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Nitric oxide mediates cyclosporine-induced apoptosis in cultured renal cells

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Nitric oxide mediates cyclosporine-induced apoptosis in cultured renal cells.

Background. The clinical use of cyclosporine (CsA) is limited by its nephrotoxicity. Apoptosis, perhaps instigated by increased nitric oxide synthase (NOS) activity, may play a role in such toxicity.

Methods. Human mesangial cells, human tubular cells, human umbilical vein endothelial cells, or murine endothelial cells were cultured with CsA at final concentrations of 0 to 1000 ng/mL for 4 to 24 hours. As inhibitors of apoptosis, 0.01 mol/L L-nitromethylarginine (L-NAME) or 1 μg/mL cycloheximide (CHX) was added, whereas 0.01 mol/L sodium nitroprusside (as a nitric oxide donor) was used as a positive control. Apoptosis was assessed by using TUNEL method and by DNA fragmentation by electrophoresis. In addition, NOS enzymatic activity, Northern blots for inducible NOS (iNOS) mRNA, and immunohistochemically demonstrable iNOS protein were evaluated.

Results. Within 12 to 24 hours, CsA significantly increased the fraction (8 to 35%) of apoptotic cells in each cell line, according to the dose. Fragmentation of DNA confirmed apoptosis. L-NAME and CHX inhibited the phenomenon, whereas sodium nitroprusside enhanced it. Each cell line significantly increased NOS activity in response to CsA, an effect blunted by L-NAME and CHX. Neither inhibitor modified the increased iNOS mRNA expression elicited by CsA. Positive staining for both iNOS and p53 proteins was observed in all cell lines incubated with CsA that were inhibited by CHX; L-NAME inhibited only p53 staining.

Conclusions. CsA induces apoptosis in various renal cell lines, and this effect is mediated by the induction of iNOS via p53. These effects may contribute to the acellular fibrosis characteristic of late CsA nephrotoxicity.

The pathologic hallmark of chronic cyclosporine (CsA) nephrotoxicity is the loss of interstitial cells and an associated progressive expansion of extracellular matrix components [1–7]. Several now widely accepted mechanisms have been proposed to explain the "fibrogenic" activity of CsA [1–7]. Although many reports examine the influence of CsA on cell survival, often with conflicting results [8–12], only one report focused on a direct influence of CsA on the survival of renal cells [13]. In this latter article, morphologically evident apoptosis was related to signs of CsA toxicity, without providing evidence of causality or any mechanistic information.

Cyclosporine is one of the most powerful known stimuli for the synthesis of endothelin and prostanoids [14–17]; in addition to their vasoconstrictive activity, these molecules activate c-fos transcription, which in turn may influence the proliferative rate of cells by inducing the transition from the G0 to the S1 phase of the cell cycle [18–20]. Of interest, these factors also favor the accumulation of matrix components; they act both to increase matrix synthesis directly and to modulate the activity of enzymes involved in matrix turnover [21–23]. More recently, the direct stimulation by CsA of transforming growth factor-β has been postulated to play a major role in fibrogenesis [24, 25].

The means whereby CsA may alter the balance between cell survival and death, resulting in progressive cell loss, remains unclear. One widely accepted hypothesis underscores the vasoconstrictive activity of CsA and posits that prolonged vasoconstriction induces a chronic relative ischemia, potentially responsible for cellular atrophy. However, recent experimental evidence suggests that apoptosis plays a critical role in the regulation of cellularity following renal injury. For example, the exuberant number of mesangial cells induced by an injection of anti-Thy 1 is cleared by apoptosis [26, 27]. Similarly, activation of apoptotic pathways occurs during recovery from poststreptococcal glomerulonephritis, characterized by decreasing numbers of both proliferating glomerular cells and infiltrating leukocytes [28, 29]. Excessive apoptotic cell death has been recently invoked in the

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pathogenesis of the "silent" loss of cells seen in progressive renal scarring [30], during the evolution of focal glomerular sclerosis [31], and in response to a reduction in renal mass [32].

A progressive loss of renal cells caused by apoptotic death was also recently demonstrated in allografted kidneys with signs of chronic transplant nephropathy [13]. Remarkably, the presence of an increase in apoptotic cells was seen in close association with the pathologic signs of CsA tubulointerstitial damage, suggesting a close, perhaps casual, relationship between the two phenomena. We recently demonstrated that CsA modulates the activity of the inducible forms of nitric oxide synthase (iNOS) in renal tissues in vivo [33]. An unpaired electron confers free-radical activity upon nitric oxide (NO), which proves to be extremely reactive with oxygen-based free radicals [34–36]. Furthermore, NO induces apoptosis by promoting de novo transcription of the pro-apoptotic tumor suppressor protein p53 [37]. Accordingly, we explored the possibility that CsA induces apoptosis in renal cells by stimulating NOS expression and thereby NO production, ultimately leading, in turn, to the expression of p53. Herein, we present evidence that this mechanism occurs in mesangial, renal cortical epithelial, and endothelial cells in vitro, and likely contributes to the reduction in the cell number, which characterizes chronic transplant nephropathy.

METHODS

Cell cultures

Mesangial and renal cortical tubular epithelial cells were obtained by explant culture of the normal portions of human kidney extirpated for renal cell carcinoma, using standard methodologies, as previously described [38, 39]. Both renal cells were grown in RPMI 1640 supplemented with 20% fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin (all from Sigma, St. Louis, MO, USA). The murine endothelial cell line t End. 1 [40], kindly provided by Professor F. Bussolino (Institute of Chemistry, University of Turin, Turin, Italy), was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, penicillin, streptomycin, and amphotericin (all from Sigma). These cells, used at the 98th to 166th passage for the experiments described herein, were derived from a thymic hemangioma expressing the polyoma middle T antigen [41, 42]. Cells of the t End. 1 line retain a wide array of the functional properties of normal endothelial cells: they proliferate at confluence without aspects of overgrowth; take up acetylated low-density lipoprotein; express CD31, vascular cell adhesion molecule-1, E-selectin, and P-selectin; respond to interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and specific endothelial cell growth factors; and produce IL-6 and chemokines [40–42]. The transformed immortal human umbilical vein cell line EC 304, kindly provided by Professor M. Simonson (Department of Medicine, Case Western Reserve University, Cleveland, OH, USA), was maintained in DMEM and supplemented with 10% FBS, penicillin, streptomycin, and amphotericin (all from Sigma). Karyotype analysis of this cell line reveals a human chromosomal constitution with a high trisomic karyotype (mode 80). Ultrastructurally, endothelium-specific Weibel-Palade bodies were identified, and immunocytochemical staining with the lectin *Ulex europaeus I* (UEA-I) and an antihuman endothelium monoclonal antibody (PH545) was positive. Angiotensin-converting enzyme activity was demonstrated. Factor VIII-related antigen, alkaline and acid phosphatase, and the epithelial marker keratin were negative.

Experimental design

Cells from each lineage were incubated with CsA at a concentration of 0 (basal), 100, 250, 500, or 1000 ng/mL for 4 to 24 hours at 37°C in a 5% CO_2 humidified atmosphere; both the selected concentration(s) and the specific duration(s) of incubation are specified in the **Results** section. For TUNEL and immunoperoxidase experiments, cells were grown in eight-well chamber slides (Lab-Tek; Miles Scientific Inc., Naperville, IL, USA); for assay of NOS activity, cells were grown in 25 cm^2 plastic flasks (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA), whereas cells were grown in 75 cm^2 plastic flasks for DNA and RNA extraction. As internal controls, some cells were coincubated with 0.01 mol/L L-nitro-methylarginine (L-NAME), 1 μg/mL cycloheximide (CHX), or 0.01 mol/L sodium nitroprusside (SNP; all from Sigma).

Terminal uridine nick 3′ end labeling

After a five minute incubation in 1% paraformaldehyde, cells were fixed in 2:1 vol/vol ethanol:acetic acid for 10 minutes at room temperature. After three washes with phosphate-buffered saline (PBS), cells were incubated with 100 U/mL terminal deoxynucleotidyl transferase (TdT), 0.5 μg/mL biotinylated uridine triphosphate in 140 mmol/L potassium cacodylate, 125 mmol/L Tris-HCl, 2.5 mmol/L cobalt chloride, pH 6.6 (all from Boehringer, Mannheim, Germany) for one hour at 37°C in a humidified chamber. After three washes, a 1:40 solution of fluoresceinated streptavidin (Boehringer) was incubated for 30 minutes at room temperature. Slides were counterstained with 0.3 μg/mL propidium iodide (Sigma) in PBS for one minute at room temperature. An epifluorescent microscope (Ernst Leitz, Inc., Rockleigh, NJ, USA) was used to detect apoptotic cells, which were quantitated by counting the number of fluorescein-positive cells relative to the total number of cells in at least 10 microscopic fields.

DNA extraction and electrophoresis

DNA was extracted from cells in culture by standard methods. Briefly, after trypsinization, cells were incubated in 1 × standard saline citrate (SSC), 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% sarcosyl, 0.1 mg/mL proteinase K (all from Sigma) at 50°C for 12 hours. After the addition of two volumes ethanol to this aqueous cell lysate, the mixture was incubated for two hours at −70°C. The pellets formed by centrifugation at 15,000 × *g* for 20 minutes at 4°C were resuspended in 10 mmol/L Tris, 1 mmol/L EDTA buffer, pH 8. The concentration of DNA was assessed by light absorbance at 260 nm. Equal quantities of DNA for each sample were separated in a Supersub Gel Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA, USA) at 200 V for three to four hours. As positive and negative controls, DNA extracted from CTL4 (IL-2 dependent) lymphocytes incubated in the presence or absence of IL-2 was used.

Nitric oxide synthase activity

After washing three times in PBS, cells were frozen in 1 mL of reaction buffer (20 mmol/L HEPES, 0.5 mol/L EDTA, 1 mmol/L dithiothreitol, pH 7.2) and homogenized on ice with three 20-second bursts in a Polytron homogenizer. Each reaction employed 100 μL of homogenate in a mixture to contain: 2 mmol/L NAPDH, 1.5 mmol/L $CaCl_2$, 1 to 100 μmol/L L-Arg, and 2.5 pCi (= 0.4 pmol/L) L-[2, 3, 4, 5-^{3}H]arginine monohydrochloride (62 Ci/mmol; Amersham International, Bucks, UK). After 30 minutes of incubation at 37°C, the reaction was stopped by adding 2 mL of 20 mmol/L HEPES, 2 mmol/L disodium EDTA, pH 6. The whole reaction mixture was applied to 2 mL columns of Dowex AG50WX-8 (Na^+ form; Aldrich, Milano, Italy), and the columns were washed with 4 mL of water. At a pH of 6, the Dowex resin binds arginine but not citrulline. The radioactivity corresponding to [^{3}H]citrulline content in 6 mL effluent was measured by liquid scintillation counting. The protein content of cells was assessed with a modified micro-Lowry method (Sigma). NOS activity was expressed as pmoles of citrulline generated/minute of incubation/mg cell protein. NOS activity was expressed as the fold increase relative to values obtained with the same cell line under basal conditions.

RNA extraction and Northern blotting

Total RNA was extracted from various cell cultures using RNA STAT-60 (TEL-TEST B, Inc., Friendswood, TX, USA). Cells grown in monolayers, with or without in vitro stimulation, were lyzed directly in a culture flask by adding extraction reagent (which contains guanidinium thiocyanate and phenol), and the cell lysate was passed through a pipette several times. The resultant homogenates were incubated for five minutes at room temperature to permit complete dissociation of nucleoprotein complexes. Next, 0.2 mL chloroform was added per milliliter of extraction reagent; the mixture was shaken vigorously for 15 seconds and incubated for two to three minutes at 4°C. Centrifugation separated the homogenates into two phases: a lower red phenol/chloroform phase and a colorless upper aqueous phase containing RNA. The aqueous upper phase was transferred to a fresh tube, and 0.5 mL isopropanol was added per 1 mL of the extraction reagent used for homogenization. This mixture was incubated at room temperature for 10 minutes and then centrifuged for 10 minutes at 12,000 × *g*. Supernatants were removed, and the RNA pellet was washed once with 75% ethanol by vortexing and subsequent centrifugation at 7500 × *g* for five minutes at 4°C. The extracted RNA in the pellet was air dried and dissolved in DEPC-H_2O for use in Northern analysis.

Total cellular RNA (20 μg) was subjected to electrophoresis on a 1% agarose/formaldehyde gel. The fractionated RNA was then blotted by the capillary method onto positively charged nylon membranes (Boehringer Mannheim), air dried for at least 30 minutes, and then baked at 120°C for another 30 minutes. The membranes were then prehybridized for two to three hours at 42°C in a solution containing 50% formamide, 5 × SSC, 5 × Denhardt's solution, 25 mmol/L KH_2P0_4, pH 7.4, containing 0.1 mg/mL sheared and denaturated salmon sperm DNA (Sigma). Next, the membranes were incubated for 16 hours (at 42°C) in hybridization solution (prehybridization solution with 10% dextran sulfate added) containing ^{32}P-labeled cDNA probes for the constitutive (c) or inducible forms of NOS (Cayman Chemical Company, Ann Arbor, MI, USA), prepared by random priming of the appropriate cDNA fragment (Oligolabelling Kit; Pharmacia Biotech, Piscataway, NJ, USA). Subsequently, the membranes were washed under stringent conditions [once for 5 min at room temperature in 2 × SSC/0.5% sodium dodecyl sulfate (SDS); twice for 20 min at room temperature in 2 × SSC/0.1% SDS; twice for 20 min at 68°C in 1 × SSC/0.1% SDS; and twice for 20 min at 68°C in 0.1 × SSC/0.1% SDS]. After a final wash, the membranes were exposed to X-OMAT AR x-ray films (Eastman Kodak, Rochester, NY, USA) at −80°C for one to three days. The membranes were also stripped and rehybridized with ^{32}P-labeled GAPDH probe (a kind gift from Dr. J.R. Sedor; Case Western Reserve University) as a control for equal loading. Autoradiographs were obtained and the bands quantitated by laser densitometry.

Immunoperoxidase staining

Cells grown in chamber slides were stained using a standard protocol. Briefly, cells were air dried and fixed in chilled acetone for 10 minutes. Endogenous peroxidase activity was inhibited by incubation for 30 minutes

in 0.025% H_2O_2 in methanol. Endogenous biotin activity was inhibited by sequential 30-minute exposures to avidin D and biotin blocking solutions (Vector Laboratories Inc., Burlingame, CA, USA). The sections were placed in dilute goat serum as a blocking agent, followed by application of 1:100 rabbit IgG anti-iNOS (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) or 1:40 rabbit IgG anti-p53 (Dako, Milano, Italy) or normal rabbit IgG at the same final concentrations for 30 minutes; antibodies were diluted in PBS containing 10% goat serum. After washing, the slides were developed with the Vectastain ABC rabbit IgG detection kit (Vector Laboratories); in accord with the manufacturer's directions, slides were sequentially exposed to biotinylated goat antirabbit IgG for one hour, then streptavidin-peroxidase for 30 minutes, and finally to 0.5 mg/mL 5,5′diaminobenzidine in 0.03% hydrogen peroxide, 0.05 mol/L Tris-buffered saline, pH 7.2, for five minutes. Three washes in PBS were employed between each step. The sections, after counterstaining with hematoxylin and mounting under cover slips, were evaluated qualitatively for the intensity of staining.

Statistical analysis

Values reported in the **Results** section represent the mean ± SD of six pooled experiments, each performed in triplicate. Statistical significance was analyzed by one-way analysis of variance (ANOVA) using a post hoc analysis with Dunnet's multiple comparison *t*-test when appropriate. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Apoptosis

Apoptotic cells and apoptotic bodies were detected frequently by in situ TdT nick end-labeling (Fig. 1) in all four lines of cultured cells after exposure to CsA, but were rarely encountered in basal cultures of the same cells in medium alone (Table 1). The dose–response curve is quite steep, as minimal to maximal rates of apoptosis were observed over a relatively narrow range of CsA doses, at least for the three lines of human cells. At a CsA dose of 100 ng/mL, the frequency of apoptosis proved to be a quite minor, but statistically significant (all $t > 2$, all $P < 0.05$), increment over basal values. However, for the three human cell lines, the frequency of apoptosis in the presence of 1000 ng/mL was not significantly higher than that seen with the 500 ng/mL dose, and the plateaus achieved approach the rates of apoptosis seen with the positive control, SNP. The murine endothelial line t End. 1 did not express peak levels of apoptosis below 1000 ng/mL, and the rate of apoptosis even at this dose was only 60% of that elicited by SNP.

Coincubation with L-NAME, a functional NOS inhibitor, reduced the frequency of apoptosis in the presence of 500 ng/mL CsA by 65 to 82% in all cell lines tested (all $P < 0.001$ vs. 500 ng/mL CsA alone; Table 1). Likewise, CHX, an inhibitor of protein synthesis, inhibited the effect of CsA on apoptosis by 88 to 94% in each of the various cell lines (all $P < 0.001$ vs. 500 ng/mL CsA alone; Table 1). Neither L-NAME nor CHX alone, in the absence of CsA, affected the basal rate of apoptosis detectable in unconditioned cells. In contrast, the incubation of each cell line with SNP, a known donor of NO, greatly increased the percentage of apoptotic cells to levels that exceeded those elicited by the highest dose of CsA tested in each of the cell lines (all $P < 0.001$ vs. the same cells under basal conditions; Table 1).

The peak frequency of apoptosis in mesangial cells was observed at 12 hours after the addition of CsA (Fig. 2), at several doses of CsA. The time course of apoptosis in both endothelial cell lines and the renal tubular cells closely resembled that in mesangial cells; half-maximal responses were observed by 12 hours, and plateaus were reached within 12 to 24 hours after the addition of CsA (data not shown).

By gel electrophoresis, DNA extracted from each cell line incubated with CsA at 250, 500, or 1000 ng/mL showed fragmentation, recognized by smears and/or multiple bands in the lower (low molecular weight) portion of the gel, similar to the typical pattern observed with the positive control and with cells incubated with SNP (Fig. 3). Such fragments were not present in the DNA from unconditioned cells or in the negative control. DNA extracted from cells coincubated with CsA in the presence of either L-NAME or CHX showed appreciably less evidence of DNA fragmentation (Fig. 3).

Nitric oxide synthase activity and mRNA expression

Cyclosporine elicited increased NOS enzymatic activity relative to the basal values in all four cell lines (Fig. 4), but the dose–response relationship was even steeper than that observed for apoptosis. In fact, compared with unconditioned cells of the same lineage, 30 to 40% increases in NOS activity were observed in response to 100 ng/mL CsA in mesangial cells, human umbilical vein endothelial cells, and murine t End.1 endothelial cells (all $t > 2.3$, all $P < 0.05$) and by 20% in renal cortical tubular cells ($t = 2$, $P < 0.05$) whereas higher doses (250 to 1000 ng/mL) evoked 300 to 1100% increases in NOS activity (all $P < 0.001$ vs. both basal and 100 ng/mL CsA), with no statistically significant differences among the different concentrations of CsA applied. As expected, coincubation of each of the cell lines in the presence of either L-NAME or CHX inhibited the CsA-induced increase in NOS activity by more than 80% (Fig. 4).

By Northern analysis, RNA from each cell line incubated with CsA showed conspicuously greater hybridization to the probe for iNOS, compared with basal con-

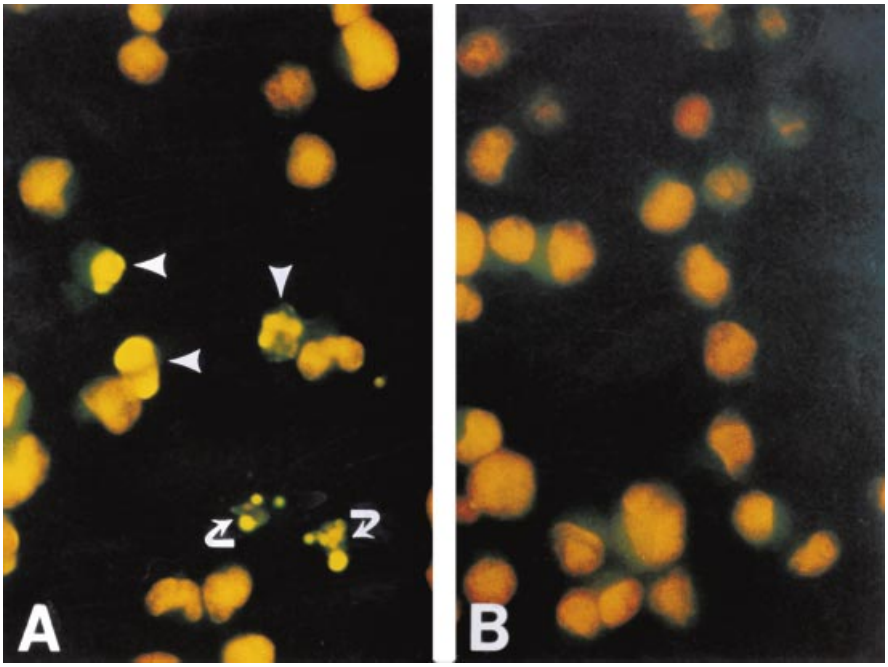


Fig. 1. By fluorescence microscopy after TUNEL, several (approximately 20%) of the murine endothelial cells incubated in the presence of 500 ng/mL cyclosporine A (CsA) for 12 hours (*A*) exhibited apoptotic (yellow) nuclei (arrowheads) or (yellow) apoptotic bodies (curved arrows). In contrast, all cells in parallel cultures incubated in medium alone (*B*) had nuclei that appeared red-orange in color.

Table 1. Apoptosis in response to cyclosporine A and influence of selected other co-stimuli[a]

Additions to culture		Cell lineage %			
Cyclosporine *ng/mL*	Other	Tubular cells	Mesangial cells	Endothelial cells (human)[b]	Endothelial cells (murine)[c]
0	none	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.3 ± 0.2
100	none	0.6 ± 0.3[d]	0.5 ± 0.1[d]	0.7 ± 0.2[d]	0.8 ± 0.3[d]
250	none	10 ± 1[e,f]	8 ± 2[e,f]	13 ± 2[e,f]	18 ± 7[e,f]
500	none	16 ± 2[e,f]	15 ± 3[e,f]	19 ± 4[e,f]	22 ± 6[e]
1000	none	18 ± 3[e]	19 ± 2[e]	21 ± 4[e]	35 ± 6[e,f]
0	L-NAME[g]	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.2
500	L-NAME	3 ± 1[d,h]	4 ± 1[d,h]	6 ± 0.5[e,h]	8 ± 2[e,h]
0	CHX[i]	0.3 ± 0.2	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.2
500	CHX	1 ± 0.5[d,h]	2 ± 1[d,h]	2 ± 0.5[d,h]	1.5 ± 0.5[d,h]
0	nitroprusside	24 ± 5[e]	26 ± 7[e]	32 ± 0.7[e]	55 ± 6[e]

[a] Data are mean ± SD percent cells positive for apoptosis, as detected by fluorescent TUNEL technique
[b] Human endothelial cells are derived from umbilical cord vein
[c] Murine endothelial cells are derived from a thymic hemangioma, immortalized with polyoma middle T
[d] $P < 0.05$ vs. the same cells under basal conditions (no additives to culture)
[e] $P < 0.001$ vs. the same cells under basal conditions
[f] $P < 0.05$ vs. the same cells at the next lower dose of cyclosporine
[g] L-nitro-methylarginine
[h] $P < 0.001$ vs. the same cells with 500 ng/ml cyclosporine alone
[i] cycloheximide

ditions (Fig. 5A). Densitometric quantitation showed increased transcription of the specific gene encoding the iNOS (Fig. 5B), while cNOS mRNA levels remained unchanged (data not shown). The addition of L-NAME or CHX to cells cultured with CsA had no effect on transcriptional levels of iNOS (data not shown).

Immunohistochemistry

Effective translation of the iNOS gene transcript induced by CsA in each cell line was demonstrated by immunoperoxidase (Fig. 6 A, B). Both glomerular and non-glomerular cells stained with antibodies specific for iNOS protein after incubation with CsA. Although cells coincubated with CsA in the presence of CHX failed to stain for iNOS (Fig. 6C), the addition of L-NAME to cells treated with CsA had no effect on the presence of iNOS protein detected by immunoperoxidase (Fig. 6D).

In parallel with the increased NOS activity, iNOS transcription, and immunoreactive iNOS protein content, each cell line treated with CsA exhibited definite and strong nuclear expression of p53, as detected by immunoperoxidase staining. The density of p53 staining increased

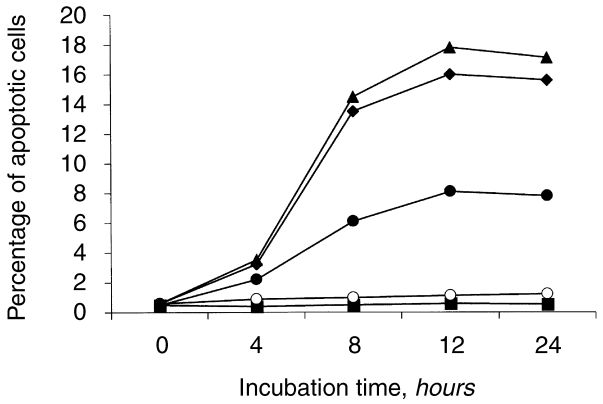


Fig. 2. Mesangial cells incubated with varying doses of CsA (as indicated) showed a similar time course for the development of apoptosis, as detected by TUNEL, although the level of the plateau varied with dose. The time course of apoptosis in cortical tubular cells and endothelial cells (not shown) was similar to that presented for mesangial cells; half-maximal responses were observed within 12 hours, and plateaus were reached 12 to 24 hours after the addition of CsA. Symbols are: (■) CsA 0 ng/mL; (○) CsA 100 ng/mL; (●) CsA 250 ng/mL; (◆) CsA 500 ng/mL; (▲) CsA 1,000 ng/mL.

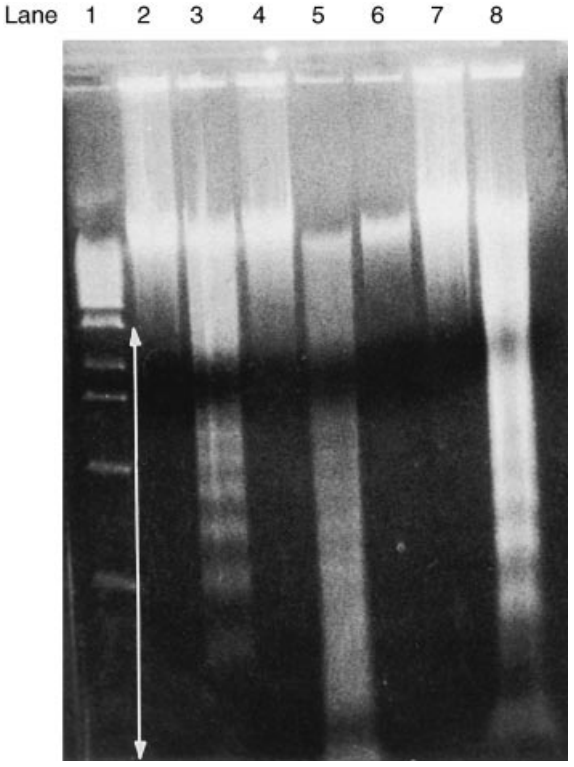


Fig. 3. DNA fragmentation is detected by agarose gel electrophoresis. In comparison to a commercial DNA calibration ladder (lane 1), low molecular weight fragments are detected as a smear or multiple bands in the lower portion of the gel (brackets) in DNA isolated from CTL4 lymphocytes cultured in the absence of interleukin-2 (IL-2; positive control, lane 3) and from mesangial cells cultured in the presence of 500 ng/mL CsA for 12 hours (lane 5) or 0.01 mol/L sodium nitroprusside (SNP; lane 8). No low molecular weight DNA is detected in CTL4 lymphocytes in the presence of IL-2 (negative control, lane 2) or in mesangial cells cultured in the absence of CsA (lane 4). The addition of either L-nitro-methylarginine (L-NAME; lane 6) or cycloheximide (CHX; lane 7) to mesangial cells in the presence of 500 ng/mL CsA markedly reduced the intensity of low molecular weight DNA fragments relative to cells cultured with CsA alone (lane 5). Similar results were observed with cortical tubular cells and endothelial cells (data not shown).

in parallel with the concentration of CsA applied to the cells (Fig. 7 A, B), whereas greatly reduced staining for p53 protein was observed when CsA-treated cells were coincubated with CHX (Fig. 7C) or L-NAME (Fig. 7D).

DISCUSSION

In this study, we demonstrate the capacity of CsA to induce apoptosis in cells of diverse lineage, adding another tile to the complex mosaic of the pathogenesis of chronic CsA nephrotoxicity. One prominent pathologic feature of chronic CsA nephrotoxicity is a progressive reduction in cell number, involving both tubular and glomerular elements. The remnant viable cells are scattered within a mass of newly formed matrix. The diminution in cell number typically occurs without signs of inflammation, mitigating the possibility that the cell loss is consequent to necrosis [1–7]. In general, apoptosis may be operative if fibrosis is associated with a derangement of cell population kinetics, when the balance between cell death and mitosis is perturbed. Accordingly, apoptosis has been recently claimed to play a role as a likely candidate for the silent loss of cells in kidneys undergoing progressive scarring [29–32].

Recently, Ito et al, using the morphological approach of terminal uridine nick 3′ end labeling (TUNEL) method, demonstrated the presence of apoptotic nuclei in biopsy specimens obtained from allografted kidneys [13]. The fourfold increase in the frequency of apoptosis in grafts with CsA nephropathy relative to the protocol biopsies of well-functioning grafts was the highest among those seen in various conditions of graft dysfunction. Noting this association, the authors proposed that CsA promotes apoptosis, but did not offer direct evidence in support of causation and offered no delineation of the operative mechanism. Indeed, although the effect of CsA upon apoptosis has been considered in a variety of cells [8–12], mostly leukocytes and especially T lymphocytes [43–46], the results remain somewhat controversial. At present, a single article reports an anti-apoptotic effect of CsA in human endothelial cells [12], and there are no published reports that directly demonstrate a pro-apoptotic activity of CsA on any indigenous renal cells.

We now document that CsA, at pharmacological concentrations, induces apoptosis in mesangial, tubular, and endothelial cells in culture. Maximal apoptosis and NOS activity were elicited by concentrations of CsA similar to the peak levels detectable at four to six hours after the last dose of drug (250 to 500 ng/mL); substantially

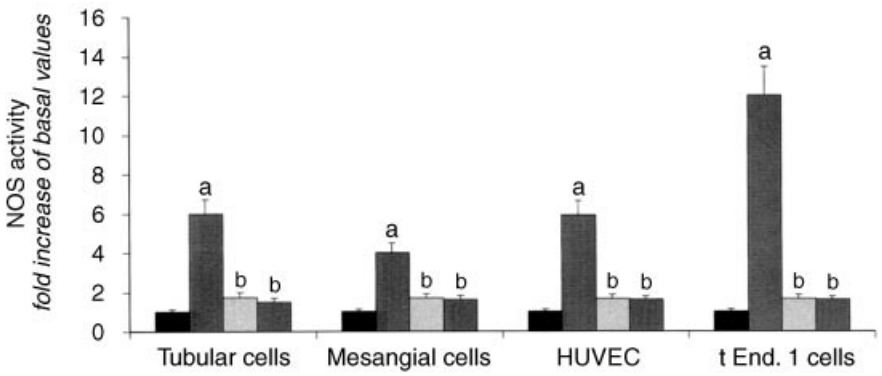


Fig. 4. Nitric oxide synthase (NOS) enzymatic activity in each of four cells lines after 12 hours of incubation with 500 ng/mL CsA is presented on the ordinate, expressed as a multiple of the enzymatic activity in the same cell line under basal conditions (no CsA added). The 4- to 12-fold levels of NOS activity elicited by CsA (*a* denotes $P < 0.001$ vs. basal conditions) were significantly decreased (*b* denotes $P < 0.001$ vs. 500 ng/mL CsA alone) by the addition of either L-NAME or cycloheximide (CHX) to the cells with CsA; inhibition by either agent uniformly exceeded 80%. Symbols are: (■) CsA 0 ng/mL; (■) CsA 500 ng/mL; (■) CsA 500 ng/mL + 0.01 mol/L L-NAME; (■) CsA 500 ng/mL + 1 μg/mL CHX.

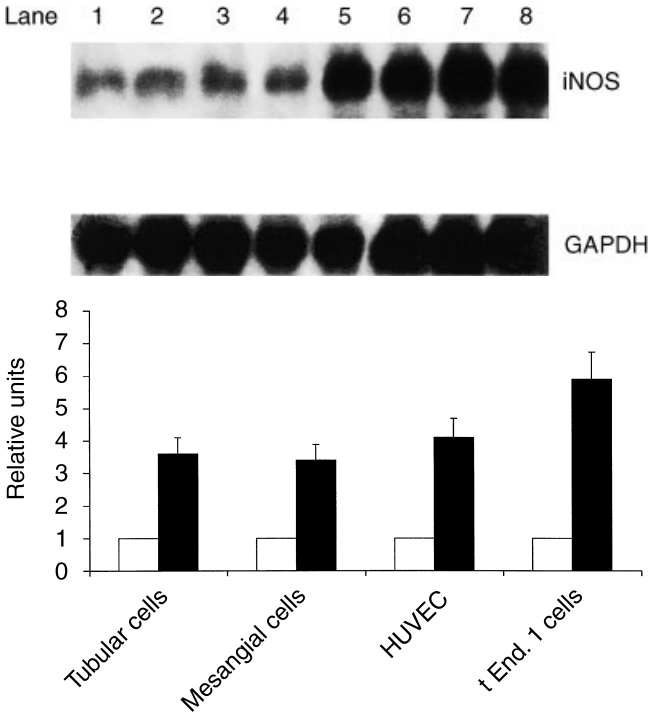
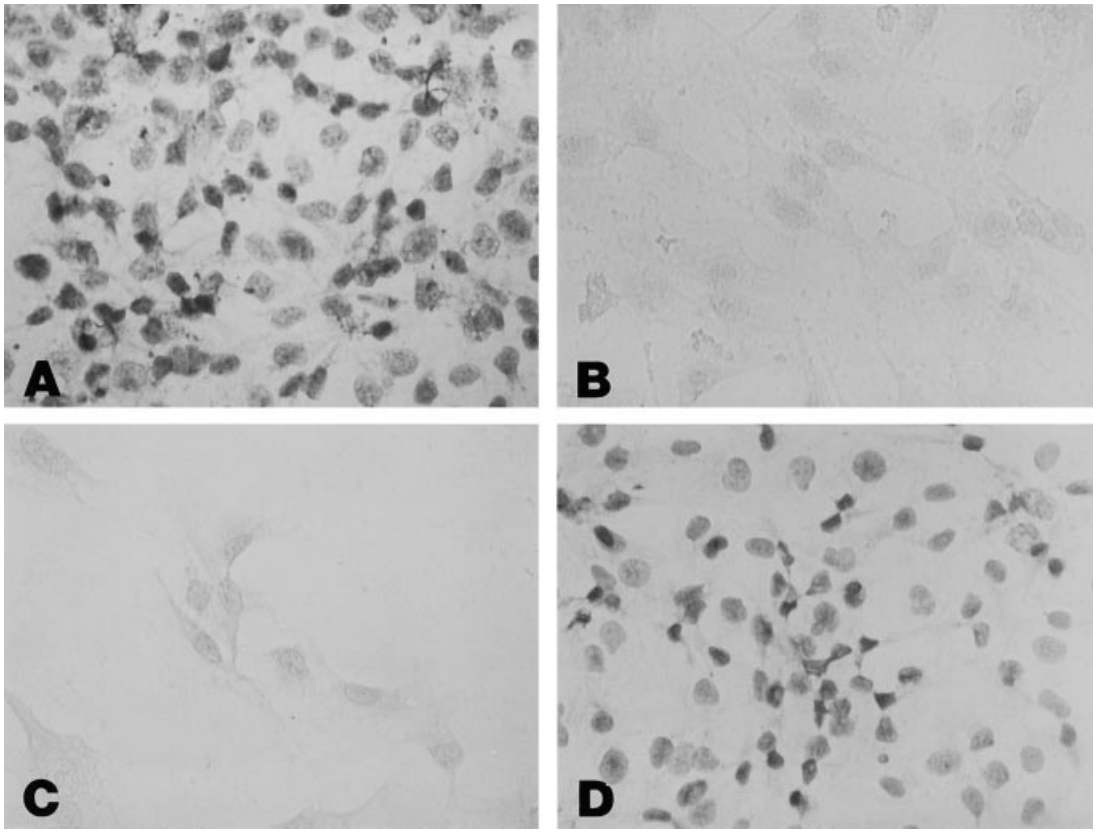


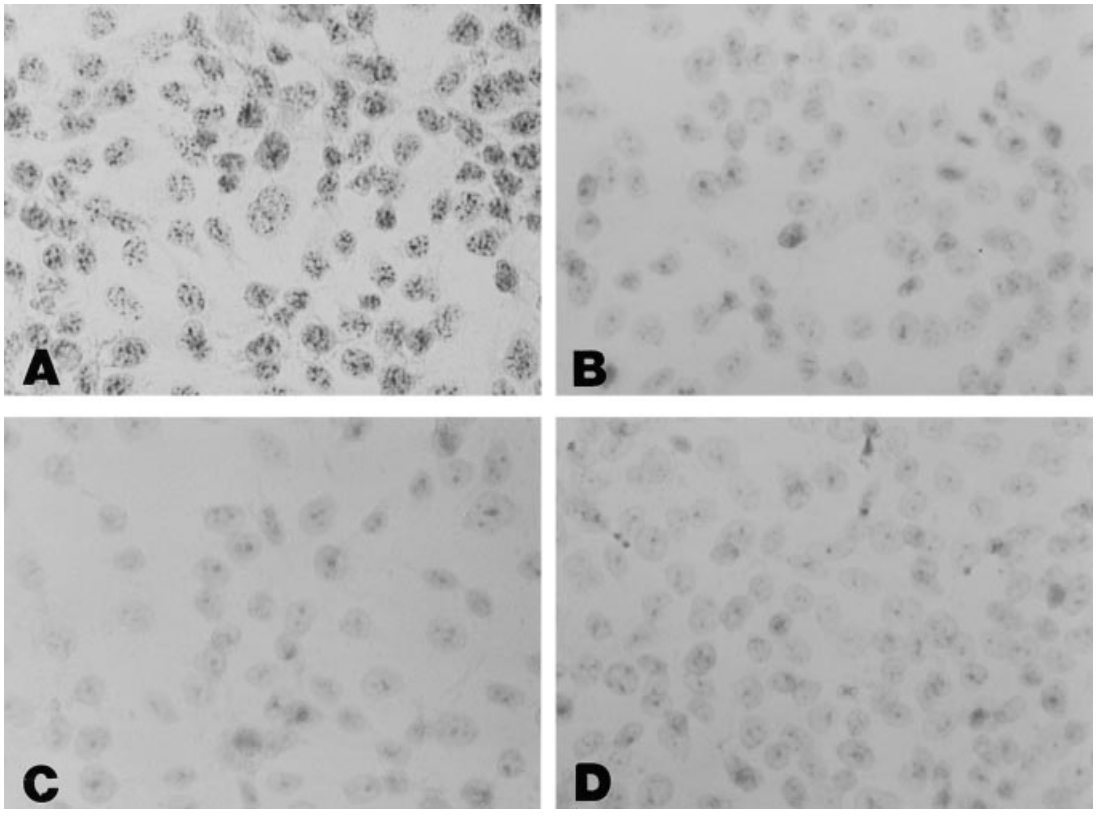
Fig. 5. By Northern blotting, hybridization (*A*) to a probe for inducible nitric oxide synthase (iNOS; upper panel) was detected in RNA from cells cultured in the absence of CsA (lanes 1 through 4), but much more intense bands developed with RNA isolated from cells incubated for 12 hours in the presence of 500 ng/mL CsA (lanes 5 through 8), whether mesangial cells (lanes 1, 5), cortical tubular epithelial cells (lanes 2, 6), murine endothelial cells (lanes 3, 7), or human endothelial cells (lanes 4, 8) are considered. Loading of RNA was equal, as detected by hybridization to the housekeeping gene, GAPDH (lower panel). Densitometric analysis (*B*) confirmed that in each cell line, iNOS RNA bands derived from cells in the presence of CsA were three to six times as intense as those developed with RNA from the same cells under basal conditions. Symbols are: (□) CsA 0 ng/mL; (■) CsA 500 ng/mL.

less CsA effect was observed at a lower concentration (100 ng/mL) that approximates the trough levels at maintenance doses in kidney transplant recipients, reached 12 hours after the last dose of the drug. Nonetheless, even at 100 ng/mL, CsA evoked significant increases in iNOS activity and apoptosis relative to cells maintained in medium alone. The apoptotic effect of CsA was detected by using two independent measures: in situ hybridization TUNEL and gel electrophoresis to disclose intranucleosomal DNA cleavage. The combination of these two methodologies allows a correct evaluation of the apoptotic phenomenon, since TUNEL analysis detects DNA breaks that expose free 3′ bases, an event that could happen not only during apoptosis but also in the early phases of the process of necrosis.

Multiple signals and metabolic events can lead to apoptosis in diverse cell types, but the morphologic features associated with this event are highly consistent [47–51]. This implies that during apoptosis, various signaling pathways converge on a common sequence of events, which has been biochemically identified [47–52]. The ability of a cell to maintain an appropriate oxidant-antioxidant balance is critical, and increased production of reactive oxygen intermediates or a reduction in intracellular scavengers (such as glutathione peroxidase, superoxide dismutase, catalase, and thioredoxin) results in apoptosis. The observation that relative ischemia enhances apoptosis in rat liver [53] supports the view that the pivotal mechanism in CsA-related apoptosis is the ischemia following prolonged vasoconstriction. Apoptosis could be favored by a decreased supply of molecular oxygen, the terminal acceptor of free electrons produced during mitochondrial oxidative phosphorylation. However, in the model reported herein, apoptosis is enhanced in cultured cells, excluding a vasoactive or ischemic mechanism and favoring a direct effect of the drug.

We recently demonstrated that CsA administered to rats in vivo modulates iNOS in kidney homogenates. The resultant increased production of NO counteracts the vasoconstriction induced by CsA, with consequent beneficial effects on renal hemodynamics [33]. However, NO carries an uncoupled electron and acts as a free radical. Furthermore, NO reacts promptly with other free radicals, such as superoxide anion producing peroxynitrite, one of the most potent peroxidant agents known


A
B
C
D


A
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[34–36]. Overall, whereas small physiological "puffs" of NO produced by constitutive NOS may favor cell viability and/or proliferation, the high and sustained amounts of NO produced by iNOS are often cytotoxic [34–36]. Indeed, NO, by acting as a free radical, is able to induce apoptosis in several cell lines, including pancreatic β cells [54] and vascular smooth muscle cells [55]. Moreover, some investigators have postulated that NO-induced intranucleosomal DNA cleavage is responsible for the destruction and dysfunction of pancreatic β cells induced by treatment with streptozotocin, which releases NO [56], and by inflammatory stimuli.

In the present work, we observed significantly increased levels of the mRNA encoding iNOS in each cell line conditioned with CsA; increased translation was demonstrated by immunoperoxidase staining, and the final protein was enzymatically active. We ascribe the increased steady-state mRNA levels to increased transcription, but we cannot exclude the alternative possibility of an increased stability of iNOS mRNA secondary to the effect of CsA. In either case, the final effect observed is increased enzymatic activity.

Several observations collectively support the conclusion that CsA promotes apoptosis via its effect on iNOS. First, the fraction of apoptotic cells peaked between 8 and 12 hours, followed by a plateau phase. These kinetics fit well with the time needed for transcription and translation of iNOS, which requires at least six to seven hours [57], and the consequential enhancement of apoptosis, which requires one to two hours [47–51]. Second, protein synthesis is required because CsA-induced apoptosis is inhibited by CHX. Third, coincubation of cells with CsA in the presence of L-NAME resulted in a significant reduction in the apoptotic rate, parallel to the marked inhibition of NOS activity, whereas the iNOS mRNA remained significantly elevated. L-NAME specifically inhibits NOS by competing with arginine, the natural substrate of NOS. Although the coincidence of an increased apoptotic rate and hyperactivity of NOS enzyme suggests a causal relationship, the nearly total reduction in apoptosis by a specific inhibitor of NOS offers direct evidence that the heightened NOS activity that is elicited by CsA leads to apoptosis. Finally, a causal role for NO in the onset of apoptosis is demonstrated by the increase in apoptotic bodies in all cell lines evaluated in response to treatment with SNP, an NO donor.

The tumor suppressor protein p53 is a key regulatory molecule in apoptosis, and the NO-related apoptotic pathway requires transcription of this gene [37]. This protein, by regulating the expression of *WAF/CIP1* genes and the related protein product p21, induces a modification of cyclin E, which in turn is responsible for the activation of the most active DNA polymerase, the delta isoform [58–60]. In our model, incubation of each of the cell lines with CsA resulted in clear and strong nuclear expression of p53, which was inhibited by blocking the NOS enzymatic activity with L-NAME. Reciprocally, expression of p53 was exacerbated by incubation of the cells with SNP.

The steep relationship between the CsA dose and apparent apoptotic response is probably due to several factors. Foremost, apoptosis is an absolute ("all or none") state; a particular cell either is or is not apoptotic. The apoptotic state is transient because both the entry into apoptosis and removal of apoptotic cells by phagocytosis occur rapidly (approximately 1 to 2 h). All of the cells evaluated herein are capable of internalizing apoptotic cells within hours. In addition, cells primed for apoptosis would not be recognized as apoptotic by TUNEL during the initial phases, prior to DNA fragmentation. Accordingly, a plateau (equilibrium) in the number of apoptotic cells is predicted from the relative rates of formation and removal of apoptotic cells, potentially limiting the "maximal" response to well below 100%, or even below 50% (Table 1). If a dose of CsA can induce apoptosis at all, the plateau will be approached quickly. Similarly, if iNOS synthesis is very rapid but transcription is short lived, the relationship between CsA dose and NOS activity response would be predictably steep.

In conclusion, the data presented herein indicate a role for apoptosis in the progressive cell loss characteristic of chronic CsA nephrotoxicity. The increase in apoptosis is mediated by an enhancement of iNOS activity that, by promoting the expression of p53, triggers a cascade resulting in the intranucleosomal cleavage of DNA. Further and detailed delineation of the molecular basis of this phenomenon could be a starting point towards avoiding CsA nephrotoxicity, while still deriving its immunosuppressive effect.

Fig. 6. Immunoperoxidase staining for iNOS protein was detected in essentially every mesangial cell cultured in the presence of 500 ng/mL CsA (*A*), but not in cells cultured under basal conditions (*B*). Although coincubation with CHX (*C*) prevented such immunohistochemical staining in cells cultured with CsA, coincubation with L-NAME (*D*) had no effect on the staining. Similar results (data not shown) were observed with tubular epithelial and endothelial cells.

Fig. 7. Immunoperoxidase staining for p53 protein was observed in all murine endothelial cells cultured incubated with 500 ng/mL CsA (*A*), but not in unconditioned cells (*B*). The staining in response to CsA was inhibited by coincubation with either CHX (*C*) or L-NAME (*D*). Similar results were observed with mesangial, tubular epithelial, and human endothelial cells (data not shown).

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