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Comparison of Early and Late Conversion of Sirolimus in Experimental Model of Chronic Cyclosporine Nephropathy

Jin Young Kim[1,*], Jung Yeon Ghee[1,*], Sun Woo Lim[1], Shang Guo Piao[1], Byung Ha Chung[1], Hye Eun Yoon[1], Hyeon Seok Hwang[1], Bum Soon Choi[1], Jin Kim[2], and Chul Woo Yang[1,2]

[1]Transplant Research Center, Convergent Research Consortium for Immunologic Disease; [2]Cell Death Disease Research Center, Department of Anatomy, The Catholic University of Korea, Seoul, Korea

*Jin Young Kim and Jung Yeon Ghee contributed equally to this work.

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Address for Correspondence:
Chul Woo Yang, MD
Department of Internal Medicine, Seoul St. Mary's Hospital, The Catholic University of Korea, 222 Banpodae-ro, Seocho-gu, Seoul 137-040, Korea
Tel: +82.2-590-2527, Fax: +82.2-536-0323
E-mail: yangch@catholic.ac.kr

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Sirolimus (SRL) is a promising drug for replacing calcineurin inhibitors. We performed this study to determine the optimal time of conversion from cyclosporine (CsA) to SRL in an experimental model of chronic CsA nephropathy. Three separate studies were performed. In the first study, SRL was given to rats with or without CsA for 4 weeks. In the second study, rats were treated initially with CsA for 1 week, and then switched to SRL (early conversion). In the third study, CsA was given for 4 weeks and then replaced by SRL for 4 weeks treatment of CsA (late conversion). The influence of SRL on CsA-induced renal injury was evaluated by assessing renal function, histopathology (interstitial inflammation and fibrosis), and apoptotic cell death. Combined CsA and SRL treatment significantly impaired renal function, increased apoptosis, and interstitial fibrosis and inflammation compared with CsA or SRL treatment alone. Early conversion to SRL did not change renal function, histopathology, or apoptosis compared with early CsA withdrawal. By contrast, late conversion to SRL significantly aggravated these parameters compared with late CsA withdrawal. In conclusion, early conversion from CsA to SRL is effective in preventing CsA-induced renal injury in a setting of CsA-induced renal injury.

Key Words: Cyclosporine; Conversion; Nephrotoxicity; Sirolimus

INTRODUCTION

Sirolimus (SRL) is a promising maintenance immunosuppressive agent for replacing calcineurin inhibitors (CNI) (1-4). SRL itself does not cause severe nephrotoxicity, but combined SRL and CNI treatment causes significant nephrotoxicity in most animal and human studies (4-8). There is an emerging consensus that conversion from a CNI to SRL is an effective strategy for reducing CNI-induced nephrotoxicity.

Clinical trials of SRL show that early conversion to SRL is effective in preserving graft function and that late conversion in recipients with poor graft function or proteinuria does not provide beneficial effects on graft function (9, 10). Therefore, early conversion to SRL after transplantation is recommended, but the optimal time for conversion has not been determined. It is also unclear whether the undesirable effects of late conversion to SRL are related to the preexisting CNI-induced renal injury or to the effect of SRL itself.

In this study, we focused on the influence of SRL on Cyclosporine (CsA)-induced renal injury. To evaluate the optimal time for conversion, we compared the influence of early and late conversion from CsA to SRL on CsA-induced renal injury in an experimental model of chronic CsA-nephrotoxicity. The results of our study show clearly that early conversion from CsA to SRL is effective in preventing CsA-induced renal injury in an experimental setting of CsA-induced renal injury.

MATERIALS AND METHODS

Animals and drugs

The institutional animal care and use committee of the Catholic University of Korea approved the experimental protocol (CUMC-2008-0075-01), and all procedures performed in this study followed the ethical guidelines for animal studies. Male Sprague Dawley rats (Charles River Technology, Seoul, Korea), initially weighing 230 to 250 g, were housed in cages (Nalge Co., Rochester, NY, USA) in a controlled-temperature and controlled-light environment, and were allowed free access to a low salt diet (0.05% sodium, Teklad Premier, Madison, WI, USA) throughout the experimental period. After starting the treatment, the rats were pair-fed and daily body weight (BW) was monitored. Animals were anesthetized with ketamine. CsA (Novartis Pharma

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Ltd, Basel, Switzerland) was diluted in olive oil (Sigma Co., St Louis, MO, USA) to a final concentration of 15 mg/mL. SRL (Wyeth-Ayerst Research, Princeton, NJ, USA) was dissolved in solution of Tween 80 (10%), N, N-dimethylacetamide (20%), and polyethylene glycol 400 (70%), to a final concentration of 0.3 mg/mL.

Experimental design

Three separate experiments were performed, as shown Fig. 1.

Experiment I: This experiment was designed to evaluate the influence of the combined treatment of SRL and CsA on CsA-induced nephrotoxicity.

Rats were randomized to four groups and treated for 4 weeks.

1) Vehicle group (VH4, n = 7): rats received a daily subcutaneous injection of olive oil (1 mL/kg) for 4 weeks.
2) VH4 + SRL4 group (n = 7): rats received a daily subcutaneous injection of olive oil and SRL (0.3 mg/kg) for 4 weeks.
3) CsA4 group (n = 7): rats received a daily subcutaneous injection of CsA (15 mg/kg) for 4 weeks.
4) CsA4 + SRL4 group (n = 7): rats received a daily subcutaneous injection of CsA and SRL (0.3 mg/kg) for 4 weeks.

Experiment II: This experiment was designed to evaluate the effect of early conversion from CsA to SLR on CsA-induced nephrotoxicity.

Rats were randomized to four groups and treated for 4 weeks.

1) VH1 + W3 group (n = 7): rats received olive oil for 1 week, after which olive oil was withheld for 3 weeks.
2) VH1 + SRL3 group (n = 7): rats received VH for 1 week and then SRL for 3 weeks.
3) CsA1 + W3 group (n = 7): rats received CsA for 1 week, after which CsA was withheld for 3 weeks.
4) CsA1 + SRL3 group (n = 7): rats received CsA for 1 week and then SRL for 3 weeks.

Experiment III: This experiment was designed to evaluate the effect of late conversion from CsA to SRL in established chronic CsA nephropathy.

Rats were randomized to four groups and treated for 4 or 8 weeks:

1) VH4 + W4 group (n = 7): rats received a daily subcutaneous injection of olive oil for 4 weeks, after which olive oil was withheld for 4 weeks.
2) VH4 + SRL4 group (n = 7): rats received a daily subcutaneous injection of olive oil for 4 weeks, and then SRL for 4 weeks.
3) CsA4 + W4 group (n = 7): rats received a daily subcutaneous injection of CsA for 4 weeks, after which the CsA was withheld for 4 weeks.
4) CsA4 + SRL4 group (n = 7): rats received a daily subcutaneous injection of CsA for 4 weeks, and then SRL for 4 weeks.

The doses and duration of administration of the vehicle (Olive

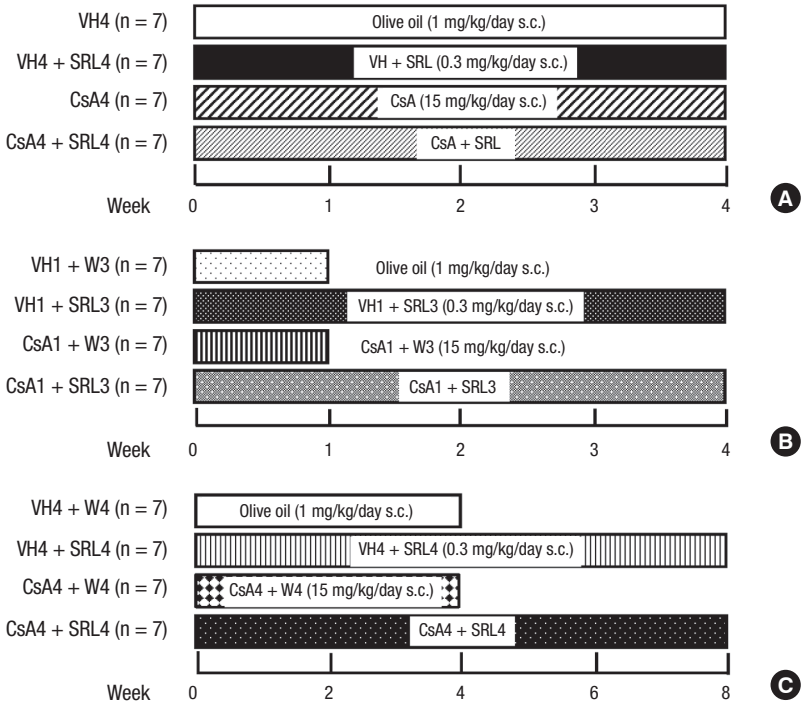

Fig. 1. Experimental design. Three separate studies were performed. (**A**) Combined sirolimus and cyclosporine, (**B**) early conversion, and (**C**) late conversion. S.C., subcutaneous injection; VH, vehicle.

oil; 1 mL/kg), CsA (15 mg/kg), and SRL (0.3 mg/kg) were chosen based on previous reports (5, 11, 12).

Measurement of renal function and whole blood CsA and SRL levels

Serum creatinine (SCr) concentration was measured using a Cobas autoanalyzer (Roche Diagnotics, Division of Hoffman-La Roche Inc., Nutley, NJ, USA). Whole-blood CsA level was measured using a monoclonal radioimmunoassay (INSCTART Corp., Stillwater, MN, USA), and whole-blood SRL concentration was measured using a microparticle enzyme immunoassay (Abbott Diagnostics, Abbott Park, IL, USA).

Preservation of kidney

The kidneys were preserved by in vivo perfusion through the abdominal aorta. In brief, the animals were perfused with 0.01 M phosphate-buffered saline (PBS) to wash out the blood. The left kidney was removed for immunoblotting analysis or RNA extraction and the right kidney was removed after perfusion with the periodate–lysine–paraformaldehyde (PLP) solution for 4 min. The kidneys were removed and cut into sagittal slices of 1-2 mm thickness and post-fixed overnight in PLP solution at 4°C. A part of the PLP-fixed tissues was embedded in wax for trichrome staining. After dewaxing, 4-μm sections were processed and stained with Masson's trichrome stain.

Measurement of interstitial fibrosis

To assess interstitial fibrosis, kidney paraffin sections were stained with Masson's trichrome stain. Tubulointerstitial fibrosis (TIF) was identified using the definition described previously (13). Briefly, a minimum of 20 fields per section was assessed using a color image analyzer (TDI Scope EyeTM Version 3.0 for Windows, Olympus, Tokyo, Japan). Randomly selected cortical fields of sections were analyzed histopathologically by two pathologists blinded to the identity of the treatment groups.

Immunohistochemistry of osteopontin (OPN) and ED-1

After dewaxing, sections were incubated with 0.5% Triton X 100/PBS solution and washed with PBS, and then incubated for 2 hr at 4°C in mouse antiserum against OPN (MPIIIB10, obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) and ED-1 (Serotec Inc., Oxford, UK). The number of ED-1-positive cells was quantified per 0.5 mm^2 area of rat kidney using a computer program (TDI Scope Eye Version 3.0 for Windows, Olympus). A minimum of 20 fields per section were assessed.

Immunoblotting of active caspase-3

Western blot analysis was performed as described previously (13). Active caspase-3 was detected by incubating for 1 hr with a specific antibody (Chemicon International, Inc., MA, USA). Antibody-reactive protein was detected using enhanced chemiluminescence (ECL, Amersham Biosciences, Little Chalfont, UK). Optical density was measured using the VH group as 100% reference and normalized by β-actin.

Northern blot analysis of OPN

Northern blotting was performed as described previously by our laboratory (11). The densitometric analysis was performed using the NIH ImagePC program for three determinations for each band, and the results were corrected to 18S.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Cells undergoing apoptosis were identified by the ApopTag in situ apoptosis detection kit (Oncor Inc., Gaithersburg, MD, USA). The number of TUNEL-positive cells was counted in 20 different fields in each section under × 200 magnification (13, 14).

Statistical analysis

The data are expressed as mean ± SEM. Multiple comparisons between groups were performed using one-way analysis of variance followed by Bonferroni post hoc testing (SPSS software version 9.0). Significance was assumed as $P < 0.05$.

RESULTS

Effect of combined treatment with SRL and CsA on chronic CsA nephropathy

Table 1 shows the basic parameters for the first experimental group. After 4 weeks, the CsA group showed deterioration of renal function, as shown by an increase in SCr concentration com-

Table 1. Effect of combined CsA and SRL treatment on basic parameters

Parameters	VH4	VH4 + SRL4	CsA4	CsA4 + SRL4
BW (g)	327 ± 6	284 ± 4	314 ± 7	228 ± 4†
SCr conc (mg/dL)	0.51 ± 0.09	0.44 ± 0.13	0.98 ± 0.03*	1.21 ± 0.04*,†
Water intake (mL/day)	16 ± 2	27 ± 3*	14 ± 2	59 ± 6†
Urine volume (mL/day)	14 ± 4	28 ± 4*	15 ± 2	60 ± 7†
CsA conc (ng/mL)	-	-	2047 ± 63	1989 ± 69
SRL conc (ng/mL)	-	12.2 ± 1.1	-	14.2 ± 1.6

Values are means ± SE. *$P < 0.01$ vs VH; †$P < 0.05$ vs CsA group. BW, body weight; SCr conc, serum creatinine concentration; CsA conc, cyclosporine concentration. SRL conc, sirolimus concentration.

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pared with the VH4 and VH4 + SRL4 groups ($P < 0.05$). As expected, the combined CsA and SRL treatment significantly impaired renal function compared with the other groups ($P < 0.05$).

Kidney tissues from CsA-treated rats had typical striped interstitial fibrosis. Tissues from rats treated with combined CsA4 and SRL4 showed more interstitial fibrosis (39 ± 4/0.5 mm^2) com-

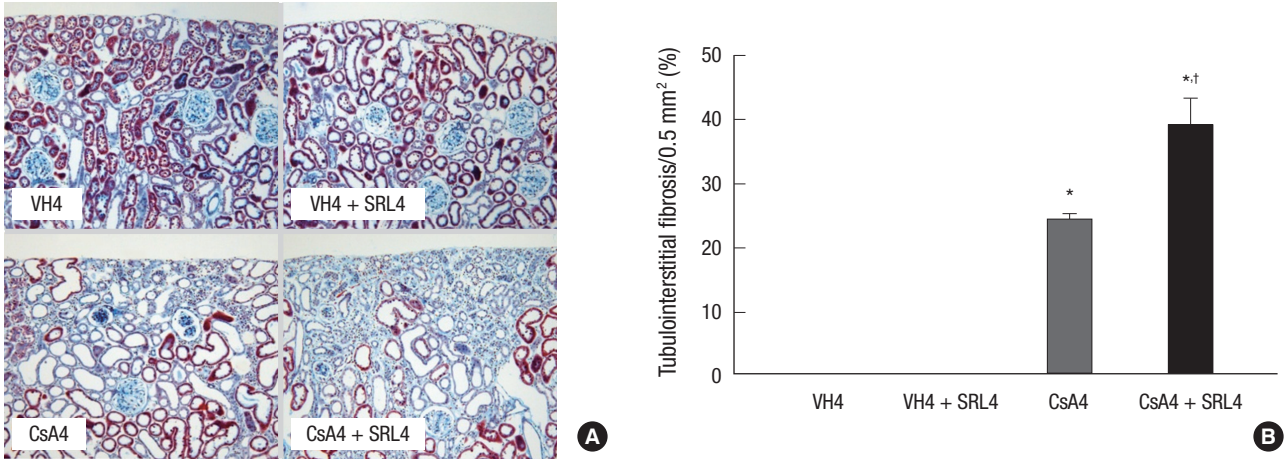

Fig. 2. Influence of combined treatment of SRL and CsA on interstitial fibrosis in chronic CsA nephropathy. (**A**) Trichrome staining. The CsA group shows typical striped interstitial fibrosis in the cortex whereas the VH4 and VH4 + SRL4 groups does not show any change. The CsA4 + SRL4 group shows further interstitial fibrosis compared with the CsA4 and VH4 + SRL4 groups (original magnification, × 200). (**B**) Quantitative analysis of TIF. Note the markedly greater interstitial fibrosis in the CsA4 + SRL4 group compared with the CsA4 and VH4 + SRL4 groups. *$P < 0.01$ vs VH4 or VH4 + SRL4 groups; †$P < 0.01$ vs CsA4 group.

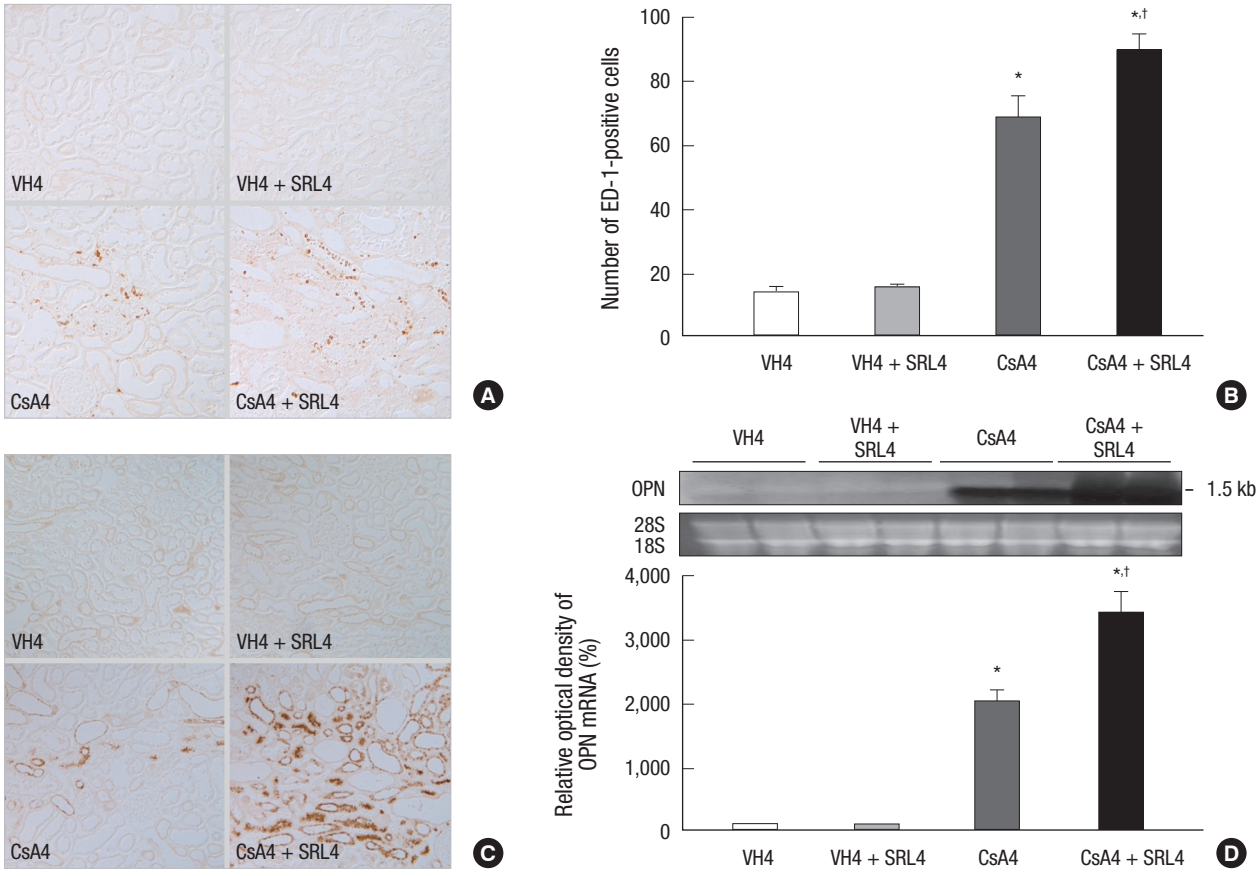

Fig. 3. Influence of combined SRL and CsA treatment on interstitial inflammation in chronic CsA nephropathy. (**A**) ED-1 immunohistochemistry. (**B**) Quantitative analysis of ED-1 positive cells. Note the significantly higher number of ED-1-positive cells in the CsA4 + SRL4 group than the CsA4 group. *$P < 0.01$ vs VH4 or VH4 + SRL4 groups; †$P < 0.05$ vs CsA4 group. (**C**) Immunohistochemistry of OPN protein. (**D**) Northern blot analysis for osteopontin (OPN) mRNA. Note the greater OPN mRNA expression in the CsA4 + SRL4 group compared with the CsA4 and VH4 + SRL4 groups. The data are presented as relative optical density with the VH4 group designated as 100% reference and are normalized to 18S. *$P < 0.01$ vs VH4 or VH4 + SRL4 groups; †$P < 0.05$ vs CsA4 group.

pared with tissues from rats treated with SRL alone (0.1 ± 0.04/0.5 mm^2) and CsA alone (24 ± 0.8/0.5 mm^2) (P < 0.01, Fig. 2).

We used immunohistochemistry to stain for ED-1 to detect macrophage infiltration in this chronic CsA nephropathy model (13, 14). As shown in Fig. 3A, ED-1-positive cells were observed rarely in the VH4 and VH4 + SRL4 groups. More ED-1-positive cells (68 ± 7/mm^2) were observed in kidneys from the CsA4 group than from the VH4 group (14 ± 2/mm^2), and VH4 + SRL4 group (15 ± 1/mm^2) (P < 0.01). The highest number of ED-1-positive cells was observed in the CsA4 + SRL4 group compared with CsA4 group (89 ± 5/mm^2, P < 0.05).

We used Northern blot analysis to measure the mRNA expression for OPN, a proinflammatory cytokine in animal models of CsA-induced renal injury (15, 16). OPN mRNA was expressed minimally in the kidneys of the VH4 and VH4 + SRL4 groups. The expression of OPN mRNA was significantly higher in the CsA4 group (1,987% ± 179%) than in the VH4 group (100% ± 0.4%) and VH4 + SRL4 group (103% ± 3%) (P < 0.01 for the CsA4 group compared with other two groups). OPN mRNA expression was much higher in the CsA4 + SRL4 group (3,315% ± 361%) than in the VH4, VH4 + SRL4 (P < 0.01), and CsA4 groups (P < 0.05, Fig. 3D). OPN protein expression followed a similar pattern (Fig. 3C).

We used TUNEL staining and active caspase-3 expression for evaluation of apoptotic cell death, one of the mechanisms involved in the injury and repair process in the chronic CsA nephrotoxicity model (17). Treatment with VH or SRL did not affect TUNEL-positive cells, which were observed rarely in the VH4 and VH4 + SRL4 groups (11 ± 0.7/0.5 mm^2 and 12 ± 0.5/0.5 mm^2, respectively). However, the number of TUNEL-positive cells was higher in the CsA4 group (54 ± 2/0.5 mm^2, P < 0.01 vs VH4 or VH4 + SRL4 groups), and even higher in the CsA4 + SRL4 group. (84 ± 2/0.5 mm^2, P < 0.01 vs VH4 or VH4 + SRL4; P < 0.05 vs CsA4, Fig. 4A, B), Immunoblotting analysis of the kidney showed a significant increase in active caspase-3 in the CsA4 group (3,541% ± 473%) compared with the VH4 (103% ± 11%) and VH4 + SRL4 group (105% ± 9%) (P < 0.01 for the CsA4 group compared with

Table 2. Effect of early conversion to SRL on basic parameters

Parameters	VH1 + W3	VH1 + SRL3	CsA1 + W3	CsA1 + SRL3
BW (g)	280 ± 3	272 ± 2	277 ± 7	253 ± 5*,†
SCr conc (mg/dL)	0.48 ± 0.09	0.44 ± 0.09	0.50 ± 0.03	0.53 ± 0.04
Water intake (mL/day)	15 ± 2	27 ± 3	14 ± 2	39 ± 6*,†
Urine volume (mL/day)	11 ± 3	16 ± 1	11 ± 2	36 ± 4*,†
SRL conc (ng/mL)	-	9.0 ± 2.2	-	11.2 ± 1.2

Values are means ± SE. *P < 0.01 vs VH1 + W3 or CsA1 + W3 group; †P < 0.05 vs VH1 + SRL3 group. BW, body weight; SCr conc, serum creatinine concentration; SRL conc, sirolimus concentration.

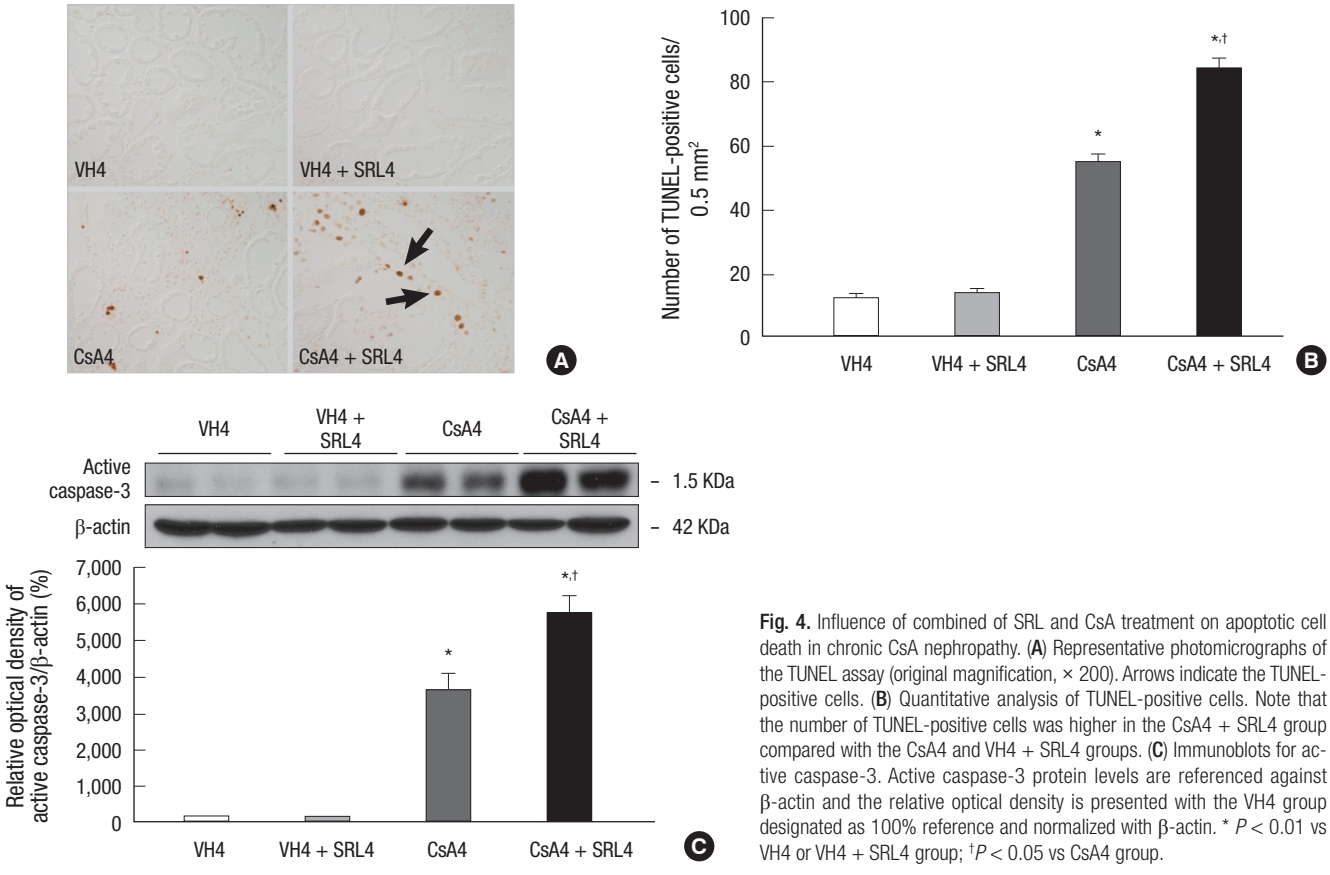

Fig. 4. Influence of combined of SRL and CsA treatment on apoptotic cell death in chronic CsA nephropathy. (**A**) Representative photomicrographs of the TUNEL assay (original magnification, × 200). Arrows indicate the TUNEL-positive cells. (**B**) Quantitative analysis of TUNEL-positive cells. Note that the number of TUNEL-positive cells was higher in the CsA4 + SRL4 group compared with the CsA4 and VH4 + SRL4 groups. (**C**) Immunoblots for active caspase-3. Active caspase-3 protein levels are referenced against β-actin and the relative optical density is presented with the VH4 group designated as 100% reference and normalized with β-actin. * P < 0.01 vs VH4 or VH4 + SRL4 group; †P < 0.05 vs CsA4 group.

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the other two groups). The expression of active caspase-3 was upregulated more in the CsA4 + SRL4 group (5,625% ± 473%) than in the CsA4 group ($P < 0.05$, Fig. 4C).

Effect of early conversion on CsA-induced renal injury

Table 2 shows the effect of early conversion from CsA to SRL on CsA-induced renal injury. Renal function did not differ between CsA early withdrawal (CsA1 + W3 group) and early conversion (CsA1 + SRL3) groups. As shown in Fig. 5, the number of ED-1 positive cells, OPN mRNA and TIF score did not differ between CsA1 + W3 and CsA1 + SRL3 groups. Fig. 6 shows the number of TUNEL-positive cells and active caspase-3 expression in each experimental group. As expected, the number of TUNEL-positive cells and active caspase-3 expression did not differ significantly between CsA1 + W3 and CsA1 + SRL3 groups.

Effect of late conversion on progression of chronic CsA nephropathy

Table 3 shows the effect of late conversion from CsA to SRL on the progression of chronic CsA nephropathy. Renal function was normalized after CsA withdrawal (CsA4 + W4 group), but late conversion of SRL (CsA4 + SRL4 group) caused deterioration of renal function as compared with the other groups ($P < 0.05$).

Fig. 7 shows the number of ED-1-positive cells in the experimental groups. The number of ED-1-positive cells was significantly higher in the CsA4 + W4 group (10 ± 1/0.5 mm^2) than the VH4 + W4 group (0.6 ± 0.1/0.5 mm^2) and VH4 + SRL4 group (0.6 ± 0.1/0.5 mm^2) ($P < 0.01$ for CsA4 + W4 compared with other two groups). Switching CsA to SRL after 4 weeks increased the number of ED-1- positive cells to 32 ± 5/0.5 mm^2 ($P < 0.01$). Northern blot analysis showed a significantly greater OPN mRNA expression in the CsA4 + W4 group (151% ± 17%) compared with the VH4 + W4 (128% ± 28%) and VH4 + SRL4 groups (89% ± 35%) ($P < 0.01$ for the CsA4 + W4 compared with the two other groups). OPN mRNA expression was the highest in the late conversion from CsA to SRL (CsA4 + SRL4) group (691% ± 510%, $P < 0.01$

Table 3. Effect of late conversion of SRL on basic parameters

Parameters	VH4 + W4	VH4 + SRL4	CsA4 + W4	CsA4 + SRL4
BW (g)	287 ± 10	273 ± 10	287 ± 10	280 ± 4
SCr conc (mg/dL)	0.69 ± 0.08	0.65 ± 0.04	0.41 ± 0.05	0.85 ± 0.38*
Water intake (mL/day)	22.6 ± 4.8	21.7 ± 5.4	23.7 ± 5.6	24.5 ± 3.1
Urine volume (mL/day)	13.3 ± 2.5	13.0 ± 4.2	11.7 ± 2.0	12.3 ± 1.9
SRL conc (ng/mL)	-	9.6 ± 1.2	-	7.2 ± 2.2*

Values are means ± SE. *$P < 0.05$ vs VH4 + W4, VH4 + SRL4, or CsA4 + W4 group. BW, body weight; SCr conc, serum creatinine concentration; SRL conc, sirolimus concentration.

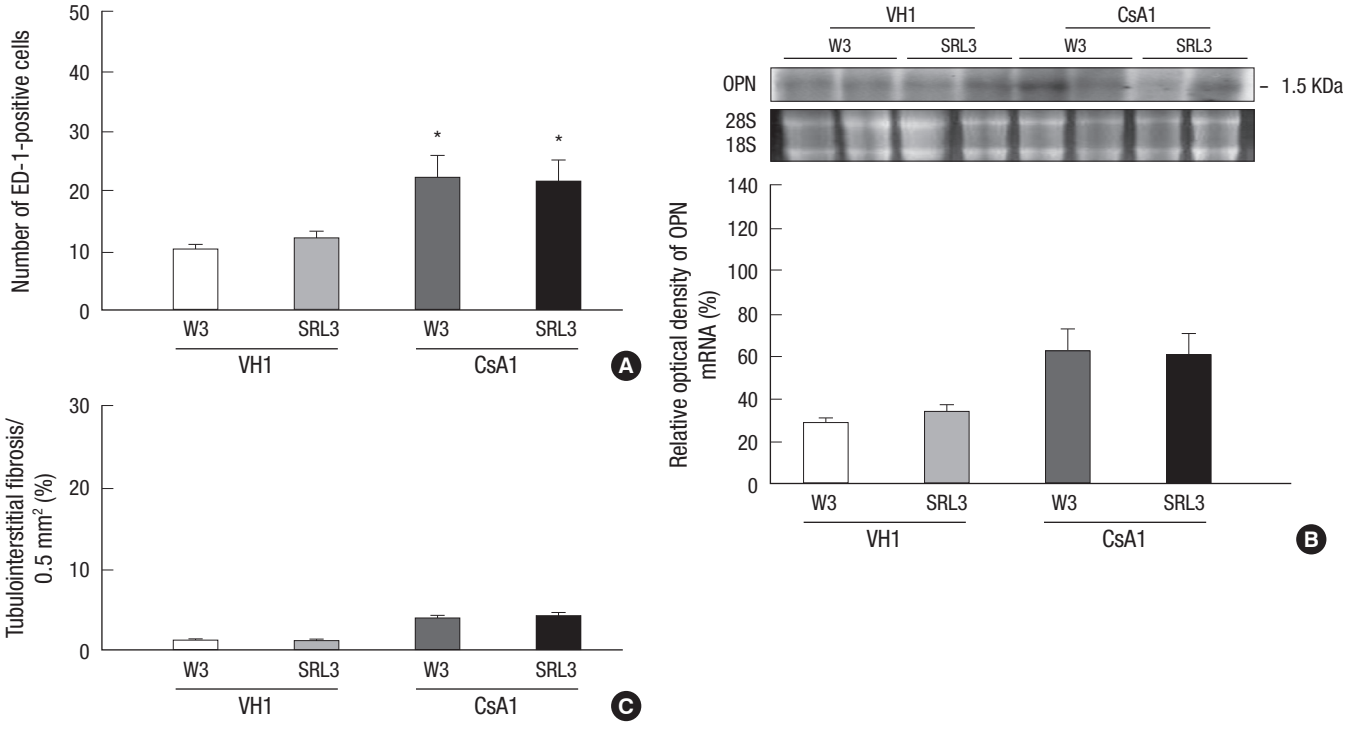

Fig. 5. Effect of early conversion from CsA to SRL on interstitial inflammation and fibrosis. (**A**) Quantitative analysis of ED-1-positive cells. There was no difference for the number of ED-1-positive cells between the CsA early withdrawal and early conversion groups. (**B**) Northern blotting for OPN mRNA. OPN mRNA did not differ between the early conversion and the CsA-early withdrawal groups. The data are presented as relative optical densities with the VH1 + W3 group designated as 100% reference and are normalized to 18S. (**C**) Quantitative analysis of TIF. The CsA early withdrawal group shows minimal striped interstitial fibrosis in the cortex. The amount of interstitial fibrosis does not differ between the CsA early withdrawal and early conversion groups.

compared with the CsA4 + W4 group, Fig. 7B).

The TIF score was higher in the CsA4 + W4 group (7.6 ± 1.1/0.5 mm^2) compared with the VH4 + W4 (1.8 ± 0.3/0.5 mm^2) or VH4 + SRL4 groups (1.7 ± 0.5/0.5 mm^2, $P < 0.01$ for the CsA4 +

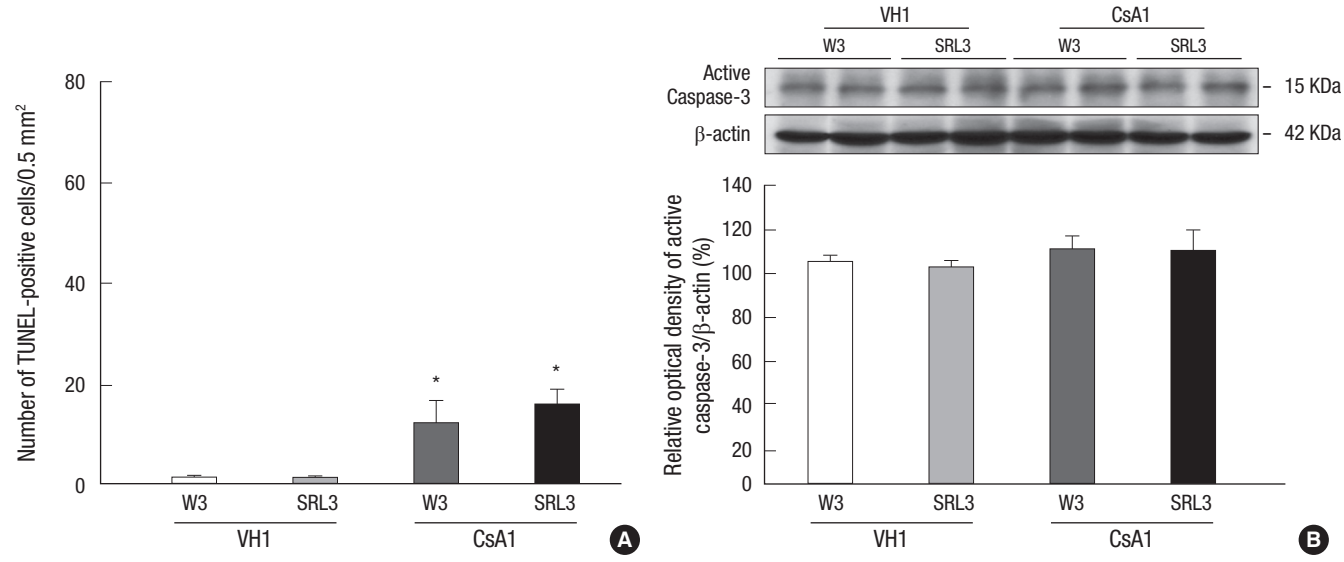

Fig. 6. Effect of early conversion from CsA to SRL on apoptotic cell death. (**A**) Quantitative analysis of TUNEL-positive cells. The number of TUNEL-positive cells is slightly higher in the CsA early withdrawal group than in the VH group (*$P < 0.01$ vs VH1 + W3 or VH1 + SRL3 group). The number of TUNEL-positive cell does not differ significantly between the CsA early withdrawal and early conversion groups. (**B**) Immunoblot analysis for active caspase-3. Active caspase -3 protein levels does not differ between the CsA early withdrawal and early conversion groups. Active caspase-3 protein levels were referenced against β-actin and the relative optical density is presented with the VH1 + W3 group designated as 100% reference and normalized with β-actin.

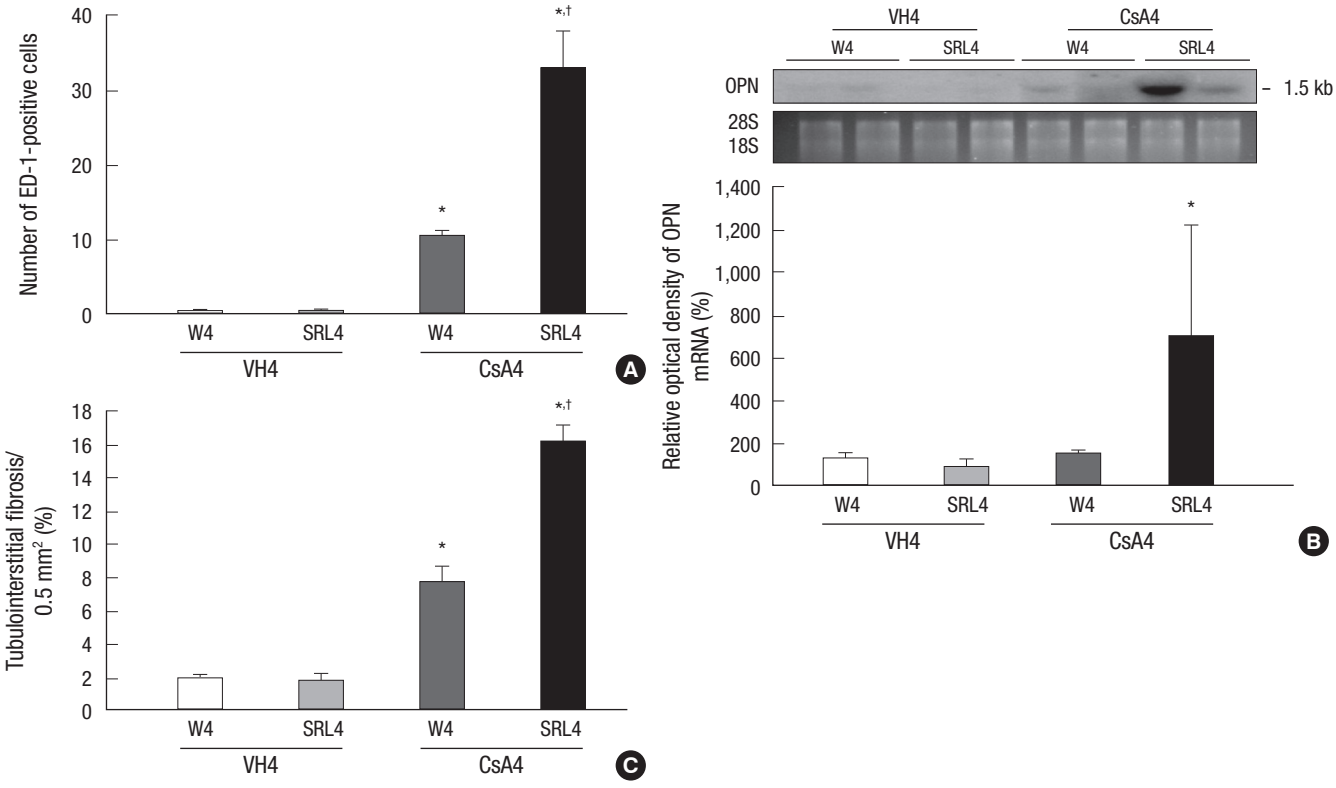

Fig. 7. Effect of late conversion from CsA to SRL on interstitial inflammation and fibrosis. (**A**) Quantitative analysis of ED-1-positive cells. The number of ED-1-positive cells is high in the CsA4 + W4 group compared with the VH4 + W4 group or the VH4 + SRL4 group. Late conversion from CsA to SRL at 4 weeks further increases the number of ED-1-positive cells compared with the CsA late withdrawal group. (**B**) Northern blotting for OPN mRNA. Late conversion from CsA to SRL significantly increases OPN mRNA expression in the CsA late withdrawal rat kidney. The data are presented as relative optical density with the VH4 + W4 group designated as 100% reference and are normalized to 18S. *$P < 0.01$ vs VH4 + W4 or VH4 + SRL4 group; †$P < 0.05$ vs CsA4 + W4 group. (**C**) Quantitative analysis of TIF. Late conversion from CsA to SRL causes greater interstitial fibrosis than that observed in the CsA late withdrawal group. *$P < 0.01$ vs VH4 + W4 or VH4 + SRL4 groups; †$P < 0.05$ vs CsA4 + W4 group.

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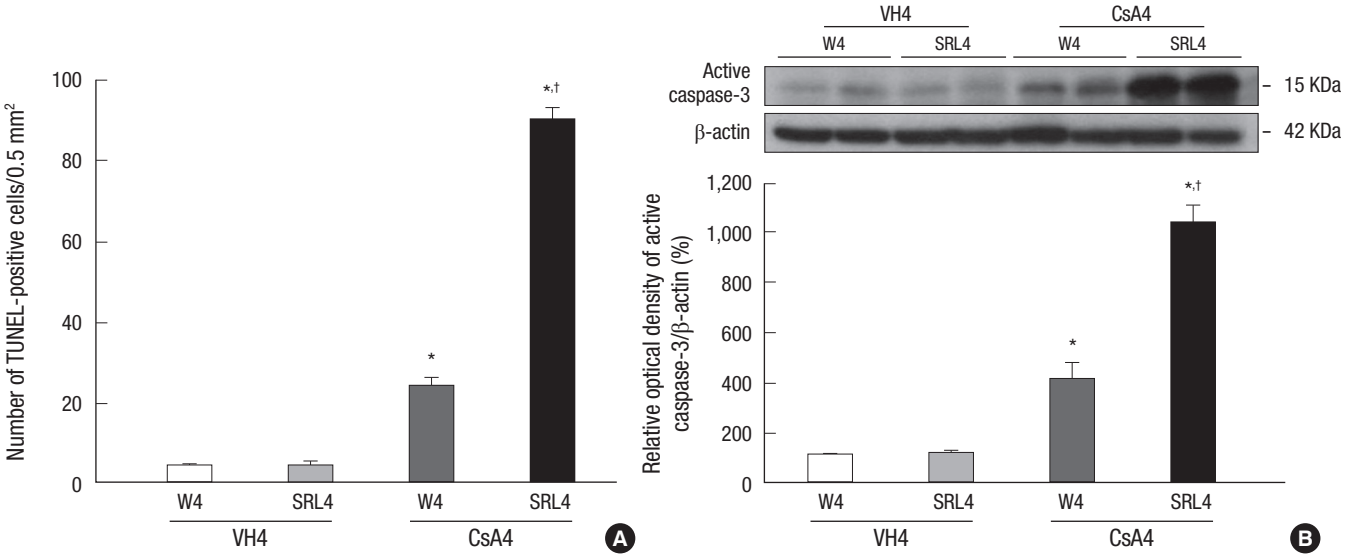

Fig. 8. Effect of late conversion from CsA to SRL apoptotic cell death. (**A**) Quantitative analysis of TUNEL-positive cells. The number of TUNEL-positive cells is much higher in the later conversion group than in the CsA-late withdrawal group. (**B**) Immunoblot analysis of active caspase-3. Note the further increase in active caspase-3 protein level in the late conversion group. Active caspase-3 protein levels were referenced against β-actin and the relative optical density was presented with the VH4 + 4W group designated as 100% reference and normalized with β-actin. *$P < 0.01$ vs VH4 + W4 or VH4 + SRL4 groups; †$P < 0.05$ vs CsA4 + W4 group.

W4 group compared with the two other groups). The TIF score was highest in the late conversion from CsA to SRL group (16 ± 1/0.5 mm^2) than in the CsA4 + W4 group (7.6 ± 1.1/0.5 mm^2, $P <$ 0.01, Fig. 7C).

Fig. 8A shows the number of TUNEL-positive cells in each experimental group. The number of TUNEL-positive cells was significantly higher in the CsA4 + W4 group (24 ± 2/0.5 mm^2) compared with the VH4 + W4 group (4 ± 0.6/0.5 mm^2) and VH4 + SRL4 group (4 ± 0.9/0.5 mm^2, $P < 0.01$ for the CsA4 + W4 group compared with the two other groups). The number of apoptotic cells was even higher in the late conversion from CsA to SRL group (89 ± 3/0.5 mm^2, $P < 0.05$ compared with the CsA4 + W4 group). Active caspase-3 protein levels were significantly higher in the CsA4 + W4 group (407% ± 70%) than the VH4 + W4 group (105% ± 5%) and the VH4 + SRL4 groups (115% ± 10%) ($P < 0.01$ for the CsA4 + W4 group compared with the two other groups). Active caspase-3 protein level was higher in the CsA4 + SRL4 group (1,029% ± 71%) compared with the CsA4 + W4 group (407% ± 70%, $P < 0.05$, Fig. 8B).

DISCUSSION

Our study was performed to determine optimal timing of conversion from CsA to SRL in experimental model of chronic CsA nephropathy. The results of our study demonstrate clearly that early conversion to SRL attenuates the progression of CsA-induced renal injury, whereas late conversion to SRL does not provide beneficial effects. This finding suggests that the conversion from SRL to CsA should occur as early as possible, before the development of chronic renal injury by CsA.

SRL by itself does not cause serious renal injury, but combined SRL and CsA treatment has a synergic effect on the development of chronic CsA nephrotoxicity (4-8). We first tested whether our experimental model of chronic CsA nephropathy produces similar results to those observed in clinical studies. SRL treatment alone did not induce significant nephrotoxicity but co-administration of CsA and SRL exacerbated CsA-induced renal injury. These results are consistent with the results of clinical studies, indicating that our model is suitable for studying SRL conversion.

To determine the optimal timing for conversion from CsA to SRL, we compared the effect of early and late conversion to SRL on CsA-induced renal injury. We chose day 7 as the time for early conversion and day 28 as the time for late conversion because CsA treatment for 4 weeks in our model induces renal dysfunction and chronic changes in kidney, whereas CsA treatment for 1 week causes minimal changes in renal function and histology (18, 19). In our current study, early conversion from CsA to SRL did not cause further significant changes in renal functional or histology alterations compared with the early CsA withdrawal group. By contrast, late conversion from CsA to SRL did not improve renal function and histopathology (9, 10). Actually, late conversion to SRL aggravated CsA-induced renal injury, as shown by a significant increase in interstitial inflammation and fibrosis, even after CsA withdrawal. This finding implies that the severity of CsA-induced renal injury is an important factor in the successful conversion to SRL.

A CONVERT study is to evaluate the effect of conversion from CsA to SRL at different time points after transplantation (6-120 months) (9), and the effect of SRL conversion was evaluated in terms of graft function or degree of proteinuria. Our experimen-

tal study is similar to CONVERT study in the point that SRL was directly converted from CsA at different stage (early and late) of CsA-induced renal injury. The results of CONVERT study showed clearly that SRL conversion is beneficial in patients with preserved graft function (baseline glomerular filtration rate more than 40 mL/min) but not in patients with a severely injured renal allograft. The results of our study were similar to those of CONVERT study. Early conversion of SRL before development of chronic change by CsA was effective in preventing CsA-induced renal injury but late conversion of SRL in established chronic CsA nephropathy failed to improve CsA-induced renal injury. This finding strengthens the rationale of CONVERT study that the effectiveness of SRL is dependent upon the status of graft function.

We further evaluated the influence of SRL conversion at the molecular level. In experimental models of chronic CsA nephropathy, CsA-induced renal injury recruits macrophages, and this recruitment is mediated by several proinflammatory cytokines (13, 20). OPN is proinflammatory mediator, and chronic CsA treatment increases OPN concentration in rat models (14-16). The CsA-induced renal injury also upregulates the expression of active caspase-3 and leads to apoptotic cell death (17, 18). In our current study, early conversion to SRL did not change macrophage infiltration and OPN protein expression after CsA withdrawal. However late conversion to SRL potentiated macrophage infiltration and OPN expression even after CsA withdrawal. In addition, early but late conversion to SRL increased the active caspase-3 expression and the number of TUNEL-positive cells compared with the CsA withdrawal group. This finding confirms the idea that early conversion to SRL is beneficial for attenuating CsA-induced inflammation and apoptotic cell death.

We used an experimental model of chronic CsA nephropathy, but our model has some limitations when translated into clinical practice. First, CsA trough levels were much higher than those targeted in clinical practice. Second, our experimental model of chronic CsA nephropathy produces minimal proteinuria, and we could not observe influence of SRL on proteinuria. Third, a follow-up period for 8 weeks has been better for defining the relationship between CsA and SRL groups in experiment III. However, the study period could not be extended, because rats cannot survive beyond 4 weeks under high dose CsA (15 mg/kg) and low salt diet. Therefore, the results of our study should be translated cautiously to clinical practice.

In conclusion, the results of our study provide a theoretical basis for a CsA sparing strategy using SRL and that it is desirable to convert SRL from calcineurin inhibitors before development of chronic CsA nephrotoxicity.

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