Sirolimus increases transforming growth factor-β1 expression and potentiates chronic cyclosporine nephrotoxicity

FUAD S. SHIHAB, WILLIAM M. BENNETT, HONG YI, SEUNG-OK CHOI, and TAKESHI F. ANDOH

Division of Nephrology, University of Utah School of Medicine, Salt Lake City, Utah; and Legacy Solid Organ and Cellular Transplantation Services, Portland, Oregon

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**Background.** Sirolimus (SRL) is increasingly being used to decrease cyclosporine (CsA) exposure. SRL is not known to be nephrotoxic and has a mechanism of action distinct from CsA. We investigated the effect of combining CsA and SRL on renal structure and function and on transforming growth factor-β1 (TGF-β1) and extracellular matrix (ECM) proteins in a model of chronic CsA nephrotoxicity.

**Methods.** Rats treated with vehicle, SRL 0.3 mg/kg/day, CsA 5 or 10 mg/kg/day, or CsA5+SRL were sacrificed at 7 or 28 days. Physiologic and histologic changes were studied in addition to TGF-β1 mRNA and protein expressions, and mRNA expression of plasminogen activator inhibitor-1 (PAI-1) and ECM proteins biglycan and types I and IV collagen.

**Results.** While SRL alone did not alter renal function and structure, it potentiated the nephrotoxic actions of CsA when used in combination with low-dose CsA5 and resulted in significant changes similar to high-dose CsA10. In addition, SRL alone increased TGF-β1 by 44% to 49% (P < 0.05 vs. VH). When used in combination with low-dose CsA5, SRL potentiated TGF-β1 mRNA and protein by 121% and 176%, respectively (P < 0.05 vs. VH and CsA5), to levels achieved with high-dose CsA10. The expression of the ECM proteins followed that of TGF-β1; a similar effect was observed with PAI-1, suggesting a decrease in ECM degradation.

**Conclusion.** Because SRL augments nephrotoxicity, caution should be exercised when it is used in combination with CsA. More studies are needed to determine the long-term clinical impact of SRL on nephrotoxicity and allograft function.

Cyclosporine (CsA) has played an important role in improving renal allograft survival [1]. However, the major side effect of long-term CsA administration continues to be chronic nephrotoxicity [2]. Chronic nephrotoxicity contributes, to a large extent, to the development and progression of chronic allograft nephropathy, an unexplained lesion that can result in renal allograft failure [3]. Chronic CsA nephrotoxicity is characterized by a decrease in glomerular filtration rate (GFR), afferent arteriopathy, and striped tubulointerstitial fibrosis [4]. Our previous studies using an experimental model of chronic CsA nephrotoxicity have shown that transforming growth factor-β1 (TGF-β1) is involved in the fibrosis of this model by increasing extracellular matrix (ECM) synthesis, and decreasing ECM degradation through increasing the activity of plasminogen activator inhibitor-1 (PAI-1) [5–8]. Apoptosis plays a role in this model where the loss of cells may prevent the ability of the kidney to remodel effectively [9]. Sirolimus (SRL) is increasingly being used as a maintenance immunosuppressive agent with designs to decrease or eliminate CsA exposure [10]. SRL binds to FK-binding protein and inhibits the mammalian target of rapamycin (mTOR), a critical regulator of cell growth and survival [11]. The immunosuppressive actions of SRL are synergistic to those of CsA because SRL influences biochemical events later in the T-cell cycle [12]. Furthermore, SRL inhibits growth factor–driven proliferation of smooth muscle cells, fibroblasts, and endothelial cells, and reduces intimal hyperplasia in models of vascular injury [13–18]. These properties on vascular changes have led many to believe that SRL may be a beneficial addition in chronic lesions of the allograft.

SRL by itself does not seem to cause significant nephrotoxicity in most animal and human studies [19–23]. When used in the absence of CsA, SRL is able to spare glomerular filtration rate (GFR) in renal allografts; however, when combined with CsA, serum creatinine levels often increase [10, 24–26]. In addition, even when subtherapeutic doses of CsA and SRL are used in combination, a synergistic effect on the development of chronic nephrotoxicity becomes evident in the rat [27]. Recent studies have also shown that SRL given around the time of renal injury can augment injury and delay repair [27–31]. The mechanism is thought to be a result of enhanced necrosis, increased apoptosis, and decreased proliferation of renal...
tubular epithelial cells [29]. Clinically, this translates into delayed graft function after kidney transplantation in addition to a newly described lesion of cast nephropathy in renal allografts of patients treated with SRL [30, 31]. It is to be noted that, in addition to ischemia reperfusion injury, CsA is probably also involved in promoting an early acute injury after transplantation by causing potent renal vasoconstriction.

There is also recent evidence to suggest that SRL can independently increase TGF-β1 expression in lymphocytes and in renal proximal tubular epithelial cells [32–34]. Moreover, patients treated with SRL have an enhanced production of TGF-β1 and type III collagen early after kidney transplantation [35, 36]. Because TGF-β possesses profound immunomodulatory and anti-inflammatory functions in the appropriate cytokine milieu, this can explain some of the immunoregulatory properties of CsA and SRL. However, it remains unclear if the enhanced production of a fibrogenic cytokine such as TGF-β will carry long-term consequences on the kidney, particularly in recipients treated with a CsA-based regimen. In this paper, we used low doses of CsA in order to examine the impact of SRL coadministration on kidney structure and function in experimental chronic CsA nephrotoxicity and on the expression of TGF-β1, PAI-1, and some ECM proteins in the kidney.

METHODS

Experimental design

Adult male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) weighing 325 to 350 g were housed 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. Animals were pair-fed and weighed daily. After one week on a low-salt diet, weight-matched animals were assigned to one of five groups of 12 animals. Six animals in each group were sacrificed at 7 days (Groups A to E), and 6 were sacrificed at 28 days (Groups F to J). The experimental groups (N = 6/group) were: Groups A and F, vehicle (VH) control; Groups B and G, SRL 0.3 mg/kg/day; Groups C and H, CsA 5 mg/kg/day (CsA5); Groups D and I, CsA 5 mg/kg/day and SRL 0.3 mg/kg/day (CsA5 + SRL); and Groups E and J, CsA 10 mg/kg/day (CsA10). Systolic blood pressure was measured by tail plethysmography (Natsume Seisakusho Co., Ltd., Tokyo, Japan), and 24-hour urine samples were collected in metabolic cages (Nalge Company, Rochester, NY, USA). The following day, rats were anesthetized with intraperitoneal ketamine, the abdomen was opened through a midline incision, and the aorta was retrogradely cannulated below the renal arteries with an 18-gauge needle. With the aorta occluded by ligation above the renal arteries, and the renal veins opened by a small incision for outflow, the kidneys were perfused with 20 mL of cold heparinized saline. The left kidney was removed and processed for light microscopy. After removing the right kidney, the cortex was dissected from the medulla, and the cortex was processed for RNA and protein analysis. After the experiment, the animals were euthanized by deep anesthesia with ketamine followed by exsanguination.

Drugs

CsA (Novartis Research Institute, East Hanover, NJ, USA) was diluted in olive oil and administered subcutaneously (sc) at a dose of 5 or 10 mg/kg/day. The vehicle group received olive oil at 1 mL/kg/day sc. SRL, obtained from Wyeth-Ayerst Research (Princeton, NJ, USA), was dissolved using a dilution of Tween 80 (10%), N-N-dimethylacetamide (20%) and polyethylene glycol 400 (70%), and was given to animals sc at 0.3 mg/kg/day.

Functional studies

Blood was collected from the jugular vein in plastic syringes transferred to metal-free tubes and chilled on ice. Plasma was harvested immediately by centrifugation at 4°C and stored at −70°C until determined. Urinary and plasma creatinine, blood urea nitrogen, and glucose levels were measured by a Cobas autoanalyzer (Roche Diagnostics, Hoffman-La Roche, Inc., Nutley, NJ, USA). CsA blood level was measured by a fluorescence polarization immunoassay (Abbott Diagnostics, Abbott Park, IL, USA). SRL blood level was measured by a microparticle enzyme immunoassay (Abbott Diagnostics).

Histology

Tissue was fixed in 10% buffered formalin and in Methyl Carnoy’s solution, and then embedded in paraffin. Sections 2 to 4 μ thick were stained with periodic acid-Schiff reagent and trichrome stain, and evaluated by light microscopy by an observer masked to the treatment groups for tubular injury, interstitial fibrosis, and afferent arteriolopathy. Findings ascribed to tubular injury included cellular and intercellular vacuolization, tubular collapse, and tubular distention.

Interstitial fibrosis consisted of matrix expansion with tubular distortion and collapse and basement membrane thickening. Arteriolopathy was characterized by expansion of the cell cytoplasm of terminal arteriolar smooth muscle cells by cosinophilic, granular material. A color-image analyzer (Nikon E400; Nikon, Inc., Tokyo, Japan; Pixera Professional digital camera, Macintosh Powerbook G3, NIH Image vs. 1.5) was used for semiquantitative scoring. The extent of tubular injury in cortical tubules was graded using the following score: 0 = no tubular injury; 0.5 = <5% of tubules injured; 1 = 5% to 20%; 1.5 = 21% to 35%; 2 = 36% to 50%; 2.5 = 51% to 65%; and 3 = >65%. Interstitial fibrosis was estimated...
by counting the percentage of injured areas per fields of cortex and medulla with a minimum of 30 fields (magnification, ×200) reviewed using the following score: 0 = normal interstitium; 0.5 = <5% of areas injured; 1 = 5% to 20%; 1.5 = 21% to 35%; 2 = 36% to 50%; 2.5 = 51% to 65%; 3 = >65%. Afferent arteriopathy was estimated by counting the percentage of juxtamedullary afferent arterioles with arteriopathy per total afferent arterioles available for examination (magnification, ×200), with a minimum of 100 glomeruli per biopsy assessed. The following score was used: 0 = no arterioles injured; 0.5 = <15% of arterioles injured; 1 = 15% to 30%; 1.5 = 31% to 45%; 2 = 46% to 60%; 2.5 = 61% to 75%; 3 = >75%.

**Northern blot analysis**

Renal tissue was finely minced with a razor blade on ice then homogenized in TRIzol reagent (Gibco BRL, Grand Island, NY, USA). RNA extraction was performed according to the manufacturer’s protocol. After resuspension in Tris-EDTA buffer, RNA concentrations were determined using spectrophotometric readings at absorbance 290 nm. Thirty micrograms of RNA were electrophoresed in each lane in 0.9% agarose gels containing 2.2 mol/L formaldehyde and 0.2 mol/L MOPS (pH 7.0) and transferred to a nylon membrane (ICN Biomedicals, Costa Mesa, CA, USA) overnight by capillary blotting. Nucleic acids were crosslinked by ultraviolet irradiation (Stratagene, La Jolla, CA, USA). The membranes were prehybridized for 2 hours at 42°C with 50% formamide, 10% Denhardt’s solution, 0.1% sodium dodecyl sulfate (SDS), 5× standard saline citrate (SSC), and 200 μg/mL denatured salmon sperm DNA. Membranes were then hybridized at 42°C for 18 hours with cDNA probes labeled with 32P-dCTP by random oligonucleotide priming (Boehringer Mannheim, Mannheim, Germany). The blots were washed in 2× SSC, 0.1% SDS at room temperature for 15 minutes, and in 0.1× SSC, 0.1% SDS at 50°C for 15 minutes. Films were exposed at −70°C for different time periods to ensure linearity of densitometric values and exposure time. Autoradiographs were scanned on an imaging densitometer (GS-700; Bio-Rad Laboratories, Hercules, CA, USA). The density of bands for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to control for differences in the total amount of RNA loaded onto each gel line. For quantitative purposes, the values were divided by the density of bands for GAPDH in the same lane. The cDNA probes used for Northern were: a mouse TGF-β1 cDNA probe (plasmid MUI5); a rat PAI-1 cDNA probe (plasmid pBluescript SK(−)); a human biglycan cDNA probe (plasmid P16); a rat procollagen α1 cDNA (plasmid pα1R1); a rat collagen IV cDNA probe (plasmid pCIV-1-PE16) (American Type Culture Collection, Manassas, VA, USA); and a rat GAPDH cDNA probe (plasmid pBluescript KS II).

### Table 1. Physiologic changes in the experimental groups

<table>
<thead>
<tr>
<th>7 days</th>
<th>Body weight g</th>
<th>Systolic plasma BP mm Hg</th>
<th>Blood creatinine mg/dL</th>
<th>Urea plasma nitrogen mg/dL</th>
<th>Glucose level ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH</td>
<td>325 ± 8</td>
<td>125 ± 8</td>
<td>0.58 ± 0.01</td>
<td>14 ± 1</td>
<td>148 ± 13</td>
</tr>
<tr>
<td>SRL</td>
<td>310 ± 7</td>
<td>120 ± 7</td>
<td>0.59 ± 0.01</td>
<td>16 ± 2</td>
<td>133 ± 10</td>
</tr>
<tr>
<td>CsA5</td>
<td>322 ± 6</td>
<td>121 ± 8</td>
<td>0.58 ± 0.01</td>
<td>18 ± 3</td>
<td>171 ± 14</td>
</tr>
<tr>
<td>CsA5+</td>
<td>311 ± 6</td>
<td>122 ± 7</td>
<td>0.65 ± 0.04</td>
<td>21 ± 3</td>
<td>141 ± 15</td>
</tr>
<tr>
<td>SRL</td>
<td>313 ± 6</td>
<td>128 ± 9</td>
<td>0.60 ± 0.01</td>
<td>24 ± 3</td>
<td>199 ± 19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>28 days</th>
<th>VH</th>
<th>402 ± 10</th>
<th>129 ± 10</th>
<th>0.60 ± 0.01</th>
<th>15 ± 1</th>
<th>117 ± 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRL</td>
<td>377 ± 12</td>
<td>125 ± 8</td>
<td>0.61 ± 0.02</td>
<td>21 ± 2</td>
<td>102 ± 5</td>
<td></td>
</tr>
<tr>
<td>CsA5</td>
<td>385 ± 9</td>
<td>125 ± 7</td>
<td>0.69 ± 0.01</td>
<td>22 ± 2</td>
<td>172 ± 13</td>
<td></td>
</tr>
<tr>
<td>CsA5+</td>
<td>361 ± 10</td>
<td>121 ± 10</td>
<td>0.95 ± 0.05</td>
<td>x 38 ± 5</td>
<td>187 ± 36</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations are: VH, vehicle placebo; CsA5, cyclosporine 5 mg/kg/day; CsA10, CsA 10 mg/kg/day; SRL, sirolimus 0.3 mg/kg/day; BP, blood pressure.**

Values for weight gain, systolic blood pressure, plasma creatinine, blood urea nitrogen, and plasma glucose levels are summarized in Table 1. Weight gain was progressive.

### Immunoassay

Frozen (−70°C) kidney tissue sections embedded in Tissue-Tek O.C.T. compound (Miles, Inc. Diagnostics Division, Elkhart, IN, USA) were processed for protein extraction. Tissue was thawed at 4°C in 5 mL lysis buffer (GITC/BME), then rinsed 3 times in 5 mL cold 4°C phosphate-buffered saline (PBS), pH 7.4. Protein extracts were prepared by homogenizing kidney tissue in 1 mL cold PBS containing 0.05% Tween 20 (PBS-T) with a glass tissue homogenizer (Kontes Glass, Vineland, NJ, USA). The homogenate was centrifuged at 4°C for 15 minutes at 15,000g, and the supernatant was collected and then centrifuged to remove cellular debris. Protein concentration in the supernatant was determined using the Micro BCA (bicinchoninic acid) assay (Pierce, Rockford, IL, USA). Latent TGF-β1 was activated to immunoreactive TGF-β1 and was detected by a commercially available TGF-β1–specific sandwich enzyme-linked immunosorbent assay (ELISA) (QuantiKine; R&D Systems, Minneapolis, MN, USA). The manufacturer’s recommendations were followed for sample activation, reagent preparation, assay procedure, and calculation of results.

### Statistical analysis

Results were expressed as mean ± standard error. All statistical analyses were calculated with SYSTAT for Macintosh, version 5.2 (SYSTAT, Inc., Chicago, IL, USA). Comparisons between groups were done using analysis of variance (Tukey-Kramer analysis followed by Tukey’s test). A P value less than 0.05 was considered statistically significant.

### RESULTS

#### Physiologic studies

Values for weight gain, systolic blood pressure, plasma creatinine, blood urea nitrogen, and plasma glucose levels are summarized in Table 1. Weight gain was progressive.
atrophy (Trichrome, magnification ×400).

Table 2. Histologic semiquantitative scoring

<table>
<thead>
<tr>
<th></th>
<th>Tubular injury 0–3+</th>
<th>Interstitial fibrosis 0–3+</th>
<th>Arteriolopathy 0–3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>SRL</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CsA5</td>
<td>0.1 ± 0.1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CsA5+SRL</td>
<td>0.3 ± 0.2</td>
<td>0 ± 0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>CsA10</td>
<td>0.3 ± 0.2</td>
<td>0 ± 0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>28 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>SRL</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>CsA5</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>CsA5+SRL</td>
<td>1.4 ± 0.2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>1.8 ± 0.2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>1.9 ± 0.1&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CsA10</td>
<td>1.3 ± 0.2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>1.6 ± 0.2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>1.7 ± 0.2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations are: VH, vehicle placebo; CsA5, cyclosporine 5 mg/kg/day; CsA10, cyclosporine 10 mg/kg/day; SRL, sirolimus 0.3 mg/kg/day. Data are mean ± SEM of 6 animals. *P < 0.05 vs. VH; †P < 0.05 vs. SRL; ‡P < 0.05 vs. CsA5.

Fig. 1. Tubulointerstitial changes. Representative photomicrographs showing the renal cortex of a salt-depleted rat given vehicle (VH), sirolimus 0.3 mg/kg/day (SRL), cyclosporine 5 mg/kg/day (CsA5), cyclosporine 5 mg/kg/day + SRL (CsA5+SRL), or cyclosporine 10 mg/kg/day (CsA10) and sacrificed at 28 days. Rats treated with either VH or SRL alone had a normal renal histology. Rats treated with low-dose CsA5 had minimal tubulointerstitial changes that were not significant. However, rats treated with higher dose CsA10, or with combination of low-dose CsA5+SRL showed similar significant tubulointerstitial changes characterized by striped interstitial fibrosis and tubular atrophy (Trichrome, magnification ×200).

Fig. 2. Arteriolopathy changes. Representative photomicrographs showing the renal cortex of a salt-depleted rat given vehicle (VH), sirolimus 0.3 mg/kg/day (SRL), cyclosporine 5 mg/kg/day (CsA5), cyclosporine 5 mg/kg/day + SRL (CsA5+SRL), or cyclosporine 10 mg/kg/day (CsA10) and sacrificed at 28 days. Rats treated with either VH or SRL did not have any arteriolopathy. Rats treated with low-dose CsA5 had only minimal arteriolopathy changes that were not significant. However, rats treated with higher dose CsA10, or with combination of low-dose CsA5+SRL showed similar significant homogenous accumulation of eosinophilic material within the smooth muscle cells of afferent arterioles (Trichrome, magnification ×400).

in all treatment groups. Although SRL-treated rats did not gain weight at the same rate as others, there were no significant differences in body weight between the groups for the entire study period. Systolic blood pressure remained similar between groups, and hypertension was not observed in any group. Similar to VH, treatment with SRL did not affect kidney function. In addition, administration of low doses of CsA5 did not result in significant changes in plasma creatinine and blood urea nitrogen compared with VH- or SRL-treated rats. However, as expected, relatively large doses of CsA10 significantly increased plasma creatinine and blood urea nitrogen levels at 28 days (P < 0.05 vs. VH and SRL). When SRL was given with low-dose CsA5 in the CsA5+SRL group, significant increases in plasma creatinine and blood urea nitrogen levels were observed at 28 days versus CsA5-treated rats (P < 0.05), and the changes were similar to CsA10-treated rats.

At the currently utilized doses and route of administration, SRL and CsA5 did not result in changes in the plasma glucose levels. However, when CsA10 was administered, a significant increase in plasma glucose was observed at 28 days (P < 0.05 vs. VH).

Histologic changes

Scores for the histologic changes observed are summarized in Table 2. Representative photomicrographs are shown in Figures 1 and 2. VH-treated rats had normal kidney histology. Treatment with low-dose CsA 5 mg/kg/day resulted in mild histologic changes with scores for tubular injury, interstitial fibrosis, and arteriolopathy of
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0.5 ± 0.1, 0.5 ± 0.1, and 0.4 ± 0.1, respectively, that did not reach statistically significance compared to VH-treated rats. Similar not significant changes were also noted in SRL-treated rats. Treatment with either SRL or with low-dose CsA5 did not result in any significant tubular injury, interstitial fibrosis, or arteriolopathy. By contrast, at 28 days, kidneys of rats treated with CsA10 developed tubular atrophy, striped interstitial fibrosis, and renal vascular lesions characterized by hypertrophied smooth muscle cells of afferent arterioles with characteristic granular cosinophilic transformation. On the other hand, the addition of SRL to low-dose CsA5 resulted in significant histologic changes of tubular injury, interstitial fibrosis, and arteriolopathy, similar to the changes seen with the higher dose of CsA10.

**Pharmacokinetic studies**

Figure 3 summarizes the results of CsA pharmacokinetics with and without SRL administration after 7 days of drug administration. CsA blood trough levels (ng/mL) were similar in rats treated with CsA5 (1010 ± 90) and with CsA5+SRL (1050 ± 150). The area under the plasma concentration time curve (AUC0-24) was also similar in the CsA5 and CsA5+SRL-treated rats (23 ± 3 and 29 ± 4 mg.h/L, respectively). These results indicate the absence of significant pharmacokinetic interactions related to CsA when CsA and SRL are coadministered subcutaneously. On the other hand, as expected, the higher CsA10 doses resulted in significantly higher (P < 0.05) CsA blood trough levels (2150 ± 230) and AUC0-24 (41 ± 5). The pharmacokinetics of SRL after 7 days with and without CsA administration are summarized in Figure 4. Although SRL blood trough levels (ng/mL) were slightly higher in the CsA5+SRL group (12 ± 2) than the SRL group (9 ± 1), the difference did not achieve statistical significance. The AUC0-24 of SRL was also not statistically different between the SRL and CsA5+SRL groups; the values were 105 ± 11 and 121 ± 18 μg.h/L, respectively. These results further indicate the absence of significant pharmacokinetic interactions related to SRL when CsA and SRL are coadministered subcutaneously.

**Expression of TGF-β1**

Compared to VH rats, SRL administration resulted in significantly higher TGF-β1 mRNA and protein levels
(P < 0.05) in the kidney. At 7 and 28 days, SRL alone increased TGF-β1 mRNA by 50% and 44%, respectively, and protein levels by 34% and 49%, respectively. The effect of low-dose CsA5 on TGF-β1 expression was similar to SRL early at 7 days (53% for mRNA and 70% for protein, P < 0.05 vs. VH), but was significantly higher than SRL at 28 days (78% for mRNA and 140% for protein, P < 0.05 vs. VH and SRL). The higher dose of CsA10 resulted in even more marked increases of 82% to 125% for mRNA and 74% to 190% for protein (7 to 28 days, P < 0.05 vs. VH); these changes were significantly higher than either SRL alone or CsA5 (P < 0.05 at 28 days). However, when SRL was added to low-dose CsA5, the result was a potentiation in TGF-β1 mRNA and protein expression in the kidney to the levels observed with the higher doses of CsA10. These values were significantly higher than either SRL alone or CsA5 (P < 0.05 at 28 days). TGF-β1 mRNA expression in the kidney in the CsA5+SRL group was 79% to 121% higher, while that of TGF-β1 protein was 76% to 176% higher when compared to VH-treated rats. The results for TGF-β1 mRNA expression are shown in Figure 5, and those for TGF-β1 protein expression are shown in Figure 6.

Expression of PAI-1

The renal expression of PAI-1, a plasmin protease inhibitor that blocks ECM degradation and that is directly stimulated by TGF-β, is shown in Figure 7. PAI-1 mRNA expression paralleled that of TGF-β1 with significant increases in PAI-1 expression with CsA5 and more marked changes with the higher dose of CsA10 (P < 0.05 vs. VH groups). Treatment with SRL alone increased PAI-1 expression at 7 and 28 days to levels that remained lower than CsA5, but which were significantly higher than VH-treated rats (P < 0.05). The combination of CsA5+SRL resulted in significant changes in PAI-1 expression that equated CsA10 and that were significantly higher than either SRL or CsA5 treatment at both time points (P < 0.05 vs. SRL and vs. CsA5).
Expression of ECM proteins

The renal expression of biglycan and of types I and IV collagen by Northern blot analysis is shown in Figure 8. These ECM proteins are directly stimulated by TGF-β and progressively increased in the CsA groups (P < 0.05 vs. VH), suggesting active ECM synthesis, with the changes being more significant with the higher doses of CsA10. Of note is that SRL increased the mRNA expression of biglycan at 28 days and of type I collagen at 7 and 28 days, but had no effect on type IV collagen at both time points. The increases with SRL were not as pronounced as with CsA5 or CsA10 and were not accompanied by any significant histologic changes (see above). However, the addition of SRL to low-dose CsA resulted in increases in the mRNA expression of the above ECM proteins, although, unlike what was observed with TGF-β1 and PAI-1, the changes did not reach the CsA10 levels. These changes were mostly seen at 28 days and were most pronounced with type I collagen and to a lesser extent with biglycan (P < 0.05 vs. CsA5) and type IV collagen (P < 0.05 vs. SRL).

DISCUSSION

SRL has potent immunosuppressive effects that are distinct from calcineurin inhibitors [10–12]. SRL was shown to reduce the incidence of acute rejection in renal transplant recipients and to achieve powerful immunosuppression in several models of transplantation. Because SRL is not known to be nephrotoxic, a potential approach to decreasing chronic CsA nephrotoxicity is to utilize a lower dose of CsA with the synergistic immunosuppressant SRL. Some of the clinical studies on the use of SRL in the setting of CsA minimization have shown an improved serum creatinine and GFR with relatively short-term follow-ups [21–23, 37–39]. However, the effect of this drug combination on long-term graft survival remains unknown. In addition, there are no detailed chronic nephrotoxicity studies on the combination of CsA and SRL.

We utilized an animal model of chronic CsA nephrotoxicity in order to study the effect of low-dose CsA administered in combination with SRL on renal structure and function. In this study, we were able to show that SRL was not nephrotoxic when used alone. On the other hand, when CsA was used in doses previously shown to cause nephrotoxicity, significant changes in the kidney were seen, including a decrease in GFR, afferent arteriopathy, and striped tubulointerstitial fibrosis. These characteristic CsA-induced changes were not observed when lower doses of CsA (half the dose that produces nephrotoxicity) were administered. However, when combined with SRL, low-dose CsA resulted in changes in renal structure and function similar to the changes observed with full-dose CsA. In other words, SRL potentiated the known nephrotoxic potential of CsA in this model. These results confirm previous observations in which CsA and SRL were synergistic in producing nephrotoxicity [27]. In addition, a number of clinical studies noted that the use of SRL with CsA was associated with a rise in serum creatinine and a worsening in GFR. These results were initially unexpected from a drug that has antiproliferative effects. SRL inhibits the proliferation of smooth muscle cells, fibroblasts, and endothelial cells [13, 14]. SRL also reduces intimal hyperplasia in immune and nonimmune models of vascular injury, and halts or partially regresses established cardiac allograft disease in rodents and aortic allograft vascular disease in primates [14–18]. The properties of SRL on the vasculature were hypothesized to be beneficial in the setting of chronic CsA nephrotoxicity, and it is based on those observations that SRL was thought to allow the minimization of CsA exposure, and
thus, nephrotoxicity. However, in the setting of experimental chronic CsA nephrotoxicity, the expected beneficial effect of SRL on the vasculature was not observed because the addition of SRL to low-dose CsA potentiated the arteriolopathy and resulted in scores similar to high-dose CsA that are known to cause significant arteriolopathy.

One possible explanation for the effect of SRL on enhancing CsA nephrotoxicity is a pharmacokinetic interaction leading to increased CsA drug concentrations and,
as a result, more CsA renal exposure [25, 40, 41]. When used clinically, SRL is administered 4 hours after CsA in an attempt to reduce the interaction between the two drugs. This is particularly true when both drugs are given orally, but is not observed when they are coadministered subcutaneously [25, 27, 40, 41]. In this study where both drugs were delivered subcutaneously, we show that CsA blood trough levels and AUC were unaffected by SRL. However, a pharmacokinetic interaction that may result in increased CsA concentrations within the kidney tissue cannot be ruled out.

Another possible explanation is that SRL increases the production of the fibrogenic cytokine TGF-β. In this study, the administration of SRL alone, without CsA, was associated with inducing TGF-β1 production. TGF-β has potent immunosuppressive properties and may be a principal mediator of the immunosuppressive effects of SRL and calcineurin inhibitors. In the early phases of immune recognition, TGF-β may be beneficial in inhibiting immune activation and may have a significant effect on inducing tolerance [42]. However, the impact of a sustained up-regulation of TGF-β1 in the kidney remains a serious concern, especially when there is abundant data linking excessive TGF-β production with the development of fibrosis [43]. In this study, while therapy with SRL alone for 28 days did not result in significant fibrosis, a longer follow-up may have been needed. These findings also confirm previous observations, in which SRL addition in vitro was found to induce TGF-β production in lymphocytes, and more recently, in proximal renal tubular cells. There is yet another property that is common to both SRL and TGF-β, and that is inhibition of cell proliferation [13–18]. The antiproliferative effect of SRL has been attributed to the inhibition of signaling events involved in cell cycle progression. However, it seems reasonable to attribute some of this effect to the antiproliferative properties of TGF-β. The other major concern is that the use of SRL in combination with low doses of CsA was associated in this model with a significant augmentation of TGF-β1 production. The net result was a TGF-β1 expression that was similar to what was observed with full doses of CsA, an effect previously demonstrated in this model. Concomitant with the increase in TGF-β1 was also an up-regulation in the expression of biglycan and collagens to levels, thus indicating active ECM synthesis. In addition, PAI-1 was similarly increased, suggesting a decrease in ECM degradation.

In other words, the effect of SRL on low-dose CsA was to restore all the components in the machinery of fibrosis previously observed with full-dose CsA. These findings are supported by another study, in which SRL increased TGF-β expression in leukocytes and in which such increases were augmented significantly when combined with CsA [32]. A more recent study in 31 patients with biopsy-confirmed chronic allograft nephropathy showed that the addition of SRL after CsA dose reduction was not associated with a reduction in TGF-β1 or collagen type III expression in the renal allograft [36]. In another study in kidney transplant recipients maintained on a CsA-based regimen, fine needle aspirates of the kidney obtained 7 days after transplantation showed a significant up-regulation of TGF-β1 synthesis in the SRL group compared with the mycophenolate mofetil (MMF) group [35].

We have previously shown that apoptosis plays an important role in the development of chronic CsA nephrotoxicity [9]. CsA, by inducing the expression of a number of proapoptotic genes, impairs the ability of the kidney to remodel effectively in response to injury. A similar effect on apoptosis was also observed with SRL [29]. Whether this effect is mediated through TGF-β, a known inducer of apoptosis, remains unclear [44]. In the setting of experimental renal injury, SRL inhibits the proliferation and increases the apoptosis of renal tubular epithelial cells [29]. Furthermore, SRL appears to induce renal injury in experimental renal models of salt depletion and ischemia [20, 29]. As a result, the combined use of SRL and CsA may augment ischemia-induced renal injury and may inhibit tissue repair. This effect is involved in prolonging the recovery from delayed graft function and in the development of a cast nephropathy lesion [30, 31]. It is to be noted that the risk of enhanced injury and decreased repair may be relevant, not only in the immediate posttransplant period, but also in later events causing renal allograft injury, such as acute rejection or urinary tract obstruction.

In de novo renal allograft recipients, new trials have shown improvements in renal function in transplant recipients taking SRL after CsA elimination [22, 23]. Other studies have successfully adopted a similar scale approach in recipients with established chronic allograft nephropathy [45]. These results, in addition to the above evidence, suggest that future regimens that involve the introduction of SRL to CsA-treated patients should eliminate rather than reduce CsA, thus avoiding any potential interference between these agents. In addition, patients on a SRL plus CsA regimen should have more prolonged follow-ups in order to assess the impact of this drug combination on long-term renal allograft function and structure.

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Reprint requests to Fuad S. Shihab, M.D., Division of Nephrology, University of Utah Medical Center, 48312 School of Medicine, 30 N. 1900 E., Salt Lake City, UT 84132. 
E-mail: Fuad.Shihab@hsc.utah.edu
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