Original Research

Expression of apoptosis-related factors in chronic cyclosporine nephrotoxicity after cyclosporine withdrawal[1]

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ABSTRACT

AIM: To examine whether the reversibility of chronic cyclosporine A (CsA) nephrotoxicity is associated with apoptotic cell death and its regulatory factors. **METHODS:** Chronic CsA nephrotoxicity was induced in Sprague-Dawley rats by administering CsA (15 mg/kg, sc) for 5 weeks, and then withdrawing it for 5 or 10 weeks. The effect of CsA withdrawal on apoptotic cell death was evaluated by an *in situ* TdT-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) assay and the expression of pro-apoptotic [(transforming growth factor-beta1 (TGF-β1) and Fas] and anti-apoptotic [epidermal growth factors (EGF) and Bcl-2] factors. **RESULTS:** Discontinuation of CsA induced significant decreases in TUNEL-positive cells in a time-dependent manner and the reduction in TUNEL-positive cells was correlated with the tubulointerstitial fibrosis score (r=0.919, P<0.01). Upregulation of TGF-β1 and Fas expression in CsA-treated rat kidneys was decreased significantly after withdrawal of CsA. In contrast, downregulated EGF and Bcl-2 expression returned to normal or supernormal levels. **CONCLUSION:** CsA withdrawal is associated with a decrease in apoptotic cell death and with changes in the expression of pro-apoptotic and anti-apoptotic molecules involved in renal wound repair. This may constitute one of the mechanisms underlying the reversibility of chronic CsA nephrotoxicity.

INTRODUCTION

Tubulointerstitial injury, characterized by striped interstitial fibrosis and tubular atrophy, is the prominent feature of chronic cyclosporine (CsA) nephrotoxicity, which is closely associated with renal dysfunction[1].

The mechanism underlying tubulointerstitial injury is multifactorial, but loss of cellularity in the areas of fibrosis by excessive apoptosis has been proposed[2].

Apoptosis is an active mechanism of cell clearance and plays an important role in the regulation of cell number during development, tissue homeostasis, and following insult[3]. In the kidney, apoptosis is beneficial in some circumstances[4], but will be deleterious if enough resident cells are lost[2]. Indeed, a pathogenic role for apoptosis has been described in a wide range of renal diseases[5-7]. It is generally accepted that CsA in-

duces renal cell apoptosis *in vivo*[2] and *in vitro*[8,9], and it is associated with the expression of those regulatory genes that favor apoptotic cell death[10,11]. Furthermore, certain intrarenal growth factors such as the pro-apoptotic factor, transforming growth factor (TGF)-β1[12], and the anti-apoptotic factor, epidermal growth factor (EGF)[13], may also affect the program of apoptotic cell death in chronic CsA nephrotoxicity.

We have recently demonstrated that chronic CsA nephrotoxicity is reversible in rats after long-term CsA withdrawal[14,15], but the mechanism underlying this phenomenon is far from clear. Therefore, the present study investigated the association between apoptotic events (apoptotic cell death and the expression of its regulatory factors) and tubulointerstitial injury in the reversibility of chronic CsA nephrotoxicity after CsA withdrawal.

MATERIALS AND METHODS

Animals and drugs Male Sprague-Dawley rats (Charles River Technology, Korea) initially weighing 200-220 g were housed in cages (Nalge Co, Rochester, NY, USA) in a temperature- and light-controlled environment and allowed free access to a low salt diet (0.05 % sodium, Teklad Premier, Madison, WI, USA) and tap water. Cyclosporine (Novartis Pharma Ltd, Basle, Switzerland) was diluted in olive oil to a final concentration of 15 g/L.

Experimental schedule The experimental protocol was approved by the Animal Care Committee of the Catholic University of Korea. Animals were classified randomly into six groups as follows:

VH5 group (*n*=5) Rats received a daily subcutaneous injection of olive oil (1 mL/kg) for 5 weeks.

CsA group (*n*=8) Rats received a daily subcutaneous injection of CsA (15 mg/kg) for 5 weeks.

VH10 group (*n*=5) Rats received a daily subcutaneous injection of olive oil (1 mL/kg) for 10 weeks.

CsAW5 group (*n*=8) Rats received a daily subcutaneous injection of CsA (15 mg/kg) for 5 weeks, and then CsA was withdrawn for 5 weeks.

VH15 group (*n*=5) Rats received a daily subcutaneous injection of olive oil (1 mL/kg) for 15 weeks.

CsAW10 group (*n*=8) Rats received a daily subcutaneous injection of CsA (15 mg/kg) for 5 weeks, and then CsA was withdrawn for 10 weeks.

Animals were killed under ketamine anesthesia at each time point. Kidney tissues were rapidly removed for morphological and molecular examinations.

Functional parameters Prior to sacrifice, rats were individually housed in metabolic cages for 24 h-urine collections, and blood samples were obtained to evaluate serum creatinine (Scr). The creatinine clearance rate (Ccr) was calculated with standard formula.

Histopathology Harvested kidney tissues were fixed in Periodate-lysine-paraformaldehyde solution and embedded in wax. After dewaxing, 4-μm sections were processed and stained with Masson's trichrome and hematoxylin. A finding of tubulointerstitial fibrosis (TIF) was defined as a matrix-rich expansion of the interstitium with tubular dilatation, tubular atrophy, tubular cast formation, sloughing of tubular epithelial cells, or thickening of the tubular basement membrane. A minimum of 20 fields per section was assessed and graded using color image analyzer (TDI Scope Eye™ Version 3.0 for Windows, Olympus, Japan). The extent of TIF was estimated by counting the percentage of areas injured per field of cortex using a scoring scale of 0-3+: 0=normal interstitium; 0.5=< 5 % injured area; 1=5 % to 15 % injured area; 1.5=16 % to 25 % injured area; 2=26 % to 35 % injured area; 2.5=36 % to 45 % injured area; 3=>45 % injured area, as previously described[14]. Histopathologic analysis was performed in randomly selected cortical fields of sections by a pathologist blinded to the identity of the treatment groups.

***In situ* TdT-mediated dUTP-biotin nick end-labeling assay** Cells undergoing apoptosis were identified by the ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD, USA). After dewaxing, the sections were treated with proteinase K, and incubated with equilibration buffer in a humidified chamber for 10 min at room temperature, followed by incubation with working-strength TdT enzyme solution in a humidified chamber at 37 °C for 2 h. The reaction was terminated by incubation in working-strength stop/wash buffer at 37 °C for 30 min. After being rinsed with phosphate-buffered saline (PBS), the sections were incubated with anti-digoxigenin peroxidase in a humidified chamber at room temperature for 30 min. The sections were then incubated with diaminobenzidine and 0.01 % H_2O_2 at room temperature for 5 min. After being rinsed with PBS, the sections were counterstained with hematoxylin and examined using light microscopy. As a positive control, slides were treated with DNase (Sigma) and the slides for the negative control were treated with buffer-lacking TdT. The number of TUNEL positive cells was counted at twenty different

fields in each section under ×200 magnification.

Immunohistochemistry The dewaxed sections were incubated with 0.5 % Triton X-100/PBS solution for 30 min and washed with PBS three times. Nonspecific binding sites were blocked with normal horse serum diluted 1:10 in 0.3 % bovine serum albumin for 30-60 min, and then incubated at 4 ℃ for 2 h in rabbit polyclonal IgG anti-EGF antibody (Upstate Biotechnology, NY, USA) diluted in 1: 2000 in a humid environment. After rinsing in Tris-buffered saline (TBS), sections were incubated in peroxidase-conjugated rabbit anti-mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 30 min. For coloration, sections were incubated with a mixture of 0.05 % 3,3'-diaminobenzidine containing 0.01 % H_2O_2 at room temperature until a brown color was visible, washed with TBS, counterstained with hematoxylin and examined under light microscopy. Similarly, immunohistochemistry for TGF-β1 (Santa Cruz Biotechnology, CA, USA) was performed in cryostat sections with a modified method. Negative controls were performed without primary antibody. Positive control tissues for anti-EGF or anti-TGF-β1 antibody were human epidermoid carcinoma or human connective tissue and epithelial mucosa.

Immunoblotting For immunoblotting analysis, kidney cortex tissue was homogenized in lysis buffer [Tris-HCl (pH 7.6) 20 mmol/L, NaCl 150 mmol/L, 1 % (w/v) sodium deoxycholate, 1 % (v/v) Triton X-100, 0.1 % sodium dodecyl sulfate (SDS), $NaVO_3$ 2 mmol/L, and freshly added 1 % (v/v) aprotinin, leupeptin 1 mg/L, pepstatin 1 mg/L, and phenylmethyl-sulfonyl-fluoride (PMSF) 1 mmol/L]. Homogenates were centrifuged at 3000 r/m in for 15 min at 4 ℃, and the protein concentration of the lysate was determined using a protein microassay of the Bradford method (Bio-RAD, Hercules, CA, USA). Protein samples were resolved on 15 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto Bio-Blot nitrocellulose membrane (Bio-RAD, Hercules, CA, USA). An equal amount of protein loading (20 μg) was verified by Ponceau S staining. The membrane was blocked for 1 h in TBS added tween-20 [TBS-T, Tri-HCl 10 mmol/L, NaCl (pH 8.0) 150 mmol/L, 0.05 % tween-20] containing 5 % nonfat powdered milk. EGF was detected by incubating for 1 h with a rabbit polyclonal anti-EGF antibody diluted 1:2000. Primary antibody incubation was followed by six washes of TBS-T. The blot was then incubated with secondary antibody (anti-rabbit Ig, horse-

radish peroxidase [HRP]) conjugate at 1:1000 (Amersham Biosciences, UK) for 1 h. Antibody-reactive protein was detected using enhanced chemiluminescence (ECL, Amersham Biosciences, UK). Optical densities were obtained using the VH group as 100 % reference and normalized with β-actin. The procedure of immunoblotting for Fas and Bcl-2 (Santa Cruz Biotechnology, CA, USA; Fas diluted 1:500, Bcl-2 diluted 1:200; secondary antibody diluted 1:1000 for both) was similar to that for EGF.

***In situ* hybridization** The 400 bp cDNA probe for EGF was cloned into pGEM 3Z (Promega, Madison, WI, USA) and linearized with *Hin*dIII to produce an antisense riboprobe (NEN, Boston, MA, USA) using T7 RNA polymerase. Antisense riboprobes were then labeled with [^{33}P]UTP and *in situ* hybridization was performed as previously described[16]. In brief, tissue sections were dewaxed, rehydrated, and then digested with Pronase E [125 mg/L in Tris-HCl (pH 7.2) 50 mmol/L, EDTA 5 mmol/L, pH 8.0] at 37 ℃ for 10 min. After 2×2 min X rinses in 0.1 mol/L phosphate buffer, pH 7.2, sections were fixed in 4 % paraformaldehyde, pH 7.4 for 10 min at room temperature and rinsed again in 0.1 mol/L phosphate buffer. Sections were then dehydrated through graded ethanol and air-dried. Sections were hybridized with ^{33}P-labeled anti-sense EGF ($5×10^5$ cpm per 25 μL hybridization buffer) in hybridization buffer (NaCl 300 mmol/L, Tris-HCl 10 mmol/L, pH 7.5, Na_2HPO_4 10 mmol/L, pH 6.8, EDTA 5 mmol/L, pH 8.0, 1×Denhardt's solution, 0.8 mg yeast RNA/mL, 50 % deionized formamide, and 10 % dextran sulfate), heated to 85 ℃ and 25 μg added to the sections. Coverslips were placed on the sections and the slides incubated in a humidified chamber with 50 % formamide/2×standard saline citrate (SSC) at 60 ℃ for 14-16 h. Slides were then washed in 2×SSC (NaCl 0.3 mol/L, $Na_3C_6H_5O_7$·$2H_2O$ 0.33 mol/L) containing 50 % formamide at 50 ℃ to remove the coverslips. The slides were again washed with 2×SSC, 50 % formamide at 55 ℃ for a further 1 h. Sections were then rinsed three times in RNase buffer (Tris-HCl 10 mmol/L, pH 7.5, EDTA 1 mmol/L, pH 8.0, NaCl 0.5 mol/L) at 37 ℃ and treated with 150 μg RNase A per mL in RNase buffer at 37 ℃ for a further 1 h, then washed with 2× SSC at 55 ℃ for 45 min. Finally, sections were dehydrated through graded ethanol, air dried and exposed to Kodak Biomax MR Autoradiography film (Eastman Kodak, Rochester, NY, USA) at room temperature for 4 d. Slides were coated with Ilford K5 emulsion (1:1 with 2 % glycerol in distilled water; Ilford,

Moberly, Cheshire, UK), stored with desiccant at room temperature for 21 d, developed in Ilford phenisol, fixed in Ilford Hypam and stained with hematoxylin and eosin (H&E). The negative controls were run as same procedure, but without EGF antisense probe or with EGF sense probe instead of antisense probe.

Northern blot analysis Northern blot was performed as previously described[15]. Briefly, kidney cortex was homogenized in RNAzol reagent (TEL-TEST, INC, Friendwood, TX, USA) and extracted. Twenty micrograms of RNA were electrophoresed in each lane in 0.9 % agarose gels containing formaldehyde 2.2 mol/L and Mops 0.2 mol/L (pH 7.0) and were transferred to a nylon membrane (NEN Life Science Products, Inc, Boston, MA, USA) overnight by capillary blotting. Nucleic acids were cross-linked by ultraviolet irradiation (Stratagene, La Jolla, CA, USA). The membranes were prehybridized at 42 ºC for 2 h with 50 % formamide, 10 % Denhardt's solution, 0.1 % SDS, 5×SSC, and 200 mg/L denatured salmon sperm DNA. They were then hybridized at 42 ºC for 12-16 h with TGF-β1 cDNA probe (plasmid MUI5) labeled with ^{32}P-dCTP by random oligonucleotide priming (Boehringer Mannheim). The blots were washed in 2×SSC, 0.1 % SDS at room temperature for 15 min and in 0.1× SSC, 0.1 % SDS at 50 ºC for 15 min. Films were exposed at -70 ºC for different time periods to ensure linearity of densitometric values and exposure time. Densitometry analysis was performed using the NIH ImagePC program, three determinations for each band, and corrected to GAPDH (%).

Statistical analysis Data were expressed as mean±SEM. All parameters were compared to the VH5 group because there was no significant difference in the vehicle-treated rats at each time point. Multiple comparisons among groups were made by one-way ANOVA with the post hoc Bonferroni test correction (SPSS 9.0 Microsoft). The Pearson single-correlation coefficient was used to compare TUNEL-positive cells with TIF scores. Statistical significance was accepted when *P*<0.05.

RESULTS

Effect of CsA withdrawal on renal function and TIF in established chronic CsA nephrotoxicity Daily treatment of rats with CsA for 5 weeks caused higher levels of Scr (Fig 1, 11.4±0.2 *vs* 6.5 ± 0.2 mg/L, *P*<0.01) and lower levels of Ccr (0.14±0.01 *vs* 0.47±0.02 mL/
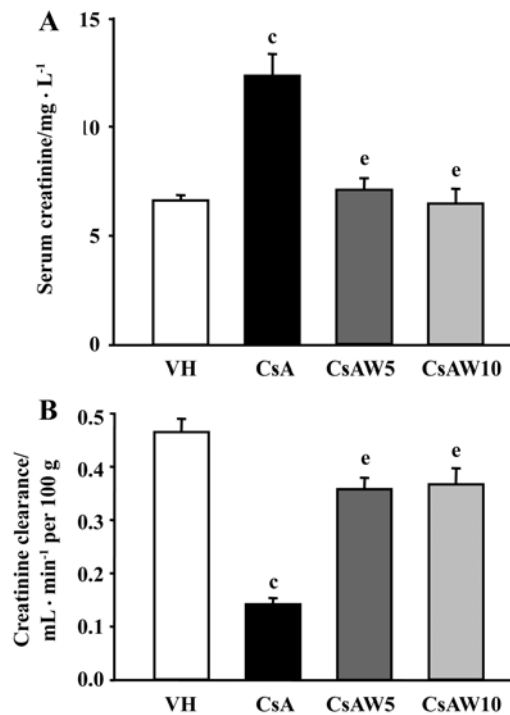

Fig 1. Serum creatinine (A) and creatinine clearance rate (B). Renal dysfunction was obvious in the CsA group. CsA withdrawal significantly improved renal function. ᶜ*P*<0.01 *vs* VH. ᵉ*P*<0.05 *vs* CsA.

min per 100 g, *P*<0.01) than those in VH-treated rats, whereas 5 or 10 weeks of CsA withdrawal normalized these parameters (Scr: CsAW5, 8.6 ±0.4 mg/L; CsAW10, 0.63±0.5 mg/L, *P*<0.05 *vs* CsA; Ccr: CsAW5, 0.31±0.03 mL/min per 100 g; CsAW10, 0.37±0.03 mL/ min per 100 g, *P*<0.05 *vs* CsA, respectively).

CsA-treated rat kidneys showed typical striped interstitial fibrosis and tubular atrophy (Fig 2). On our scoring system, a significant increase in the TIF score was observed in the CsA group compared with the VH group (1.8±0.2 *vs* 0.22±0.04, *P*<0.01), but 5 weeks of discontinued CsA caused a significant decrease in the TIF score compared with the CsA group (1.0±0.07, *P*<0.01 *vs* CsA). A further decrease was observed after 10 weeks of withdrawal relative to the CsAW5 group (0.5±0.07, *P*=0.034 *vs* CsAW5).

Effect of CsA withdrawal on TUNEL-positive cells in established chronic CsA nephrotoxicity TUNEL-positive cells were rarely observed in VH-treated rat kidneys (11±1), but their numbers were elevated in CsA-treated rat kidneys (Fig 3, 71±8, *P*<0.01 *vs* VH). Following 5 weeks of CsA withdrawal, TUNEL-positive cells were significantly reduced compared with
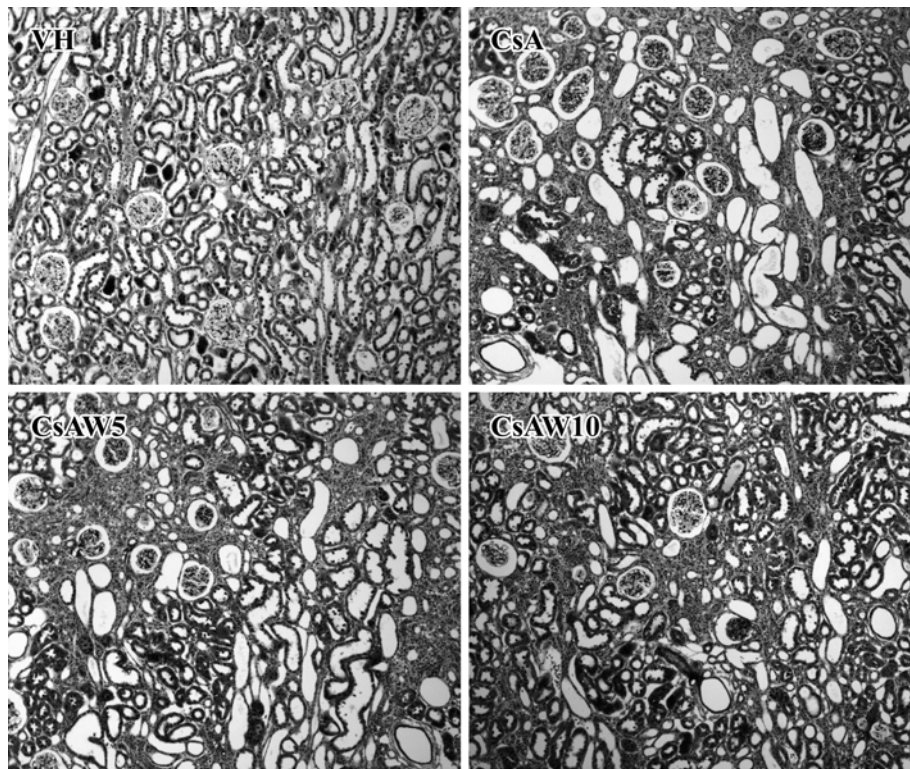
Fig 2. Degree of renal TIF and quantitative analysis. Representative photomicrographs showing that striped interstitial fibrosis, tubular atrophy, inflammatory cell infiltration, and expansion of the interstitium were pronounced in the kidneys with CsA treatment, whereas lesions were significantly decreased after 5 weeks of CsA withdrawal. Further decrease was observed after 10 weeks. Trichrome staining, ×100. cP<0.01 *vs* VH. eP<0.01 *vs* CsA. hP<0.05 *vs* CsAW5.

the CsA group (41±5, $P<0.01$ *vs* CsA), and a further decrease was observed when CsA was withdrawn for 10 weeks (26±2, $P<0.05$ *vs* CsAW5).

Correlation analyses between TUNEL-positive cells and TIF To define the relationships between TUNEL-positive cells and TIF, linear regression analysis was performed on the experimental groups (Fig 3). The reduction in TUNEL-positive cells during CsA with-

drawal correlated well with the TIF score ($r=0.919$, $P<0.01$).

Effect of CsA withdrawal on Fas and Bcl-2 proteins in established chronic CsA nephrotoxicity Immunoblotting analyses of the kidney cortices revealed a significant increase in Fas protein in the CsA group compared with the VH group during the 5-week treatment period (Fig 4A; 253 %±35 % *vs* 104 %±3 %,
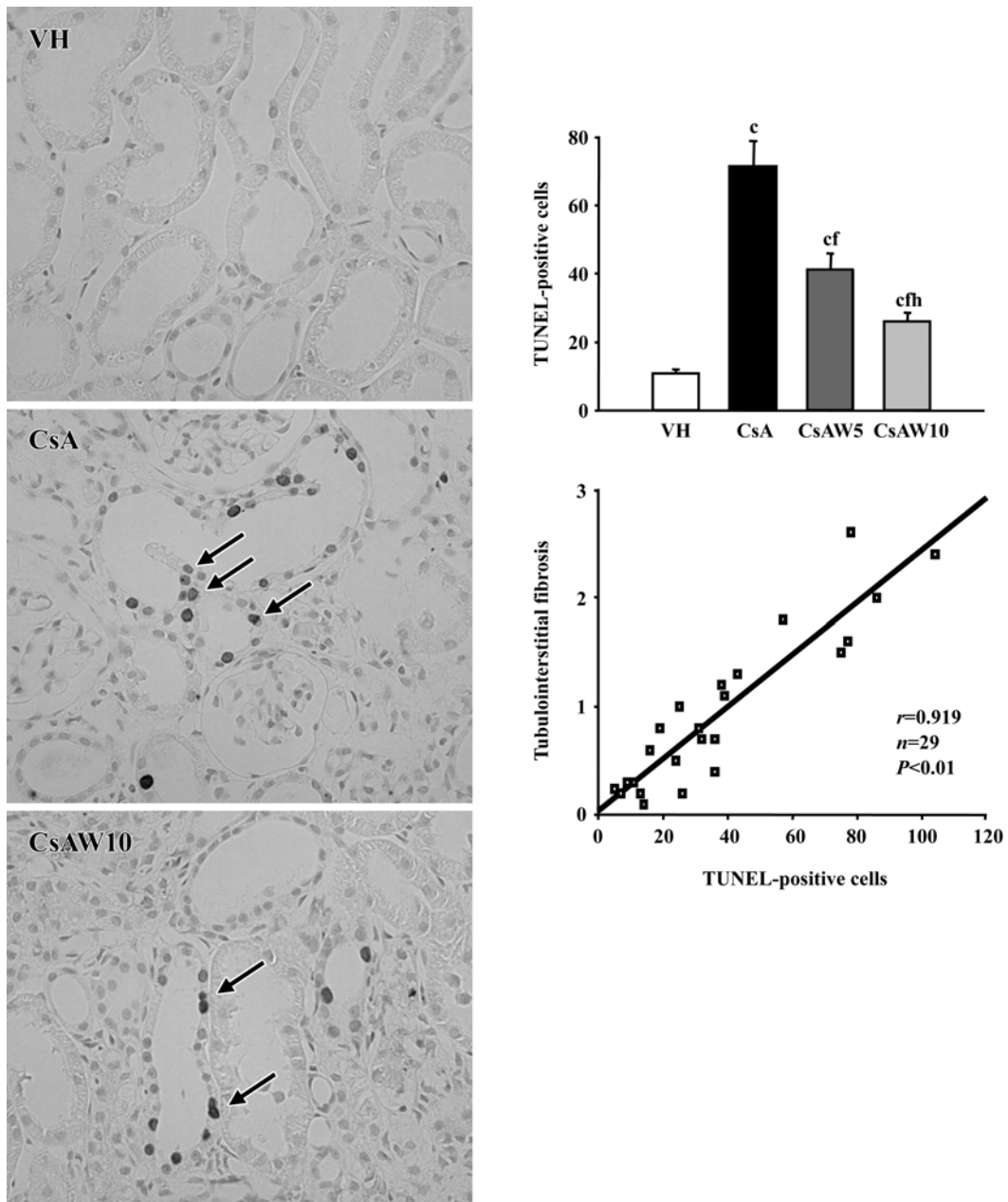
Fig 3. Representative photomicrographs of TUNEL assay, quantitation, and the relationship between TUNEL-positive cells and TIF scores. The number of TUNEL-positive cells increased in CsA-treated rat kidneys, whereas their numbers decreased after five weeks of CsA withdrawal, with a further decrease after 10 weeks. There was a close relationship between TUNEL-positive cells and TIF scores. Correlation analysis was performed in the experimental groups (*n*=29). TUNEL-positive cells are denoted by arrows. TUNEL assay, ×400. [c]$P<0.01$ *vs* VH. [f]$P<0.01$ *vs* CsA. [h]$P<0.05$ *vs* CsAW5.

$P<0.01$). This upregulation of Fas protein expression was significantly reduced after 5 weeks of CsA withdrawal (163 %±17 %, $P<0.05$ *vs* CsA), with further decrease after 10 weeks (106 %±12 %, $P<0.05$ *vs* CsAW5). In contrast, the dramatic suppression of Bcl-2 protein expression (Fig 4B, 52 %±9 %, $P<0.01$ *vs* VH) in CsA-treated rat kidneys returned to normal-(124 %±12 %, $P<0.01$ *vs* CsA) or supernormal levels

(411 %±68 %, $P<0.01$ *vs* CsAW5) following CsA washout.

Effect of CsA withdrawal on intrarenal EGF expression in established chronic CsA nephropathy
In situ hybridization and immunohistochemical detection of EGF are shown in Fig 5. In the VH group, EGF mRNA and protein were constitutively expressed in the distal convoluted tubules and in the thick ascending limb
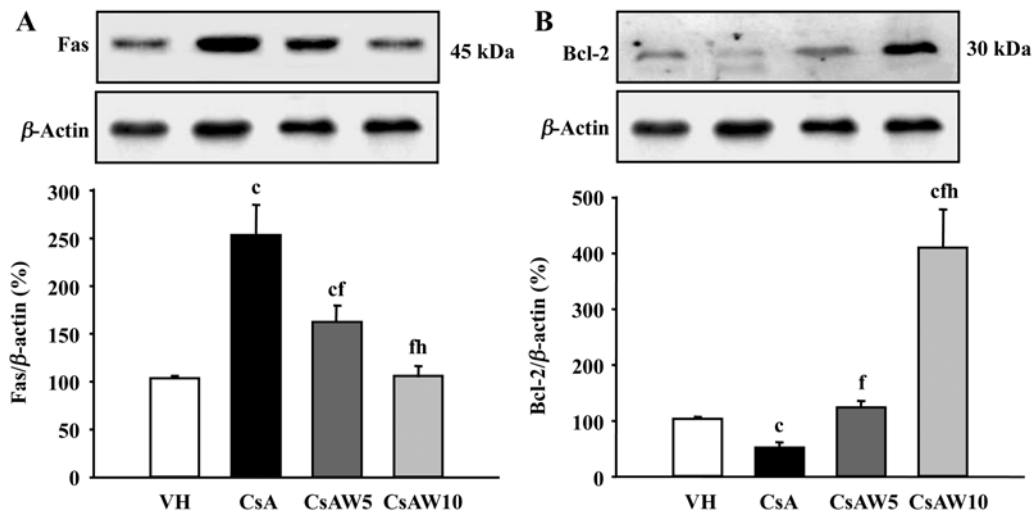
Fig 4. Immunoblotting of Fas (A) and Bcl-2 (B) proteins and densitometric analysis. CsA treatment resulted in the upregulation of Fas protein expression and the downregulation of Bcl-2 protein expression. CsA withdrawal time-dependently reversed these trends. [c]$P<0.01$ *vs* VH. [f]$P<0.01$ *vs* CsA. [h]$P<0.05$ *vs* CsAW5.

of Henle. In the CsA group, however, both EGF mRNA and protein were almost undetectable, although their expression was dramatically increased in both tubules as we have reported previously. Using immunoblotting and densitometric analysis, we found that suppressed EGF expression in the CsA-treated rat kidneys (19 %±3 %, $P<0.01$ *vs* VH) returned to normal (120 %±13 %, $P<0.01$ *vs* CsA) or supernormal levels (213 %±26 %, $P<0.05$ *vs* CsAW5) following the discontinuation of CsA treatment (Fig 6).

Effect of CsA withdrawal on TGF-β1 expression in established chronic CsA nephropathy As shown in Fig 7, TGF-β1 mRNA expression was higher in CsA-treated rat kidneys than that in VH-treated rat kidneys (641 %±40 % *vs* 104 %±9 %, $P<0.01$). This expression was reduced by CsA washout (395 %±41 %, $P<0.01$ *vs* CsA), and a further decrease was observed after washout over 10 weeks (254 %±35 %, $P<0.05$ *vs* CsAW5). Consistently, TGF-β1 protein expression was rarely observed in VH-treated rat kidneys, whereas TGF-β1 immunoreactivity was markedly upregulated in CsA-treated rat kidneys and was mainly localized to areas of injured tubules and interstitium (Fig 8). With CsA withdrawal, TGF-β1 immunoreactivity was significantly reduced.

DISCUSSION

The present study clearly demonstrates that, in established chronic CsA nephrotoxicity, CsA withdrawal is associated with a decrease in TUNEL-positive cells and a concomitant reversal of the expression of pro-apoptotic (TGF-β1 and Fas) and anti-apoptotic (EGF and Bcl-2) factors. These phenotypic changes correlate better with the regression of renal TIF. Our findings imply that the apoptosis pathway may be the predominant mechanism involved in the repair process associated with chronic CsA nephrotoxicity during CsA withdrawal.

CsA induces renal cell apoptosis directly[8,9] and indirectly[2], and the available evidence indicates that increased apoptotic cell death is strongly associated with tubulointerstitial injury[2,10,11]. The major finding of this study is that CsA withdrawal caused a significant decrease in the number of TUNEL-positive cells in a time-dependent manner. This was accompanied by improved renal function, and correlated with recovery from TIF (r=0.919, $P<0.01$). Our findings are consistent with a clinical trial that showed that a reduction in CsA dose led to a decrease in TUNEL-positive cells in patients with chronic allograft nephropathy[17], suggesting that a decrease in apoptotic cell death is involved in the reversibility of chronic CsA nephrotoxicity.

Bcl-2 protein protects cells from a variety of stimuli that induce apoptosis, such as growth factor deprivation and DNA-damaging drugs[18,19]. On the other hand, Fas promotes the apoptosis of many cell types when it forms cross-links with Fas-L[20]. We have shown, as have others, that chronic CsA treatment upregulates the expression of Fas and downregulates that of Bcl-2 ex-
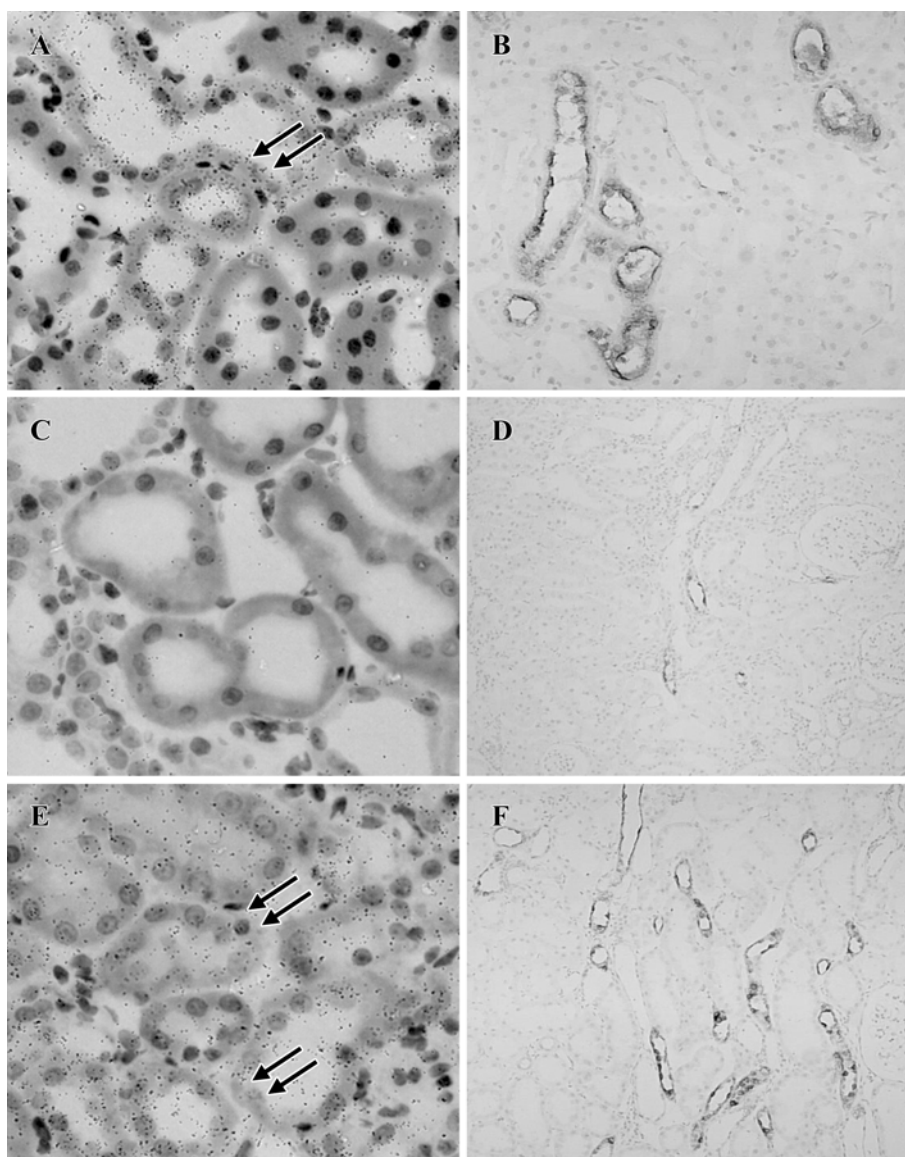
Fig 5. Representative photomicrographs of *in situ* hybridization (left panel) and immunohistochemistry (right panel) to detect epidermal growth factor (EGF). Both EGF mRNA and protein were present in VH-treated rat kidneys (A and B, arrows), whereas their expression decreased significantly when CsA was administered to rats (C and D). Following CsA withdrawal, EGF returned to normal or supernormal levels (E and F, arrows). *In situ* hybridization, ×400; immunohistochemistry, ×400 in B, ×100 in D and F.

pression in association with apoptotic cell death[10,11,21]. In the present study, we observed that CsA withdrawal reversed the patterns of Bcl-2 and Fas protein expression, and that this was more pronounced after 10 weeks of CsA withdrawal. Therefore, in this study, it is likely that the CsA withdrawal may have restored the balance between the expression of pro-apoptotic Fas and anti-apoptotic Bcl-2 in favor of cell survival.

EGF is a renotrophic growth factor that is implicated in recovery from renal injury and protection from apoptotic cell death[22]. Indeed, exogenous EGF accel-

erates renal tubular cell regeneration and lessens the severity of acute renal failure after ischemia[23] or exposure to nephrotoxins[24]. In the normal kidney, EGF is constitutively expressed in the distal convoluted tubules and in the thick ascending limb of Henle[13], where it is thought to act in a paracrine or autocrine manner to modulate a variety of cellular functions[25,26]. We have previously demonstrated that renal tubular EGF expression is reduced to almost undetectable levels in diabetic nephropathy[16,27] and chronic CsA nephrotoxicity[13], and that the loss of endogenous EGF parallels an increase in
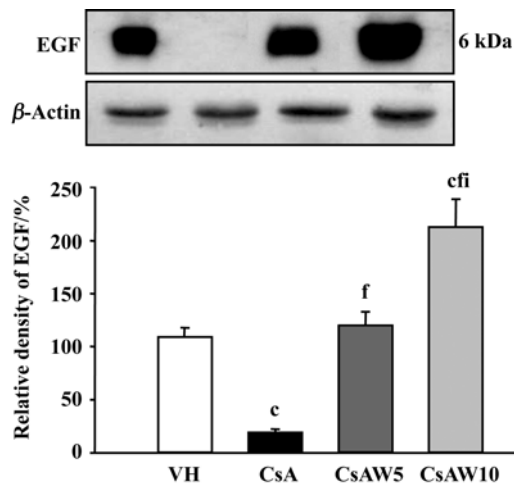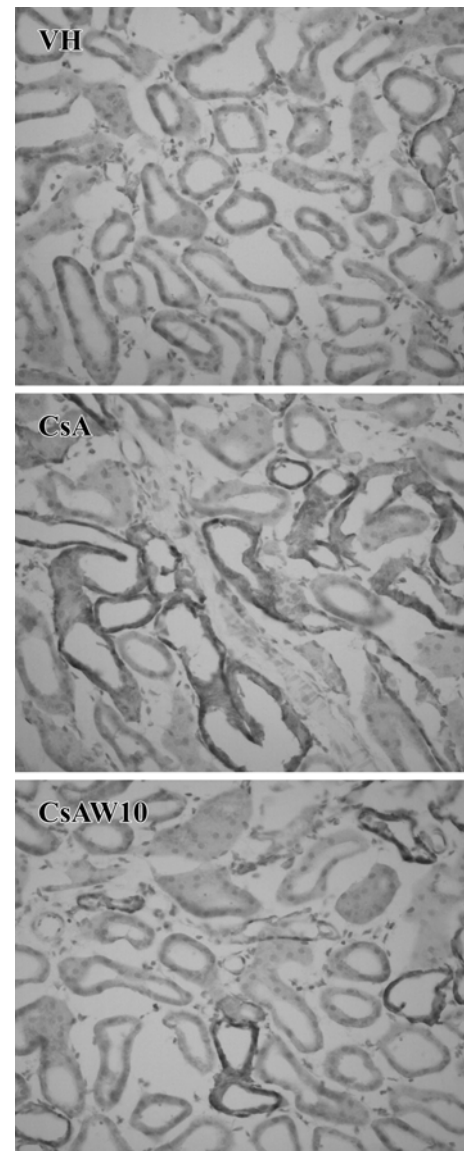
Fig 6. Immunoblotting of epidermal growth factor and densitometric analysis. Consistent with the results of *in situ* hybridization and immunohistochemistry, immunoblotting demonstrated that CsA suppressed EGF protein expression, whereas CsA withdrawal time-dependently increased it. [c]P<0.01 *vs* VH. [f]P<0.01 *vs* CsA. [i]P<0.01 *vs* CsAW5.
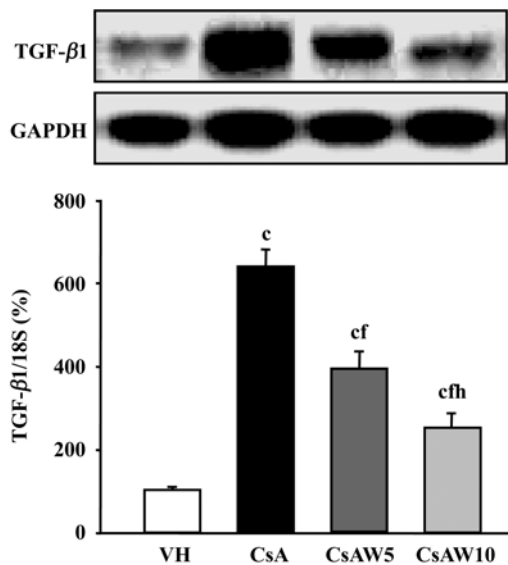
Fig 7. Northern blotting of transforming growth factor-β1 mRNA and quantitative analysis. The upregulation of TGF-β1 mRNA expression seen in CsA-treated rat kidneys was significantly reduced after CsA withdrawal in a time-dependent fashion. [c]P<0.01 *vs* VH. [e]P<0.01 *vs* CsA. [h]P<0.05 *vs* CsAW5.

Fig 8. Representative photomicrographs of immunohistochemical detection of transforming growth factor-β1 protein. CsA treatment for five weeks induced the upregulation of TGF-β1 protein expression, which was mainly localized to injured tubules and interstitium. However, TGF-β1 expression and immunoreactivity decreased after CsA withdrawal. Immunohistochemical staining, ×400.

apoptotic cell death and tubulointerstitial injury. In the present study, we found that the suppressed EGF mRNA and protein expression (to 18 % of control levels) induced in CsA-treated rat kidneys returned to normal (6.3-fold *vs* CsA) or supernormal (2.0-fold *vs* VH) levels after CsA withdrawal. Our observations suggest that CsA withdrawal may be associated with restoration of intrarenal EGF favoring renal cell survival and tubular regeneration.

In contrast to EGF, TGF-β is a pleiotropic growth factor that is implicated much strongly in kidney disease progression, most likely through its actions on the synthesis and degradation of the extracellular matrix[28-30]. Regardless of its profibrogenic capacity, TGF-β may also contribute to renal disease progression by its potent pro-apoptotic activity[31-34]. Studies *in vivo* have

shown that upregulation of TGF-β was closely linked to apoptotic cell death in chronic CsA nephrotoxicity, and that this was abrogated by the administration of anti-TGF-β antibody[12]. In this study, CsA withdrawal decreased TGF-β1 mRNA and protein expression in a time-dependent way. This finding allows us to propose that the decrease in TGF-β1 expression induced by CsA withdrawal is related to the attenuation of apoptotic cell death.

Our model of chronic CsA nephrotoxicity is characterized by the activation of the renin-angiotensin system (RAS)[35-37]. It is well known that angiotensin II (Ang II) induces renal cell apoptosis *in vivo*[2,13,16] and *in vitro*[34,38-40], and that a blockade of Ang II by either angiotensin-converting enzyme inhibitors or Ang II receptor antagonists may inhibit apoptotic cell death[16,41,42]. In our previous study, we reported that long-term CsA discontinuation effectively decreased RAS activity, as assessed by plasma renin activity and intrarenal renin immunoreactivity[14,15]. Therefore, attenuation of apoptotic cell death by the discontinuation of CsA treatment may be the consequence of a reduction in RAS activity.

In summary, the present study demonstrates that long-term CsA withdrawal decreases renal cell apoptosis and balances the expression of anti-apoptotic factors (EGF and Bcl-2) and pro-apoptotic factors (TGF-β1 and Fas). This is closely associated with the reversal of TIF of established chronic CsA nephrotoxicity. This study provides a rationale to better explain the natural etiology of chronic CsA nephrotoxicity.

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