Thromboxane Receptor Blockade Attenuates Chronic Cyclosporine Nephrotoxicity and Improves Survival in Rats with Renal Isograft

Norberto Perico, Magda Rossini, Ornela Imberti, Barbara Malanchini, Raul Plata Cornejo, Flavio Gaspari, Tulio Bertani, and Giuseppe Remuzzi

ABSTRACT

The question of whether pharmacological inhibition of the thromboxane A2 activity prevents cyclosporine-induced chronic renal dysfunction in a Lewis rat model of renal isograft was addressed. Transplanted animals were given a daily oral dose of cyclosporine (20 mg/kg; N=15), cyclosporine (20 mg/kg) and the thromboxane A2 receptor antagonist GR32191 (3 mg/kg twice daily, by gavage; N=15), or the vehicle alone (N=12). Treatments were started the day of kidney transplant, and animals were monitored for 1 year. Cyclosporine-treated animals developed renal insufficiency, as documented by serum creatinine levels of 0.49 ± 0.09, 0.95 ± 0.12, and 1.38 ± 0.15 mg/dL before and after 6 and 12 months of observation, respectively. Cyclosporine and GR32191 used in combination partially but significantly prevented the deterioration of renal function (serum creatinine, basal, 0.52 ± 0.06; month 6, 0.68 ± 0.04; month 12, 0.93 ± 0.10 mg/dL). At the end of the study, GFR, as inulin clearance, was significantly lower in rats given cyclosporine (0.28 ± 0.09 mL/min/100 g) than in rats given cyclosporine plus GR32191 (0.45 ± 0.05 mL/min/100 g) or than in vehicle-treated animals (0.56 ± 0.07 mL/min/100 g). Similar results were obtained for the effective RPF, measured as p-aminohippurate clearance. At the same time points, comparable to whole-blood cyclosporine levels were found in rats receiving cyclosporine alone and in those given cyclosporine plus GR32191. More than 50% of the animals on cyclosporine alone died from uremia before the end of the observation period. By contrast, rats receiving cyclosporine in combination with GR32191 had a prolonged survival. These findings indicate that limiting the functional consequence of excessive thromboxane A2 synthesis in a rat isograft model prevents the long-term consequences of cyclosporine renal toxicity and increases the survival of transplanted animals.

Key Words: GFR, RPF, renal insufficiency

Cyclosporine (CsA) given to prevent rejection has probably contributed to the good results of the last 10 yr in transplantation programs (1–3). However, with increasing use, CsA side effects have been increasingly recognized (4). The most relevant complication of CsA is the renal toxicity (4–6), which is likely a consequence of its deleterious effect on renal vessels, as convincingly documented in experimental animals (7) and humans (8,9). By promoting renal vasoconstriction (7,8,10), CsA reduces RBF, which in turn causes a fall in GFR. Evidence has been accumulating to indicate that thromboxane A2 (TxA2)—a potent vasoconstrictor—participates in CsA-associated renal ischemia. Relevant to this concept are data that CsA increased the urinary excretion of TxA2 metabolites both in experimental animals (11–16) and in humans (17,18), whereas pharmacological inhibition of TxA2 synthase or TxA2 receptor blockade (19,20) prevented or reversed CsA-associated renal failure. Most studies involving pharmacological manipulation of the TxA2 pathway with the aim of reducing CsA-associated renal dysfunction ended with encouraging results (12,13,18–20). However, all of these were short-term studies and were performed in normal animals. Whether treatment with drugs that reduce the synthesis of TxA2 or prevent its binding to specific receptors might reduce CsA nephrotoxicity on long-term experiments has not been established so far. Here we took advantage of a model of renal grafting in the rat to answer the following questions: (1) whether pharmacological manipula-
tion of the TxA2 pathway prevents CsA-induced chronic renal dysfunction; (2) whether such an approach would improve animal survival.

METHODS

Male inbred Lewis rats [Charles River Italia S.p.A., Calco, Italy] weighing 200 to 250 g at the start of the experiment were used. Animals were housed in climate-controlled rooms on a 12-h light-dark cycle, were fed standard rat chow (Altromin-Rieper, Vandoeles, Italy), and had free access to tap water. This study was conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Design

Left kidneys from male Lewis rats were orthotopically isografted into rats of the same sex and strain as described below. All renal transplant rats were divided as follows: group 1 (N = 15) rats were given a daily oral dose of CsA (20 mg/kg) (Sandoz, Basel, Switzerland) by gavage for 12 months after renal transplantation; group 2 (N = 15) animals received a daily oral dose of CsA (20 mg/kg) in combination with TxA2 receptor antagonist GR32191 (Glaxo, Greenford, United Kingdom), 3 mg/kg twice daily by gavage for 12 months, starting the day of kidney transplantation; group 3 (N = 12) rats were given by gavage the vehicle (polyoxyethyleneated castor oil) in which CsA was dissolved. The dose of GR32191 was chosen on the basis of previous findings showing the efficacy of this drug in preventing CsA nephotoxicity in isografted rats over a short-term observation period (20). Moreover, at this dose, GR32191 was effective in rats in antagonizing the biological activity of a stable TxA2 mimetic, U-46619, on renal function (20, 21).

Blood samples were collected basally (before treatment) and at monthly intervals from the tail vein for determination of hematocrit, serum Na concentration, and serum creatinine level. At the same time intervals, 24-h urine samples were collected in metabolic cages for the measurement of urine output and urinary Na excretion. During the observation period, systolic blood pressure was measured every month in all rats by the tail cuff method (22).

At the end of the experimental period, renal clearance studies were carried out and GFR and RPF were determined in the three groups of rats. At the completion of the hemodynamic study, blood (2 mL) was collected from the femoral vein and CsA concentrations were measured by HPLC.

To evaluate whether the thromboxane receptor antagonist GR32191 may influence the pharmacokinetics of CsA, additional experiments were performed in normal male Sprague-Dawley rats. Animals were given CsA orally (group 4, 20 mg/kg/day; N = 6) or CsA plus GR32191 (group 5, 3 mg/kg twice daily; N = 6) for 30 days. At day 30, CsA pharmacokinetics were determined in the two groups of animals by collection of blood samples from the tail vein in EDTA-containing tubes at 0.5, 1, 2, 3, 4, 6, 10, 14, and 24 h after the last dose of CsA. Samples were stored at −20°C until HPLC analysis. As pharmacokinetic parameters, the area under the curve from time equals zero to 24 h [AUCl (0 → 24)], the average CsA concentration at the steady state (C∞), the maximum concentration (Cmax), and the time of maximum observed concentration (Tmax) of blood CsA were determined.

Kidney Transplantation

Rat renal transplants were performed as previously described (20, 23). Briefly, in anesthetized donor rats, the left kidney was carefully removed by transecting the ureter near the bladder junction, avoiding injury to the blood supply. Then, an aortic segment with the renal artery was cut and made into a carrel patch. Similarly, the renal vein was separated from the renal artery and transected near the inferior vena cava. The kidney was then placed in cold saline before transplantation. In the recipient, a left nephrectomy was performed. The donor kidney was orthotopically transplanted by end-to-end renal vein anastomosis. The renal artery was then anastomosed to the aorta with the small patch of donor aorta. A ureterovesical anastomosis was performed. Finally, the native right kidney of the recipient was removed. Ischemic time ranged from 20 to 30 min.

Renal Clearance Studies

Inulin and p-aminohippurate (PAH) clearance experiments were performed as previously described (12). Rats were anesthetized with thiopental sodium (50 mg/kg body wt; i.p.) and were placed on a constant-temperature table. After tracheostomy, a polyethylene catheter was inserted in the left femoral artery for blood sampling and for monitoring the arterial blood pressure by a Statham pressure transducer and a writing recorder (Battaglia Rangoni, Bologna, Italy). Catheters were also placed in the left femoral vein and in the bladder. A solution of 0.9% NaCl containing 5% inulin and 0.2% PAH was infused i.v. as a priming load, followed by a continuous infusion at the rate of 2 mL/h. After 40 to 50 min of equilibration, three timed clearance periods of about 30 min each were started. Urine was collected from the bladder, and blood samples were drawn at the midpoint of each clearance period to quantify inulin and PAH concentrations. At the conclusion of the experiments, the abdomen was opened and a blood
sample was carefully taken from the left renal vein for determination of PAH concentration and calculation of the PAH extraction ratio \( (E_{PAH}) \). Inulin concentrations in plasma and urine samples were determined by the resorcinol method (24). PAH concentration was determined by the method of Smith et al. (25). GFR was calculated as inulin clearance by the following formula:

\[
GFR = \frac{U_m \cdot V_u}{P_m}
\]

where \( U_m \) and \( P_m \) are urine and plasma inulin concentrations, respectively, and \( V_u \) is the urine flow rate. PAH clearance \( (C_{PAH}) \) was corrected with \( E_{PAH} \) to give true estimates of RPF. Renal vascular resistance was estimated by dividing mean arterial pressure by RBF, calculated as: \( C_{PAH} / [E_{PAH} \cdot (1 - \text{Hct})] \), where Hct is hematocrit.

**Analytical**

Renal function throughout the study was assessed by measuring serum creatinine concentration. Serum was obtained by tail vein puncture and then by leaving the collected blood samples at 37°C for 30 min. The sera were frozen and kept at -20°C until assayed by the alkaline picrate method (26). Serum and urinary Na concentration were measured by an ion-selective electrode (Beckman E4A® Electrolyte Analyzer; Beckman, Brea, CA). Urinary Na excretion was calculated from the standard formula.

For blood CsA measurement, the extraction procedure and HPLC analysis were performed by the method of Kahn et al. (27) with slight modifications. The assay was performed on a whole-blood sample, and cyclosporine D (1 µg) was the internal standard. Isocratic liquid chromatographic separation was carried out on HPLC by using a mobile phase of acetonitrile:methanol:water:ammonium sulphate at a proportion of 520:160:320:1, respectively. The \( C_{ss} \) was calculated by the formula:

\[
\text{AUC}_{ss}(0 \rightarrow 24) = C_{ss} \cdot 24
\]

where \( \text{AUC}_{ss} (0 \rightarrow 24) \) is the steady-state area under the curve by trapezoidal rule during the 24-h dosing interval.

**Histological Studies**

At the end of the 1-yr observation period, renal tissue specimens were fixed in Dubosq-Brazil fluid, dehydrated in alcohol, and embedded in paraffin. Sections (3 µm thick) were stained with Masson’s trichrome, hematoxylin-eosin, and periodic acid-Schiff reagent (PAS stain) and were examined by light microscopy. Sections including superficial and juxtamedullary glomeruli were evaluated. Glomerular, tubular, interstitial, and vascular changes were graded from 0 to 4+. All biopsies were analyzed by the same pathologist who was unaware of the experimental groups.

**Statistical Analysis**

All results are expressed as the mean ± SD. Data were analyzed by one-way or two-way analysis of variance as appropriate. Significance level of difference between individual group means, subjected to the analysis of variance, was established using Duncan or Tukey-Cicchetti test for multiple comparisons (28). For survival comparison, Fisher’s exact test was used. Comparison of pharmacokinetic parameters between the two groups of animals was performed with the two-tailed *t* test. Statistical significance level was defined as \( P < 0.05 \).

**RESULTS**

Animals failed to gain weight during the first week after kidney isograft. Thereafter, body weight gain was fairly comparable in CsA-treated rats, in animals given CsA and GR32191, or in those receiving vehicle alone (Figure 1).

Water and food intake, urinary Na concentration, and systolic blood pressure were comparable between the three groups of rats at all times during the study.

Similarly, there was no significant difference in urinary Na excretion in the three groups of animals, despite a tendency to increase in rats given CsA or CsA plus GR32191 starting 4 months after transplantation (CsA: basal, 2.39 ± 0.28; month 12, 2.52 ± 0.26 mEq/day; CsA plus GR32191: basal, 2.33 ± 0.36; month 12, 2.53 ± 0.27 mEq/day; vehicle: basal, 2.29 ± 0.30; month 12, 2.36 ± 0.18 mEq/day). Figure 2 shows renal function, measured as serum creatinine, in the three groups of rats over the entire experimental period. A progressive increase in serum

![Figure 1. Serial values for body weight in renal isograft rats treated with CsA, CsA plus GR32191 (CsA + GR32191), or vehicle alone for 1 yr after transplant. Values are means ± SD.](https://example.com/figure1.png)
creatinine as given with basal value was observed in rats given CsA alone, and the difference was statistically significant after 30 days of treatment (basal, 0.49 ± 0.09; day 30, 0.80 ± 0.07 mg/dL; P < 0.01). Then, serum creatinine progressively increased to the end of the experimental period (month 6, 0.95 ± 0.12; month 12, 1.38 ± 0.15 mg/dL). Rats given CsA plus GR32191 still had an increase in serum creatinine 30 days after surgery as compared with basal (0.52 ± 0.06 versus 0.61 ± 0.03 mg/dL; P < 0.05), but the increment was less than in animals given CsA alone (P < 0.01). Thereafter, serum creatinine increased slightly over the entire observation period (month 6, 0.68 ± 0.04; month 12, 0.93 ± 0.10 mg/dL). Rats given the vehicle alone did not show a significant change in serum creatinine values during the first 3 months after transplantation (basal, 0.50 ± 0.04; day 30, 0.51 ± 0.03 mg/dL). In these animals, a modest and age-related increase in serum creatinine was found throughout the study (month 12, 0.78 ± 0.06 mg/dL).

Figure 3 shows renal clearance studies performed at the end of the observation period in surviving animals. The group of animals given CsA had the lowest values of GFR (0.28 ± 0.09 mL/min/100 g) and RPF (1.30 ± 0.10 mL/min/100 g). The group on CsA plus GR32191 had significantly [P < 0.05] higher values of GFR (0.45 ± 0.05 mL/min/100 g) and RPF (1.75 ± 0.13 mL/min/100 g) as compared with the group of animals on CsA alone. However, the association of CsA and GR32191 did not normalize GFR and RPF values, which remained significantly lower as compared with those in the group of animals given the vehicle alone (GFR, 0.56 ± 0.07 mL/min/100 g; RPF: 2.23 ± 0.20 mL/min/100 g). The filtration fraction had a tendency to be lower in rats given CsA alone as compared with that in the other two groups, but the difference did not reach statistical significance (CsA, 0.21 ± 0.05; CsA plus GR32191, 0.26 ± 0.02; vehicle, 0.25 ± 0.01). Renal vascular resistance averaged 78.0 ± 6.0, 58.2 ± 4.5, and 45.7 ± 5.1 mm Hg/mL/min/100 g in animals given CsA. CsA plus GR32191, and vehicle, respectively.

At the end of 1-yr follow-up, blood levels of CsA were numerically higher in animals given CsA plus GR32191 (1.269 ± 179 ng/mL) than in rats receiving CsA alone (1.107 ± 214 ng/mL), but the difference did not reach statistical significance.

At the same time point in rats given CsA alone, light microscopy examination showed glomerular hypercellularity (1.25 ± 0.5) and expansion of the mesangial matrix (0.87 ± 0.25). Tubulointerstitial changes were also found characterized by tubular vacuolization (0.75 ± 0.28) and marked interstitial fibrosis (2.00 ± 1.15) associated with inflammatory reaction (1.75 ± 0.95). Significant vascular lesions that showed diffuse glomerular thrombosis were found in only one of these animals. By contrast, no significant changes were observed at the glomerular level in rats given CsA plus GR32191. In these animals, only mild tubular damage (0.75 ± 0.28) was present without significant glomerular, interstitial, or vascular lesions. All animals given vehicle alone showed only minimal tubular vacuoization.

Survival curves are shown in Figure 4. Eight of 15 rats of the CsA group died 4 to 10 months after surgery, whereas only 2 of 15 animals on CsA plus GR32191 died at 6 and 9 months after surgery. This difference was statistically significant (P < 0.05). No animal died in the vehicle group over the 1-yr obser-
CsA Nephrotoxicity in Renal Isograft Rats

Figure 4. Survival curves of renal isograft rats showing higher mortality for 15 rats on CsA as compared with the 15 rats on CsA plus GR32191 (CsA + GR32191), as a long-term administration.

Discussion

The results of this study indicate that rats undergoing renal isograft that were given CsA for 1 yr developed progressive renal insufficiency. In addition, more than 50% of the animals died from uremia before the end of the observation period. By contrast, rats receiving a TxA2 receptor blocking agent in association with CsA were significantly protected from renal insufficiency and had a prolonged survival.

These findings are in line with those of previous experimental (29–32) and human (8,33–35) data, which showed that protracted treatment with CsA is associated with chronic renal insufficiency. It has been suggested that CsA induces constriction and occlusion of the afferent arteriole with downstream glomerular damage that may be irreversible (8,9,36). Thus, tridimensional reconstruction of the glomerular tuft in heart graft recipients given CsA for more than 2 yr revealed that 40% of glomeruli were affected by global or segmental sclerosis (9).

In the last few years, considerable efforts have been devoted to understand the mechanisms by which CsA impairs RBF. The consistent and marked increase in urinary excretion of TxA2 metabolites (11–18) leads to the hypothesis that excessive TxA2 synthesis, likely from renal and nonrenal sources (14,16), plays a major role in CsA-induced renal ischemia. For that reason, pharmacological manipulations have been attempted to modulate the renal eicosanoid pathway in an attempt to ameliorate GFR (12,13,18–20). Most results obtained in rats treated chronically with CsA have been encouraging. A TxA2 synthase inhibitor prevented the increased urinary TxB2 and ameliorated GFR by 40% (12). In similar experiments, a TxA2 receptor antagonist ameliorated GFR and normalized RBF (19). K-MAP, an agent that increases the urinary prostaglandin I2 and reduces TxB2 urinary excretion, ameliorated RBF (37).

It has to be mentioned that in all of the above experiments CsA was given to normal animals. This makes it difficult to extrapolate the results of such studies to the human condition in which CsA is given after transplantation with the aim of preventing rejection. Ideally, one would like to see whether, in a model of organ grafting, TxA2 synthesis is increased as a consequence of the chronic use of CsA, and, if so, it would be important to provide evidence that pharmacological manipulation of the TxA2 pathway ameliorates GFR and improves animal survival. Such experiments are critically important with respect to renal transplantation, because renal ischemia, which is invariably associated with kidney graft surgery, acts synergistically with CsA in inducing and maintaining the ischemic injury (38). These experiments, however, cannot be performed in models of renal allograft because of the associated confounding factor represented by ongoing rejection. It has been known for many years that during rejection the inflammatory cells infiltrating the kidney may release considerable amount of TxA2, which is excreted in the urine (39–41).

A way to overcome the problem is to use a model of renal transplantation in syngenic animals. By such an approach, all of the phenomena related to the rejection are avoided, whereas the effect of ischemia associated with renal transplant surgery remains (42). The significant protection from the progressive fall in GFR obtained by combining CsA with the TxA2 receptor antagonist confirmed that TxA2 is a likely mediator of CsA-associated renal failure. In addition, these findings are consistent with data that
a TxA₂ synthase inhibitor acutely improved renal allograft function in patients who were taking CsA as a part of their immunosuppressive regimen and who had elevated serum creatinine and no clinical evidence of rejection (18).

Another relevant finding in animals with renal isograft receiving CsA for a prolonged period of time is the considerable mortality. Death in these rats is due to CsA-induced end-stage renal failure because all animals on vehicle alone survived to the end of the observation period. These results may offer a contribution to the controversial issue of whether the chronic nephropathy associated with CsA in humans with organ transplantation may lead to end-stage renal failure. Progressive renal failure occurs in cardiac allograft recipients given high-dose or low-dose CsA for more than 12 months (8), as documented by the development of end-stage renal disease in 5 of 73 patients. Similarly, 2 of 30 patients undergoing single- or double-lung transplantation and receiving CsA as a part of their immunosuppressive therapy progressed to end-stage renal failure and required dialytic therapy (43).

The beneficial effect of the TxA₂ receptor antagonist was not related to lowering blood CsA concentration. Indeed, at the end of a 1-yr observation period, the whole-blood trough level of CsA in animals on CsA and GR32191 was numerically higher than that in rats on CsA alone.

We conclude that the combination of CsA and TxA₂ receptor blocking given to animals with renal transplantation significantly reduces renal toxicity and improves renal survival as compared with that in animals given CsA alone.

ACKNOWLEDGMENTS

We thank Glaxo Ltd, Greenford Middlesex, United Kingdom, through the courtesy of Dr. A. McAllister for kindly providing GR32191. We are also indebted to Dr. Romer, Sandoz Ltd, Basel, Switzerland, for the gift of cyclosporine. This study was in part supported by CNR, Grant no. 90.00135.PF70, Progetto Finalizzato "Biotecnologie e Biosstrumentazione." M. Rossini and O. Iberti are recipients of fellowships from the Associazione Italiana Donatori Organi. Laura Piccoli helped prepare the manuscript.

REFERENCES


