Renal tubular epithelial cell death and cyclosporin A

Rene C. Bakker, Cees van Kooten, Marion E. van de Lagemaat-Paape, Mohamed R. Daha and Leendert C. Paul

Department of Nephrology, Leiden University Medical Centre, Leiden, The Netherlands

Abstract

Background. The pathogenesis of chronic cyclosporin A (CsA) nephrotoxicity is largely unknown. In this study we examined whether CsA produces cell death through necrosis or apoptosis of either cultured human proximal tubular epithelial cells (PTEC) or the porcine tubular cell line LLC-PK1.

Methods. Primary isolates of human PTEC and LLC-PK1 cells were treated for various time periods with CsA at concentrations of 0.01–100 μg/ml. Apoptosis was studied by the assessment of annexin binding and propidium iodide uptake, the measurement of cellular DNA content and cell cycle analysis, and by the evaluation of nuclear morphology. Cell death was studied by the trypan blue exclusion method. Hypoxic conditions were simulated through chemical ATP depletion.

Results. In human PTEC, cell death was observed at CsA concentrations higher than 10 μg/ml; at these concentrations PTEC died as a result of necrosis and the toxicity of its vehicle Cremophore EL, and not as a result of CsA inducing apoptosis. The addition of cycloheximide to relieve a possible block in the apoptotic process had no effect on human PTEC, but did result in apoptosis of LLC-PK1. In human PTEC, CsA did not augment cell death induced by chemical ATP depletion.

Conclusions. The results of this in vitro study do not support the hypothesis that CsA directly induces cell death of proximal tubular epithelial cells.

Keywords: apoptosis; cell culture; cyclosporin A; nephrotoxicity; proximal tubular epithelial cell; transplantation

Introduction

Cyclosporin A (CsA) is one of the most widely used drugs in organ transplant patients [1]. CsA-based immunosuppressive regimens are associated with 1-year success rates for kidney transplants of ~90% [2], but a major drawback is CsA renal toxicity. Acute CsA nephrotoxicity is characterized by renal vasoconstriction and is largely reversible upon dose reduction [3]. An irreversible decline in kidney function may also be observed after long-term CsA use and is associated with structural changes such as interstitial fibrosis, tubular atrophy, arteriolar hyalinosis and glomerulosclerosis [4].

The exact pathogenesis of chronic CsA nephrotoxicity remains unknown [1]. Morphological studies reported proximal tubular epithelial cell vacuolization and inclusion bodies early after transplantation during CsA treatment, and animal and human studies have found an increase in the urinary excretion of the proximal brush border enzyme N-acetyl-β-D-glucosaminidase [5]. Moreover, the urinary excretion of β2-microglobulin is enhanced during CsA therapy, suggesting proximal tubular cell damage [6]. It has recently been hypothesized that a high concentration of CsA directly induces tubular cell necrosis and that a lower therapeutic concentration of the drug promotes apoptosis [7]. In both human and animal studies, a higher rate of tubular cell apoptosis has been described during CsA exposure [8,9]; however, it is still not clear whether this increased apoptotic activity is the result of a direct toxic effect of CsA or the result of an indirect mechanism such as ischaemia. The aim of the present study was to examine whether CsA directly induces cell death of cultured proximal tubular epithelial cells by either necrosis or apoptosis.

Materials and methods

Materials

CsA was obtained as Sandimmune®, containing Cremophore EL and alcohol as vehicle (2:1) (Novartis Pharma...
B.V. Arnhem, The Netherlands), and as a powder (Sigma, St Louis, MO, USA), which was dissolved in ethanol. The mouse monoclonal antibody anti-Fas15 was a gift from Professor L.A. Aarden (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Antimycin A (AA), 2-deoxy-D-glucose (DOG) and cycloheximide (CHX) were obtained from Sigma.

**Cell cultures**

All cell cultures were performed in an incubator using a humidified 5% CO$_2$-95% air mixture at 37°C. Human primary proximal tubular epithelial cells (PTEC) were obtained from pre-transplant renal biopsies as described previously [10]. In brief, small fragments of pre-transplant biopsies were placed in 25 cm$^2$ flasks (Costar, Cambridge, MA, USA) coated with a matrix of type I bovine collagen (Sigma) and decocomplemented foetal calf serum (FCS; Gibco BRL, Breda, The Netherlands) in Dulbecco’s modified Eagle’s medium (DMEM)/HAM-F12 at a ratio of 1:1 (Seromed, Biochrom KG, Berlin, Germany) supplemented with insulin (5 µg/ml), transferrin (5 µl/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), tri-iodothyronine (4 pg/ml) and epidermal growth factor (10 ng/ml) (all from Sigma). Medium was replaced every 3 days. The cells grown from the biopsied tissue showed the characteristic morphology of tubular cells and immunofluorescence staining confirmed their proximal descent (Figure 1A and B). Subculturing of these cells was performed in the same type of medium using 25 and 75 cm$^2$ flasks (Costar) coated with FCS only. PTEC between passage 2 and 7 were used for the experiments. The porcine cell line LLC-PK$_1$ was kindly provided by Dr Michael P. Ryan (Department of Pharmacology, University College Dublin, Ireland). The cell line was originally obtained from the ATCC (Manassas, VA, USA), and cells have the characteristics of renal PTEC [11]. LLC-PK$_1$ cells were subcultured in 75 cm$^2$ flasks (Costar) using DMEM culture medium (Seromed) supplemented with 10% (v/v) decocompleted FCS. LLC-PK$_1$ cells were used between passage 210 and 230.

**Fluorescence-activated cell sorter (FACS) analysis**

For FACS analysis, cells were harvested by brief trypsinization to prevent proteolysis of surface receptors. After the cells were washed twice with FACS buffer (1% BSA, 1% decocompleted normal human serum, 0.02% sodium azide in PBS), 10$^5$ cells were incubated with specific monoclonal antibodies against either alanine aminopeptidase (CD13) or Thy-1/CD90 (AS02, Dianova-Hamburg, Germany). After incubation for 45 min at 4°C, cells were washed twice with FACS buffer and subsequently incubated with goat anti-mouse Ig-PE (DAKO) for 30 min at 4°C. Finally, the cells were washed, fixed with 1% paraformaldehyde, and assessed for fluorescence using a FACSscan and LYSIS-II software (Becton Dickinson, Mountain View, CA, USA).

**Cell treatments**

For viability and apoptosis assays, cells were washed with PBS, trypsinized and seeded at concentrations of 1.5 x 10$^5$ (human PTEC) or 0.5 x 10$^5$ (LLC-PK$_1$ cells) in 24-well plates (Greiner, Frickenhausen, Germany) coated with FCS, and grown for 24 h to assure culture subconfluence. They were then washed with PBS and treated for 24 h with CsA dissolved in culture medium, in a humidified incubator supplying a 5% CO$_2$-95% air mixture at 37°C. CsA-containing solutions were prepared by direct dilution of the clinical formulation Sandimmune$^{	ext{R}}$ (CsA 50 mg/ml in Cremophore EL and ethanol (2:1)) in culture medium or by dissolution of CsA powder (Sigma) in absolute ethanol (5 mg/ml), with further dilutions made in culture medium. The final concentrations achieved were checked by a radioimmunoassay and the biological activity was measured in an OKT$_3$

---

**Fig. 1.** (A) Morphological appearance of primary cultures of human PTEC. The characteristic ‘dome’ is the cell layer that has been lifted from the solid surface as a result of active ionic transport processes. (B) FACS analysis using a monoclonal antibody against alanine aminopeptidase (CD13), a cell surface marker that distinguishes proximal from distal TEC but not from fibroblasts, and an antibody against Thy-1/CD90 that is present on fibroblasts but not on TEC. The grey area under the curve represents cells that were incubated with the specific monoclonal antibody, while the white area represents cells that were incubated with the secondary antibody only (see Materials and methods). The strong staining for CD13 and the absence of staining for CD90 confirms the proximal descent of the cultured TEC.
T-cell proliferation assay. Inhibition of T cell proliferation was found with CsA dilutions up to 0.01 μg/ml.

To induce a state resembling tissue hypoxia in vivo, cultured cells were ATP depleted with the use of glucose-free culture medium and the addition of 2 μM Antimycin A, an inhibitor of the mitochondrial respiratory chain, and 5 mM 2-deoxy-D-glucose, an inhibitor of glycolysis.

**Evaluation of cell viability**

Cell viability was evaluated using the trypan blue exclusion assay. In brief, spontaneously detached cells and cells obtained after trypsinization were pooled and tested visually for their ability to exclude the dye. Cells that stained with trypan blue were considered dead.

**Detection of apoptosis**

After the culture supernatant was harvested, cells were washed in PBS and trypsinized to single cell suspensions. Trypsin was subsequently inactivated by the addition of culture medium supplemented with 10% FCS. PBS and the cell suspension were pooled with the supernatant and pelleted by centrifugation for 5 min at 230 g.

For morphological assessment, cells were fixed with 1% paraformaldehyde and kept on ice for at least 10 min. Cytospin specimens were prepared, stained for 3 min with Hoechst 33258 and evaluated by fluorescent microscopy.

For the assessment of phosphatidylserine externalization, cells were washed in 1 ml annexin buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4 adjusted at 4°C), resuspended in 50 μl FITC–annexin V (Nexins Research, Leiden, The Netherlands) (1:250 in binding buffer) and incubated for 15 min in the dark on ice. Prior to measurement, 100 μl of propidium iodide (PI; Molecular Probes, Leiden, The Netherlands) diluted in annexin buffer (final concentration 1 μg/ml) was added. Labelled cells were analysed on a FACScan using the Lysis II software. The percentage of cells binding FITC–annexin V and/or PI was calculated using the WinMDI2.7 software. Cells that were negative or positive for both dyes were considered live or dead, respectively, while apoptotic cells were only positive for FITC–annexin V and/or PI was shown. A significant increase in the number of annexin V, PI positive cells (71% vs medium control 6.8%) was noted when a CsA concentration of 100 μg/ml was used. In the apoptosis control, significantly more cells stained for annexin V alone or for both dyes.

Subsequently, the DNA content of the cells was examined after 24 h of incubation with CsA and the stage of the cell cycle was analysed (Figure 3). A significant increase in the number of cells with a reduced DNA content (sub-G₀/G₁ fraction) was observed only at the highest CsA concentration (100 μg/ml). Next, we incubated PTEC for 24 h with increasing concentrations of CsA, and tested for dead cells by the use of the trypan blue exclusion method or for apoptosis by the evaluation of nuclear morphology (Figure 4). As expected, a concentration-dependent increase in cell death was observed at concentrations > 10 μg/ml. However, no increase in the number of apoptotic cells was found (Figures 4 and 5).

The proximal tubular descent of the cells growing out the tissue was confirmed by their characteristic epitheloid cell shape, their ability to form domes (Figure 1A) and their immunofluorescence staining for alanine aminopeptidase (CD13), a cell-specific marker for PTEC. Isolates were cultured for 24 h in the presence of increasing concentrations of the clinical formulation Sandimmune®, and tested for their ability to bind annexin V and to take up PI. As an apoptosis control we used the mouse monoclonal antibody anti-Fas15 (1 μg/ml) combined with cycloheximide (10 μg/ml) dissolved in culture medium, as described previously [12].

At CsA concentrations of 10 μg/ml or lower, no significant increase in either annexin V binding or PI uptake was observed (Figure 2). Similar results were obtained when the number of cells seeded in the wells was reduced to 25%, the incubation period with CsA was extended to 72 h, or 20% (v/v) serum was added to the media during CsA exposure (results not shown). A significant increase in the number of annexin V, PI positive cells (71% vs medium control 6.8%) was noted when a CsA concentration of 100 μg/ml was used. In the apoptosis control, significantly more cells stained for annexin V alone or for both dyes.

Subsequently, the DNA content of the cells was examined after 24 h of incubation with CsA and the stage of the cell cycle was analysed (Figure 3). A significant increase in the number of cells with a reduced DNA content (sub-G₀/G₁ fraction) was observed only at the highest CsA concentration (100 μg/ml). Next, we incubated PTEC for 24 h with increasing concentrations of CsA, and tested for dead cells by the use of the trypan blue exclusion method or for apoptosis by the evaluation of nuclear morphology (Figure 4). As expected, a concentration-dependent increase in cell death was observed at concentrations > 10 μg/ml. However, no increase in the number of apoptotic cells was found (Figures 4 and 5).

The effect of CsA vs its vehicle on the viability of primary isolates of human PTEC

Next we compared the cytotoxicity of CsA or its vehicle Cremophore EL on human PTEC. PTEC were incubated for 24 h with high concentrations of Sandimmune, starting at 10 μg/ml, or its vehicle at comparable dilutions. Cell death was determined by the trypan blue exclusion assay (Figure 6). The vehicle itself exerted a profound cytotoxic effect, which was at least equal to the effect of Sandimmune at comparable dilutions. To examine the toxicity of CsA alone, CsA powder was dissolved in alcohol and diluted further in culture medium. PTEC were incubated with increasing concentrations of CsA for 24 h. Concentrations up to 10 μg/ml did not result in an increased rate of cell death, as assessed by the trypan blue method or in an increase of cells that displayed morphological signs of apoptosis (data not shown). A higher concentration could not be tested because...
Fig. 2. The effect of Sandimmune® on apoptosis or cell death of PTEC, as assessed by flow cytometric analysis of FITC-annexin V binding and PI staining. Cells were treated for 24 h with medium, the anti-Fas15 monoclonal antibody combined with cycloheximide 10 μg/ml (A), or increasing concentrations of Sandimmune (B). Bottom-right quadrants: cells with externalized phosphatidylserine but still with an intact cell membrane, indicative of cells in early apoptosis. Top-right quadrants: cells positive for both dyes, i.e. late apoptotic or necrotic cells.

Fig. 3. The effect of Sandimmune® on the cell cycle of human PTEC. Human PTEC were incubated for 24 h with the anti-Fas15 monoclonal antibody combined with cycloheximide (A), medium (B), or increasing concentrations of CsA (C–G), and the DNA contents of the cells were analysed on a FACScan. An increase in the number of cells with reduced DNA content was observed at a Sandimmune concentration of 100 μg/ml and in the apoptosis control.
of the inability to dissolve CsA. These results suggest that in primary isolates of human PTEC, the acute cellular toxicity of CsA at concentrations >10 μg/ml is mainly the result of vehicle toxicity and is not caused by the drug.

The effect of combined CsA and cycloheximide on apoptosis in primary isolates of human PTEC and LLC-PK1 cells

The addition of cycloheximide may relieve the resistance to a pro-apoptotic stimulus in PTEC [12], therefore we examined the effect of CsA plus cycloheximide on both human PTEC and the porcine proximal tubular cell line LLC-PK1, for which a pro-apoptotic influence of CsA has been described [7]. Human PTEC or LLC-PK1 were incubated with 1 μg/ml of the clinical formulation Sandimmune combined with cycloheximide (10 μg/ml) or cycloheximide alone for 24 h, and apoptosis was evaluated by nuclear morphology (Figure 7A). In human PTEC, the addition of cycloheximide did not increase the number of cells with apoptotic nuclear morphology, whereas the combination produced apoptosis in 27.5 ± 3.5% of LLC-PK1 cells. Cycloheximide alone, however, induced a comparable degree of apoptosis, disclosing a difference in the regulation of apoptosis between primary isolates of human PTEC and LLC-PK1 cells. Similar results were obtained for PTEC when CsA concentrations of >10 μg/ml were used. A dose–response curve of CsA, with or without 10 μg/ml cycloheximide added to the medium, did not reveal pro-apoptotic features of CsA in LLC-PK1 cells (Figure 7B). Depriving LLC-PK1 cells of serum for 24 h did not change the results, and neither did treatment with CsA dissolved in ethanol. The addition of 20% (v/v) serum to the medium of human PTEC did not prime these cells to enter apoptosis during simultaneous exposure to CsA and cycloheximide (data not shown).

The effect of CsA on cell viability during chemical ATP depletion

Due to the renal vasoconstrictor potential of CsA in vivo and the demonstrated direct inhibitory effect of the drug on the ATP production of isolated mitochondria [3,13], we decided to examine cell death in human PTEC that were cultured under simulated hypoxic conditions and co-exposed to CsA. PTEC were therefore subjected to chemical ATP depletion using glucose-free culture medium, to which 2 μM Antimycin A and 5 mM 2-deoxy-D-glucose were added. The viability of cells was assessed by the trypan blue exclusion assay and apoptosis was evaluated by examining nuclear morphology. After 3, 6, and 24 h of ATP depletion a significant increase in the number of dead cells was observed. The amount of cell death, however, was not influenced by the addition of 1 μg/ml CsA (Figure 8). No increase in apoptosis was noted at any time point or condition (data not shown). The use of a CsA concentration of 10 μg/ml did not change the results.

Discussion

In this study we examined the influence of CsA on the viability of cultured proximal tubular epithelial cells by measuring cell death through either necrosis or apoptosis. Human cells derived from primary isolates and an immortalized porcine cell line were used. Because CsA may be concentrated in renal tissue in vivo [14], and the corresponding levels in vitro have not been determined conclusively, we also examined concentrations that appear supraphysiological (up to 100 μg/ml). The results show that when CsA is used at concentrations as seen in vivo there is no effect on cell viability. Also, preconditioning for apoptosis by either ATP depletion or co-treatment with cycloheximide did not reveal any pro-apoptotic activity of CsA. At very high concentrations of CsA (>10 μg/ml), as used in the clinical formulation of Sandimmune, cultured human PTEC die as a result of necrosis due to vehicle toxicity.

The exact pathogenesis of chronic CsA nephrotoxicity has remained elusive [1]. Histopathological studies have suggested a toxic effect of the drug on afferent arterioles and tubular epithelial cells, as exemplified by hyaline changes in these vessels, morphological alterations in proximal tubular epithelial
cells and a higher rate of tubular apoptosis assessed by the TUNEL assay [4,8]. Evidence has also been presented to show that CsA may directly stimulate various cells in the kidney to locally produce profibrogenic growth factors [15].

Whether human PTEC are a direct target for CsA toxicity remains controversial. In the past, seemingly contradictory results have been obtained using cultured tubular epithelial cells and CsA concentrations achieved in vivo. Two studies reported loss of viability of cultured human PTEC after CsA exposure at CsA concentrations of 0.05 or 1 μg/ml [16,17], whereas another study did not, despite the fact that higher drug concentrations (up to 10 μg/ml) were used [18]. This variance might be explained by differences in the experimental protocols. In the first study [16], PTEC were deprived of essential culture supplements before incubation with CsA, whereas in the second study [17] human PTEC were of foetal origin.

The results of our in vitro study do not support the hypothesis that CsA induces apoptosis of human PTEC directly, as no increase in apoptosis was found over the full range of CsA concentrations tested (0.01–100 μg/ml). These findings are at variance with three reports that examined apoptosis induced by low concentrations of CsA in unspecified human tubular epithelial cells [19], pig proximal tubular epithelial cells [20] or LLC-PK1 cells [7]. The reason(s) for these discrepant results are not yet clear. Two of these previous studies [19,20] examined primary isolates of tubular cells, but used serum in their culture media. In contrast, we did not add serum during the isolation or subculture period of human PTEC in order to prevent undesired outgrowth of non-tubular cells [10]. Differences in the primary cells studied may also be responsible for the variance. In another set of experiments we incubated human PTEC with tacrolimus, for which a similar histopathological pattern of nephrotoxicity has been described as for CsA. Likewise, no loss of cellular viability could be found, with 5 μg/ml the highest concentration tested (data not shown).

To relieve resistance to pro-apoptotic stimuli, cycloheximide has been used successfully in cell culture systems in the past [12]. For PTEC, Fas ligation alone is not sufficient to induce apoptosis, but in combination with cycloheximide, apoptosis is readily detectable.
In our study, co-treatment of CsA and cycloheximide did not unmask a putative pro-apoptotic influence of CsA. Interestingly, we found a difference in regulation of apoptosis between primary isolates of human PTEC and the LLC-PK1 cell line. In LLC-PK1 cells, treatment with cycloheximide alone resulted in an increase in the number of cells entering apoptosis. This indicates that LLC-PK1 cells are a less suitable model for human PTEC when apoptosis is studied.

In the present study we also decided to analyse the effect of CsA on PTEC that were chemically depleted of ATP, because CsA induces renal vasoconstriction in vivo and has an inhibitory effect on the ATP production of isolated mitochondria [13]. We demonstrate that CsA does not affect cell death of ATP-depleted cultured human PTEC. However, this does not exclude the possibility that in vivo tissue hypoxia due to vasoconstriction or obstruction of afferent renal arterioles is still responsible for tubular cell apoptosis during CsA treatment, as has been suggested by the results of a study in salt-depleted rats [9]. In this study, CsA treatment produced an increase in apoptosis in tubular cells, which was partially reversed by co-treatment with losartan, an angiotensin II type 1 receptor antagonist, or with L-arginine, a substrate for nitric oxide synthetase.

We conclude that the cellular viability of cultured adult human PTEC is not influenced by short-term exposure to CsA at physiological concentrations during normoxic or simulated hypoxic experimental conditions. At very high drug concentrations, cultured human PTEC die as a result of cell necrosis, an effect that might solely be based on vehicle toxicity.

References


---

**Fig. 7.** The effect of Sandimmune® combined with cycloheximide on apoptosis of human PTEC and LLC-PK1 cells (A). Human PTEC and LLC-PK1 cells were treated for 24 h with cycloheximide (10 μg/ml) with or without Sandimmune® 1 μg/ml, and apoptosis was evaluated by examining nuclear morphology. (B) Dose-response curve of 24-h Sandimmune® treatment of LLC-PK1 in the presence of cycloheximide. In LLC-PK1 cells, cycloheximide treatment produced a significant increase in apoptosis. However, no effect of CsA either alone or in combination with cycloheximide was noted. Results are expressed as the mean ± SD of experiments performed in triplicate wells (n = 3) (A), or the mean ± SEM of experiments performed in duplicate (n = 3) (B). *P < 0.05; **P < 0.01.

**Fig. 8.** The effect of chemical ATP depletion and Sandimmune® treatment on the viability of human PTEC. Cells were exposed for 3, 6 and 24 h to Antimycine A (2 μM) and 2-deoxy-D-glucose 5 mM with or without Sandimmune® 1 μg/ml dissolved in glucose-free culture medium. Cell death was measured by the trypan blue exclusion assay. Results are expressed as the mean ± SEM of experiments performed in triplicate wells (n = 5).

Received for publication: 11.8.01
Accepted in revised form: 14.2.02