurotoxicity and essential hypertension with CsA [5]. Hypomagnesemia is also common in transplant patients receiving CsA and has been proposed as an important factor in the pathogenesis of CsA nephrotoxicity [6]. Recent studies suggest that oxygen free radicals may be involved in tissue injuries produced by magnesium deficiency [33].

In this study, despite a lack of the ability to inhibit calcineurin,

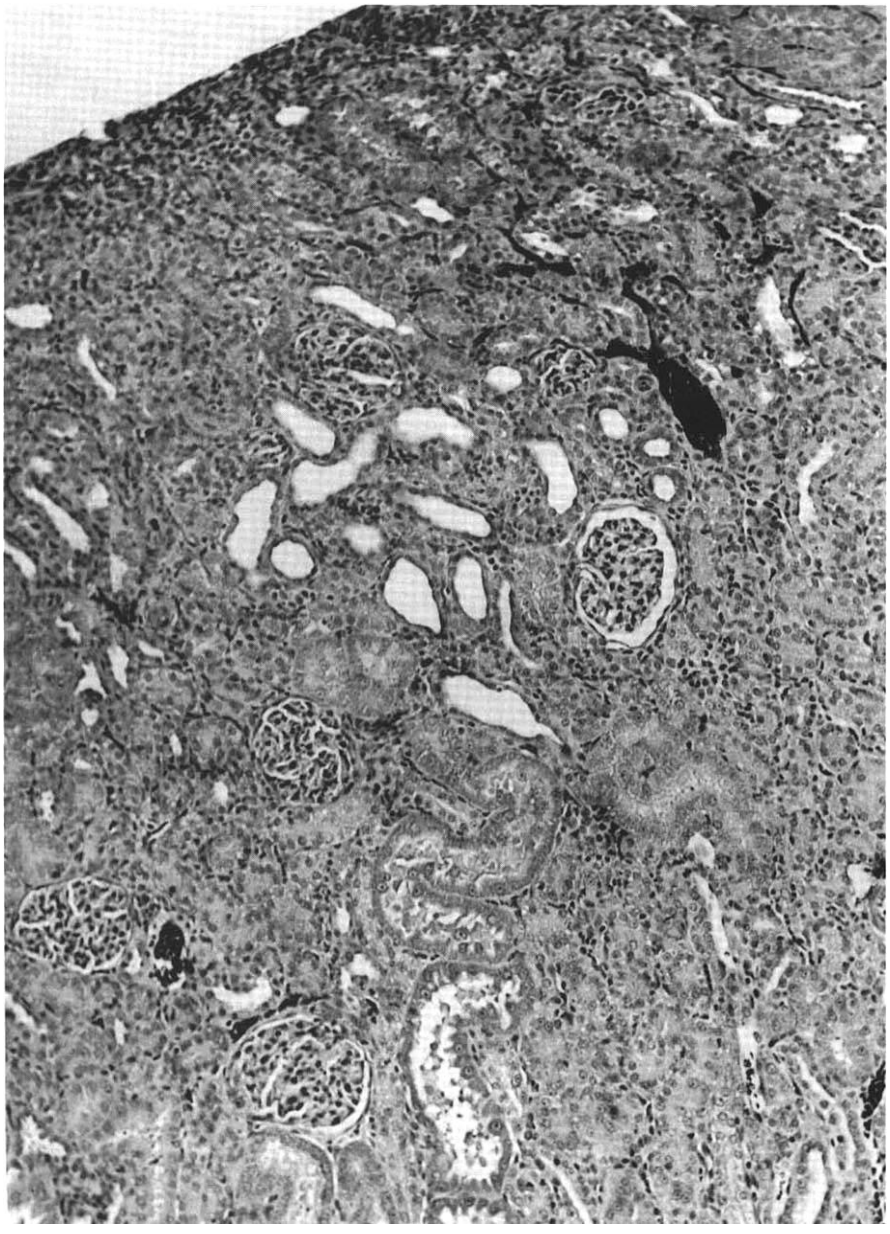

Fig. 1. *Histologic changes in experimental rapamycin nephrotoxicity in a sodium depleted rat.* Micrograph showing the renal cortex of a sodium depleted rat given rapamycin 3 mg/kg/day for two weeks. Most of the cortical tubules are collapsed. Only a few segments near the center of the field have normal caliber. Among the few tubules with patent lumina, most are lined by flattened epithelium. The interstitium contains small number of lymphocytes (Periodic-acid Schiff, ×200).

RAPA clearly induced hypomagnesemia associated with inappropriately high fractional excretion of magnesium, and histologic evidence of partial tubular collapse, vacuolization in proximal tubules and nephrocalcinosis that were similar to the changes observed in the CsA and FK treated animals. In this RAPA model, we found that the histologic changes were accompanied by tubular functional changes namely decreased medullary concentrating ability (U_{Osm} and TcW) and increased tubular enzymuria (AAP) and FE_{Mg}, while the kidney injury was not associated with decreased RBF nor GFR. Since all three drugs induced significant hypomagnesemia with inappropriately high FE_{Mg}, a single mechanism of tubular injury distinct from calcineurin inhibition is possible. The precise mechanism of hypomagnesemia and tubular injury induced by CsA, FK and RAPA remains to be elucidated, however, tubular injury can be dissociated from renal hemodynamic changes as has previously been shown for CsA [20]. The magnesiuria caused by CsA is thought to be associated with a physiological defect in mTAL, which is the major site for renal magnesium reabsorption [34]. In our study, when individual animals in all groups were compared by linear regression, a significant correlation could be demonstrated between the injury score and urinary osmolality, FE_{Mg}, plasma magnesium or urinary AAP. Thus, hypomagnesemia and the tubular injury are clearly associated, although cause and effect has yet to be proven.

The common belief is that the acute form of CsA injury is a consequence of CsA induced renal vasoconstriction [35]. According to this notion, the arteriolar vasoconstriction with resultant impairment in RBF leads to acute tubular injury. However, more recent studies have suggested that there can be a dissociation between the functional and histologic injuries induced by CsA.

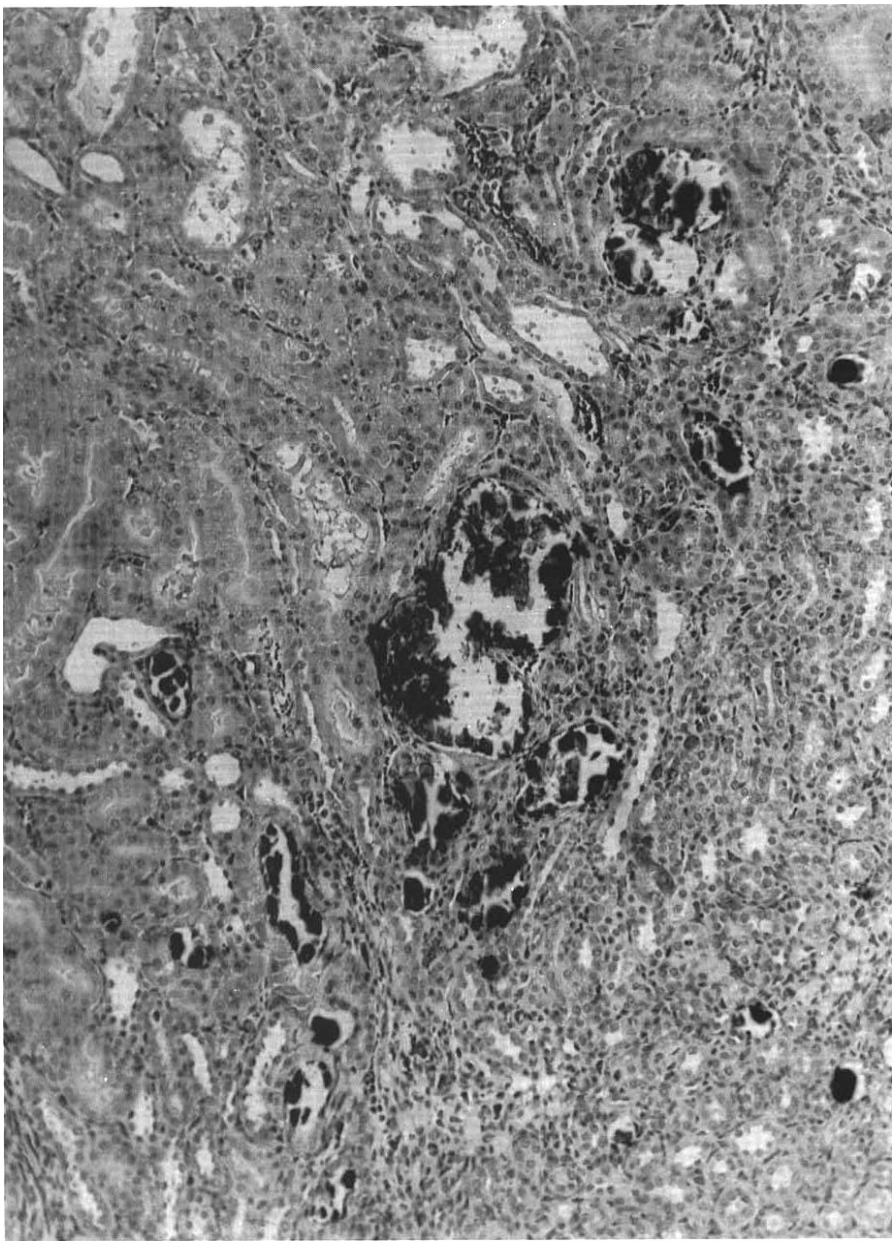

Fig. 2. *Day 14 rapamycin treated animal with typical nephrocalcinosis in juxtamedullary region, showing numerous intratubular calcifications (Periodic-acid Schiff, ×200).*

CsA withdrawal led to improved GFR but the degree of tubular atrophy and tubulointerstitial fibrosis actually progressed [36]. Blockade of the renin-angiotensin system with losartan or enalapril strikingly prevented CsA induced tubulointerstitial fibrosis without improving GFR [20]. These results suggest that the mechanism promoting structural injury in chronic CsA nephrotoxicity can be dissociated from those causing glomerular dysfunction. Although our RAPA model is an acute model, there may also be a dissociation between the functional and histological injuries induced by RAPA.

It is unclear why we found renal histopathologic lesions in animals treated with RAPA compared to previous reports. One factor may be the sodium-depletion during which the renin-angiotensin system is activated. Recently, several investigators used a reproducible animal model of chronic CsA and FK nephrotoxicity based on the observation that sodium depletion exacerbates CsA nephrotoxicity [37–40]. In this model, CsA treatment in rats on a low salt diet induced a significant decrease in GFR and histological changes similar to those described in patients on long-term CsA therapy. Our salt depletion model could be used to assess the presence and pathogenesis of any chronic RAPA induced nephrotoxicity.

One criticism of these animal models are the high doses of the drugs. The daily CsA dose given in our model (15 mg/kg) is higher than that employed in human transplantation (4 to 8 mg/kg) [41]. CsA blood levels achieved in this experiment (3250 ng/ml) are much higher than those the peak levels observed in humans (1000 ng/ml in the acute phase). However, high CsA doses and blood

levels are also necessary to overcome graft rejection in experimental rodent transplantation and thus the relative therapeutic window between efficacy and toxic side effects is similar to humans. The daily FK dose given in our model (6 mg/kg) is 20 times higher than that used in transplant patients (0.15 to 0.3 mg/kg), mainly because bioavailability of FK in rats is much lower than that of humans [42]. However, FK blood levels achieved in our study (10 ng/ml) are within the therapeutic window for humans (10 to 20 ng/ml). We could not determine RAPA blood levels due to technical difficulties. Since RAPA is homologous to FK in chemical structure and pharmacokinetic properties, it is assumed that RAPA blood levels are in the same range as FK. Obviously a simple transposition of the rodent dose-response relationship to humans is impossible.

In summary, we have demonstrated that CsA and FK strikingly decrease urinary excretion of nitric oxide, renal blood flow and GFR, whereas RAPA does not. In contrast, we have shown that all these three drugs cause significant hypomagnesemia associated with inappropriately high fractional excretion of magnesium, suggesting renal magnesium wasting, and lesions in the rat kidneys consisting of partial tubular collapse, vacuolization in proximal tubules and nephrocalcinosis accompanied by tubular functional changes namely decreased medullary concentrating ability (U_{Osm} and TcW) and increased tubular enzymuria (AAP). These studies suggest that, while these three immunosuppressants all induced hypomagnesemia associated with renal tubular injury, only the calcineurin inhibitors (CsA and FK) produced glomerular dysfunction in an acute experimental model of nephrotoxicity. The mechanism of hypomagnesemia and tubular injury induced by these immunosuppressive drugs is unclear but seems independent of calcineurin. On the other hand the mechanism of renal vasoconstriction might be related to inhibition of calcineurin phosphatase.

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