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Apoptosis and necrosis: Mechanisms of cell death induced by cyclosporine A in a renal proximal tubular cell line

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Apoptosis and necrosis: Mechanisms of cell death induced by cyclosporine A in a renal proximal tubular cell line.

Background. The mechanisms of cyclosporine (CsA)-induced nephrotoxicity are not fully understood. While hemodynamic changes may be involved *in vivo*, there is also some evidence for tubular involvement. We previously showed direct toxicity of CsA in the LLC-PK_1 renal tubular cell line. In the current study we examined mechanisms (apoptosis or necrosis) of cell death induced by CsA in the LLC-PK_1 renal proximal tubular cell line. The possible role of the Fas (APO-1/CD95) antigen-Fas ligand system in the mediation of CsA-induced cell death was also investigated.

Methods. Cells were treated with CsA (0.42 nM to 83 μM) for 24 hours and alterations in DNA and protein synthesis and membrane integrity were examined. Flow cytometry was used to investigate: (*i*) alterations in the DNA content and cell cycle; (*ii*) the forward (FSC) and side (SSC) light scattering properties (indicators of cell size and granularity, respectively); (*iii*) the externalization of phosphatidylserine (PS) as a marker of early apoptosis using FITC-annexin V binding; and (*iv*) expression of the apoptotic Fas protein. DNA fragmentation in apoptotic cells was also determined by the TUNEL assay.

Results. CsA (all doses) caused a block in the G_0/G_1 phase of the cell cycle as indicated by a decrease in DNA synthesis and supported by an increase in the % of cells in the G_0/G_1 phase with concurrent decreases of those in the S and G_2/M phases. The effect on protein synthesis appeared to be much less. Lower doses of CsA (4.2 nM) caused the appearance of a "sub-G_0/G_1" peak, indicative of reduced DNA content, on the DNA histogram that was paralleled by a reduction in cell size and an increased cell granularity and an increase in FITC-annexin V binding. DNA fragmentation was evident in these cells as assessed using the TUNEL assay. Higher doses of CsA increased cell size and decreased cell granularity and reduced membrane integrity. Expression of Fas, the cell surface molecule that stimulates apoptosis, was increased following low dose CsA exposure.

Conclusions. These results indicate that CsA is directly toxic to LLC-PK_1 cells with reduced DNA synthesis and cell cycle blockade. The mode of cell death, namely apoptosis or necrosis, is dose dependent. Fas may be an important mediator of CsA induced apoptosis in renal proximal tubular cells.

Key words: CsA, nephrotoxicity, cell cycle blockade, proximal tubular cells.

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The immunosuppressive agent cyclosporine A (CsA) is widely used to treat allograft rejection and in the treatment of various autoimmune disorders [1]. A major limiting factor in the use of CsA is nephrotoxicity which still remains a significant problem [2]. CsA nephrotoxicity is associated with acute changes in renal hemodynamics followed by irreversible tubular injury [3]. Morphological evidence suggests that the early sublethal tubular damage is confined to the S3 segment of the proximal tubule. This includes swelling of the endoplasmic reticulum and appearance of large vacuoles [4]. Whether these tubular alterations are secondary to hemodynamic changes or reflect direct tubular toxicity of CsA has not been established. With *in vivo* experiments it is difficult to distinguish between these possibilities. We have previously shown, using an *in vitro* model system, that CsA is directly toxic to renal tubular cells in culture with a relative site selective action to the proximal tubular region of the nephron [5, 6]. In addition, low doses of CsA were seen to cause sustained elevations in intracellular calcium prior to a loss in cellular viability.

Cellular injury can trigger a variety of responses including adaptation, repair, proliferation and cell death in the form of either apoptosis or necrosis. Apoptosis is a genetically controlled form of cell death distinct to necrosis that can serve as a molecular selection process for both normal physiological processes and during toxic damage and disease states [7, 8]. It is characterized by cell shrinkage, chromatin condensation and systematic cleavage of DNA into intranucleosomal length fragments by endonuclease activity that may be calcium or magnesium dependent [9]. Apoptosis has been implicated as an important pathogenic mechanism in renal disease [10] and has been shown to occur both during renal development and following renal injury such as that observed in acute renal failure and chronic glomerulonephritis. The renal proximal tubular epithelial cell has been shown to be a target for apoptotic cell death following ischemic or toxic injury [11, 12], and indeed apoptosis has been shown to play a crucial role in the growth and differentiation of proximal tubular cells in primary culture [13].

The Fas (APO-1/CD95) antigen-Fas ligand system mediates apoptotic cell death in a number of cell types and Fas up-regulation has been observed in glomerulonephritis [14]. Furthermore, agonistic anti-Fas antibodies have been shown to induce glomerular cell apoptosis in mice *in vivo* [15].

It has been postulated that the form of cell death recruited by the cell following toxic injury is very much dose dependent [16]. Cell death induced in proximal tubular epithelial cells by the nephrotoxic agent, cisplatin, was shown to be concentration dependent, with low concentrations inducing apoptosis and higher concentrations inducing necrosis [17].

We have examined the mechanisms of cell death induced by CsA in the LLC-PK_1 renal proximal tubular cell line. The studies demonstrated that high concentrations of CsA (μM range) induce necrosis whereas lower concentrations (nM range) induce cell death with characteristics typical of apoptosis. Fas may be an important mediator of CsA induced apoptosis in renal proximal tubular cells.

METHODS

Materials

Cyclosporine A was a generous gift from Novartis Pharmaceutical Ltd. The mouse monoclonal Fas antibody was obtained from Dr. Hugh McGlynn, University of Ulster, Coleraine, Northern Ireland, who had originally obtained it as a gift from Itoh and co-workers [18]. The experiments were also repeated and replicated using a commercially available rabbit polyclonal Fas antibody (Santa Cruz Biotechnology). All other reagents were of the highest available purity from commercial sources.

Cell culture

LLC-PK_1 cells were obtained from ATCC (USA). Cells were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, incubated at 37°C in 95% O_2/5% CO_2 and grown to confluency in 75 cm^2 flasks (Costar).

Cell treatment

CsA was prepared as a stock solution (4.2 mM) by dissolving the powder in 100% ethanol. For DNA and protein synthesis assays, cells were seeded into 24 well plates (Greiner) at a density of 2×10^4 cells/ml. Twenty-four hours later they were treated with CsA (0.42 nM to 83 μM) or with vehicle alone (1:100 ethanol) for 24 hours. For flow cytometry cells, were cultured in 25 cm^2 costar flasks (1×10^6 cells/flask).

Estimation of DNA and protein synthesis

DNA and protein synthesis were measured by ^{3}H-thymidine and ^{14}C-leucine incorporation, respectively. Cells were incubated in 1 ml of DMEM containing either 0.25 μCi ^{3}H-thymidine/ml or 0.25 μCi ^{14}C-leucine/ml for four hours at 37°C. Uptake was terminated by washing 4 times in ice-cold PBS and the cells were solubilized in 2% (wt/vol) SDS. The radioactivity was determined by liquid scintillation counting.

Flow cytometric DNA analysis

Single cell suspensions were prepared by treating the cells with a trypsin/EDTA mixture in DMEM. Spontaneously detached cells present in the culture medium were also included in the analysis. Cell suspensions (1×10^6) were pelleted, resuspended in 200 μl of PBS and fixed for at least 30 minutes at 4°C in 2 ml of 70% ice-cold ethanol. Cells were washed twice in PBS, resuspended in 800 μl PBS to which was added 100 μl of 1 mg/ml RNAse A (Sigma) and 100 μl of 400 μg/ml propidium iodide (PI), and allowed to incubate for 30 minutes at 37°C. Cells were analyzed on a FACStar Plus Flow Cytometer (Becton Dickinson, Aalst, Europe) with laser excitation at 488 nm using a 639 nm band pass filter to collect the red PI fluorescence. Forward (FSC) and side (SSC) light scatter were also recorded as indices of cell size and granularity, respectively. The percentages of cells in the various phases of the cell cycle namely G_0/G_1, S and G_2/M were assessed using the CELL-FIT™ program on the flow cytometer.

Determination of cell injury

Cellular viability was measured by assessing trypan blue exclusion and lactate dehydrogenase (LDH) release as previously described [16, 19].

TUNEL staining

DNA fragmentation was assessed using an Apoptosis Detection System, Fluorescein, from Promega Corp. (USA). In brief, cells were cytospun onto glass slides and fragmented DNA of apoptotic cells was measured by the catalytic incorporation of fluorescein-12-dUTP at the 3′-OH ends using the enzyme Terminal deoxynucleotidyl Transferase [20]. The fluorescein-12-dUTP-labeled DNA was visualized using a fluorescent microscope.

FITC-annexin V binding

FITC-annexin V binding was determined using an apoptosis detection kit (NX50) commercially available from R&D Systems (Europe). In brief, cells were resuspended in $1 \times$ binding buffer [21] at a concentration of 1×10^6 cells/ml. One hundred microliters of the cells (1×10^5) were incubated with FITC-conjugated annexin V (1 μg/ml) for 15 minutes at room temperature. Labeled cells were analyzed on a FACStar Plus flow cytometer with laser excitation at 488 nm using a 530 nm band pass filter to collect the FITC fluorescence. Membrane integrity was determined immediately after flow cytometric analysis using trypan blue exclusion as described above.

Fas flow cytometric immunofluoresence

Single cells suspensions (1×10^6) were washed in 1 ml of PBS. They were incubated for 30 minutes on ice with 1:100 monoclonal (mouse) anti-Fas antibody diluted in PBS. Following three washes in PBS the presence of bound antibody was detected by incubating with 1:100 goat anti-mouse FITC conjugated antibody (DAKO, UK) in PBS for 30 minutes on ice. Labeled cells were washed twice in PBS and analyzed on a FACStar Plus flow cytometer with laser excitation at 488 nm using a 530 nm band pass filter to collect the FITC fluorescence. The appropriate controls were maintained by the omission of the primary antibody or by its replacement with pre-immune serum.

Western blot analysis of Fas expression

Twenty micrograms of LLC-PK_1 whole cell extracts were resolved on 12% SDS-PAGE. The gel was blotted to nitrocellulose using a semi-dry transfer system. For the detection of Fas expression, the blot was then blocked for one hour at room temperature with PBS containing 5% (wt/vol) milk fat proteins/PBS. Following three washes in PBS and in PBS-0.05% (vol/vol) tween-20 (PBS-T) the blot was incubated for two hours with 1:100 polyclonal (rabbit) anti-Fas antibody (Santa Cruz Biotechnology) diluted in 3% milk fat proteins/PBS. The blot was washed again and incubated with a goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Promega Corp., USA). The blot was developed using enhanced chemiluminesence (Boehringer Mannheim).

Data analysis

Results are expressed as the mean ± SEM from three to six independent experiments. Data were statistically analyzed by one-way ANOVA followed by the Dunnett post-test and $P < 0.05$ was deemed significant.

RESULTS

DNA and protein synthesis

CsA treatment for 24 hours in LLC-PK_1 cells significantly decreased DNA synthesis in a dose dependent manner as assessed using ^{3}H-thymidine incorporation (Fig. 1A). Cells were treated on day 2 of culture in the log phase of growth and therefore appeared very sensitive to CsA induced alterations in DNA synthesis. Previous studies from our laboratory have shown that necrosis, as assessed using neutral red uptake and MTT conversion, was not evident in LLC-PK_1 cells treated with doses of CsA less than 0.42 μM [5]. However, in this study, very low doses of CsA (below 0.42 μM) were observed to significantly inhibit DNA synthesis, indicating that DNA damage is a very sensitive indicator of CsA induced nephrotoxicity.

CsA also caused dose dependent decreases in protein synthesis as measured using ^{14}C-leucine incorporation (Fig. 1B). However, much higher doses of CsA (21 μM or greater) were required to inhibit protein synthesis than were required to effect DNA synthesis, suggesting that protein synthesis is less sensitive to CsA induced damage.

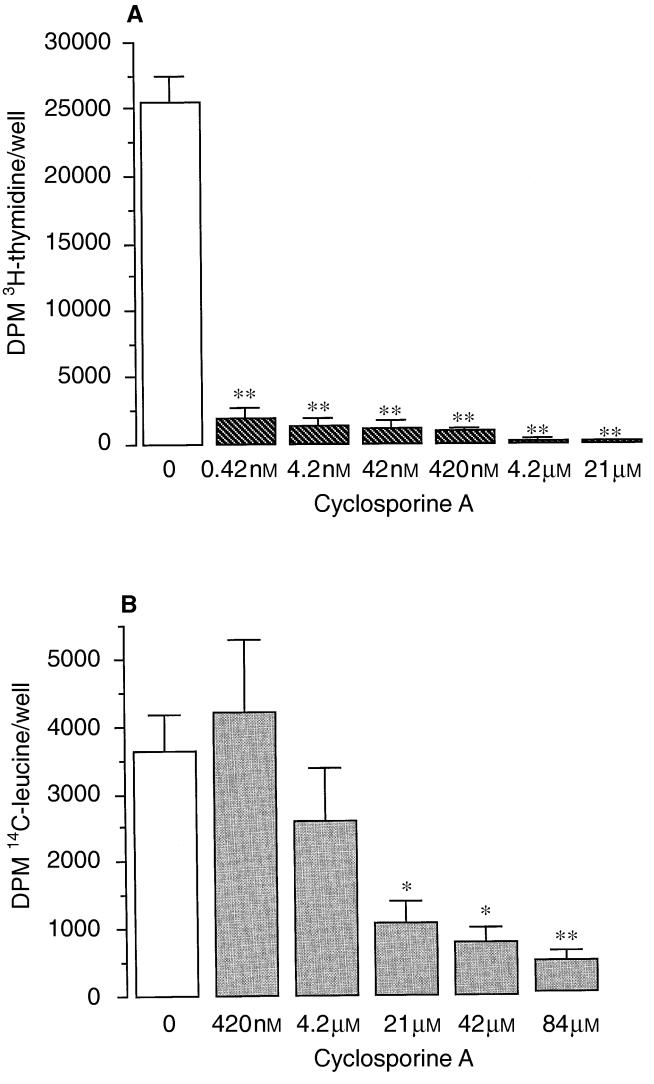


Fig. 1. Effect of cyclosporine (CsA) on DNA and protein synthesis in LLC-PK_1 cells. (*A*) ^{3}H-thymidine incorporation was used as an index of DNA synthesis. (*B*) ^{14}C-leucine incorporation was used as an index of protein synthesis. In both cases results are expressed as the DPM of radioisotope incorporated per well and represent the mean ± SEM of 3 independent experiments each performed in quadruplicate. $^*P < 0.05$, $^{**}P < 0.01$.

Flow cytometric cell cycle analysis

Flow cytometric analysis of the cell cycle of LLC-PK_1 cells provided compatible evidence for the decrease in DNA synthesis observed by ^{3}H-thymidine incorporation. CsA treatment for 24 hours caused dose dependent increases in the percentage of cells in the G_0/G_1 (resting) phase that was paralleled by decreases in the percentages of cells in the S (DNA synthesis) and G_2/M (mitotic/dividing) phases (Table 1). For example, at 4.2 nM CsA, the following

Table 1. Dose-response effects of cyclosporine (CsA) on the cell cycle and apoptosis in LLC-PK_1 cells

Treatment	% Cells in each phase of the cell cycle excluding apoptotic cells			
Cyclosporine A	% G_0/G_1	% S	% G_2/M	% Apoptosis
Control	47 ± 4	28 ± 3	28 ± 5	3 ± 1
0.42 nM	65 ± 9[a]	22 ± 4	13 ± 5	N/D
4.2 nM	64 ± 4[a]	18 ± 4	18 ± 1	21 ± 5[b]
42 nM	67 ± 6[a]	16 ± 2[a]	17 ± 4	14 ± 1[b]
420 nM	64 ± 4[a]	16 ± 1[a]	20 ± 3	12 ± 2[b]
4.2 μM	77 ± 3[b]	12 ± 3[b]	11 ± 1[b]	8 ± 1[b]
21 μM	85 ± 1[b]	5 ± 1[b]	10 ± 1[b]	6 ± 1

Abbreviations are in the Appendix. LLC-PK_1 cells were treated on day 2 of culture with 0.42 nM to 21 μM CsA for 24 hr. The percentage of cells in each phase of the cell cycle excluding apoptotic cells was analysed using the CELLFIT™ program on a BD FACStar Plus Flow Cytometer. Results are expressed as the percentage of cells in the appropriate phase (% of 10,000 events) and represent the mean ± SEM of 3 to 6 independent experiments. The percentage of apoptotic cells was quantified as the percentage of total cells displaying reduced DNA stainability in the sub-G_0/G_1 peak.

[a] $P < 0.05$, [b] $P < 0.01$

changes from control were detected: G_0/G_1 phase, 47 ± 4 to 64 ± 4; S phase, 28 ± 3 to 18 ± 4; and G_2/M, 28 ± 5 to 18 ± 1 (Table 1). It can be seen that very low doses of CsA (including 0.42 nM) were capable of mediating the cell cycle changes seen, providing further evidence for the importance of DNA damage in CsA induced toxicity.

Cellular injury assays

The cellular injury induced by representative high (42 μM) and low (4.2 nM) doses of CsA was also assessed by investigating trypan blue exclusion and LDH release (Fig. 2). A dose of 42 μM CsA caused a 20% decrease in membrane integrity with only 80.1 ± 3.3% of cells excluding trypan blue after CsA (42 μM) exposure ($N = 6$, $P < 0.01$, Fig. 2A). No significant alteration in membrane integrity was observed in cells treated with 4.2 nM CsA, as indicated by the finding that 97.5 ± 0.6% ($N = 6$) of cells excluded trypan blue. Furthermore, 42 μM CsA caused a significant increase in LDH release (1.4 ± 0.03-fold over basal, $P < 0.01$, Fig. 2B) whereas 4.2 nM CsA caused no significant increase in LDH release in LLC-PK_1 cells.

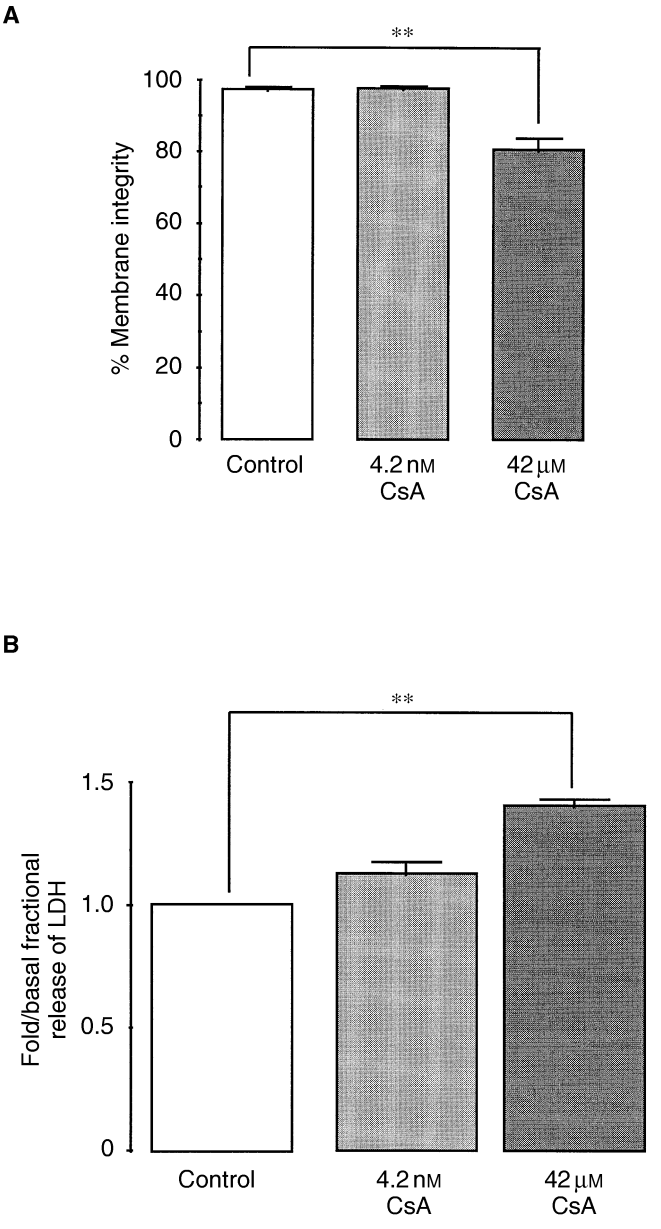


Fig. 2. Effect of cyclosporine (CsA) on trypan blue exclusion and LDH release in LLC-PK_1 cells. LLC-PK_1 cells were exposed to 4.2 nM and 42 μM CsA for 24 hours. (*A*) For trypan blue exclusion (% membrane integrity) cells were trysinized, mixed with trypan blue and viewed under a light microscope. Cells that were blue were scored as having an altered membrane integrity. (*B*) Fractional release of LDH was determined by the equation S/(S + C); where S = LDH in the supernatant and C = LDH in the monolayer, after cell lysis. Results represent the mean ± SEM of 3 to 6 independent experiments. $^{**}P < 0.01$.

Flow cytometric assessment of apoptosis using DNA analysis and light scatter measurements

Figure 3 panels A, B and C depicts flow cytometric DNA histograms where the amount of fluorescence emitted is directly proportional to the amount of DNA present in the cells. In all cases cellular debris was gated out on the basis of cell scatter prior to analysis. CsA treatment for 24 hours with a low dose (4.2 nM) was seen to cause the appearance of a "sub-G_0/G_1" peak on the DNA histogram (Fig. 3B), which was absent in both the control cells (Fig. 3A) and the cells treated with 4.2 μM CsA (Fig. 3C). This "sub-G_0/G_1" peak represents a population of cells with reduced DNA stainability that is most likely due to DNA fragmentation. The gates for the "sub-G_0/G_1 peak" were established by using cells that were treated with cisplatin, a known inducer of apoptosis in renal cells [17]. Cisplatin (50 μM) was observed to cause the appearance of a "sub-G_0/G_1" peak that represented 40% of the total cell population (results not shown).

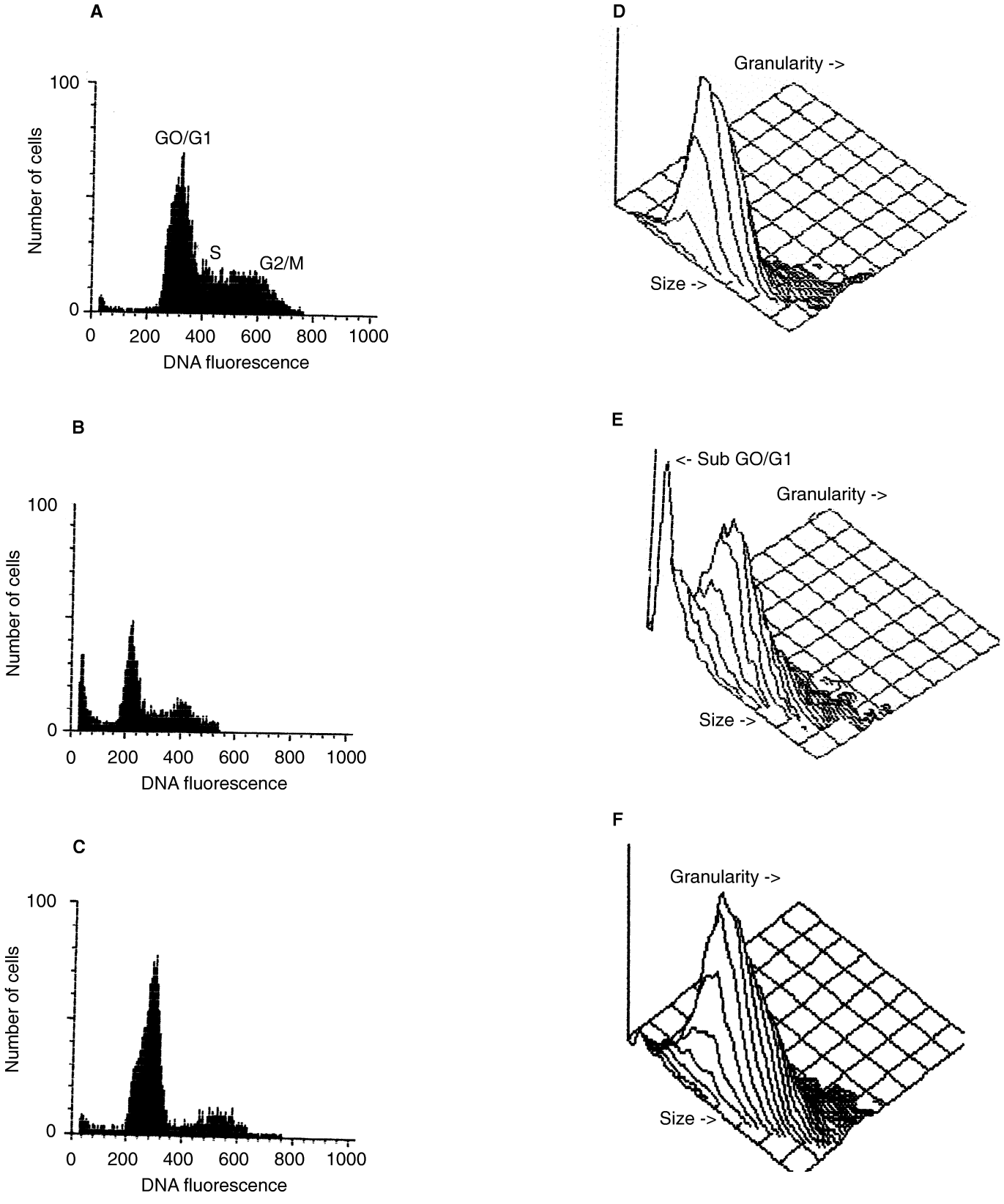


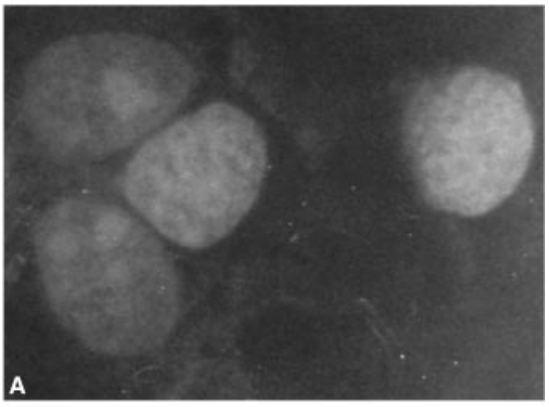
Fig. 3. Flow cytometric analysis of the effect of cyclosporine (CsA) treatment for 24 hours on the DNA profile, size and granularity of LLC-PK$_1$ cells. Results from one representative experiment that represents 10,000 events (cells) are shown. (*A*) DNA profile of control cells. (*B*) DNA profile of 4.2 nM CsA treated cells. (*C*) DNA profile of 4.2 μM CsA treated cells. (*D*) Light scattering properties of control cells; FSC, size and SSC, granularity. (*E*) Light scattering properties of 4.2 nM CsA treated cells. (*F*) Light scattering properties of 4.2 μM CsA.

Table 2. Time-dependent induction of apoptosis by cyclosporine (CsA 4.2 nM) in LLC-PK_1 cells

Time *hr*	% Apoptosis
0	3 ± 1
2	4 ± 1
4	3 ± 1
6	5 ± 1
8	5 ± 1
12	6 ± 1
24	14 ± 3[a]

LLC-PK_1 cells were treated for 2 to 24 hr with 4.2 nM CsA. The percentage of apoptotic cells was determined by PI staining and was quantified as the percentage of cells displaying reduced DNA stainability in the sub G_0/G_1 peak. Results are expressed as the mean ± SEM of 3 independent experiments.

[a] $P < 0.01$



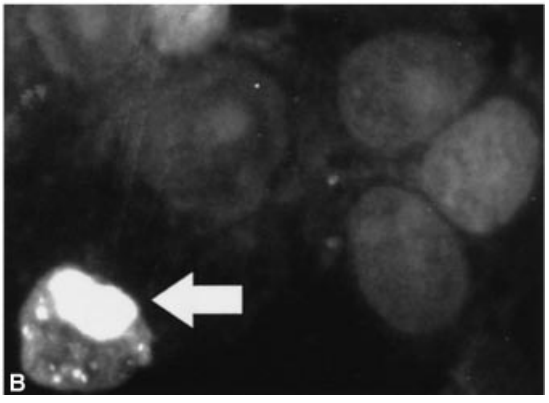


Fig. 4. Apoptosis detection following cyclosporine (CsA) treatment using the TUNEL assay. Cells were treated for 24 hours with 4.2 nM CsA and apoptotic cells were detected using the TUNEL assay. Arrows indicate TUNEL positive cells. (*A*) Cells treated with vehicle alone. (*B*) Cells treated for 24 hours with 4.2 nM CsA.

On examination of the light scattering properties of the control cells (Fig. 3D) one main population could be observed that appeared to have a normal distribution. However, analysis of the cells treated with 4.2 nM CsA (Fig. 3E) revealed two cell populations: one major population and also a subpopulation. The major population had similar characteristics to that of the control cells. The subpopulation displayed a decrease in forward light scatter (FSC) and an increase in side scatter (SSC) indicative of cell shrinkage and increased granularity, respectively, both indices representing characteristics of apoptotic cell death. The DNA fluorescence of this subpopulation when gated appeared to be equal to that of the "sub-G_0/G_1" peak seen in Figure 3B. This subpopulation was not apparent in the cells treated with 4.2 μM CsA (Fig. 3F).

Time dependent induction of apoptosis by 4.2 nM CsA

Table 2 shows a time course for the induction of apoptosis as assessed by DNA analysis and appearance of the "sub-G_0/G_1" peak in LLC-PK_1 cells. It can be seen that CsA induced apoptosis in a time dependent manner, with the maximum apoptosis occurring at 24 hours post-treatment (14 ± 3% of cells, $N = 3$, $P < 0.01$).

TUNEL staining

The fragmented DNA of apoptotic cells was measured by the catalytic incorporation of fluorescein-12-dUTP at the 3′-OH ends using the enzyme terminal deoxynucleotidyl transferase. TUNEL positive cells were not present in the control cells (Fig. 4A). However, in cells treated for 24 hours with 4.2 nM CsA, fluorescence (arrow) indicative of the fluorescein-12 dUTP incorporation into apoptotic cells was apparent (Fig. 4B).

Use of flow cytometric light scatter measurements to discriminate between viable, apoptotic and necrotic cells

The comparative effects of high (42 μM) and low (4.2 nM) doses of CsA on the light scattering properties of the LLC-PK_1 cells are shown in a representative experiment in Figure 5. In the control cells (Fig. 5A), the majority of cells (81%) were in one major population that was taken to represent viable cells. Approximately 6% of control cells appeared in a region of increased SSC and decreased FSC, indicative of increased granularity and decreased cell volume, respectively, and which was termed the "apoptotic" region. Treatment with 4.2 nM CsA (Fig. 5B) increased the proportion of cells in this "apoptotic" population to 15%. In contrast, treatment with the higher 42 μM dose (Fig. 5C) caused an appearance of a third population (35% of total population) with increased FSC and decreased SSC. This population, which has an increased cell size and a decreased cell granularity, was described as "necrotic."

FITC-annexin V binding

The externalization of phosphatidylserine was assessed by measuring FITC-annexin V binding by flow cytometry (Fig. 6). One representative experiment out of three is shown. It can be seen that treatment with CsA (4.2 nM) for

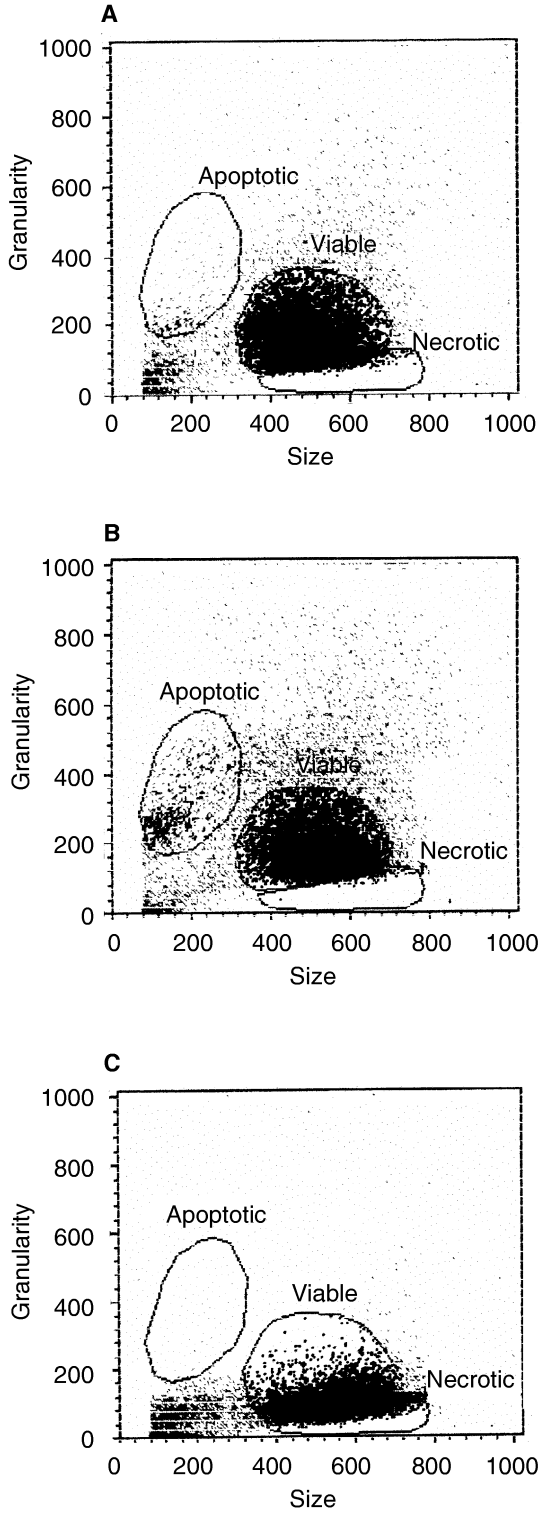


Fig. 5. Flow cytometric assessment of the effects of a low and high dose of cyclosporine (CsA) on the size and granularity of LLC-PK$_1$ cells. Forward light scatter (FSC) and side light scatter (SSC) of the laser beam were used as indices of size and granularity, respectively. Results from one representative experiment which represent 10,000 events (cells) are shown. (*A*) Control. (*B*) 4.2 nM CsA. (*C*) 42 μM CsA.

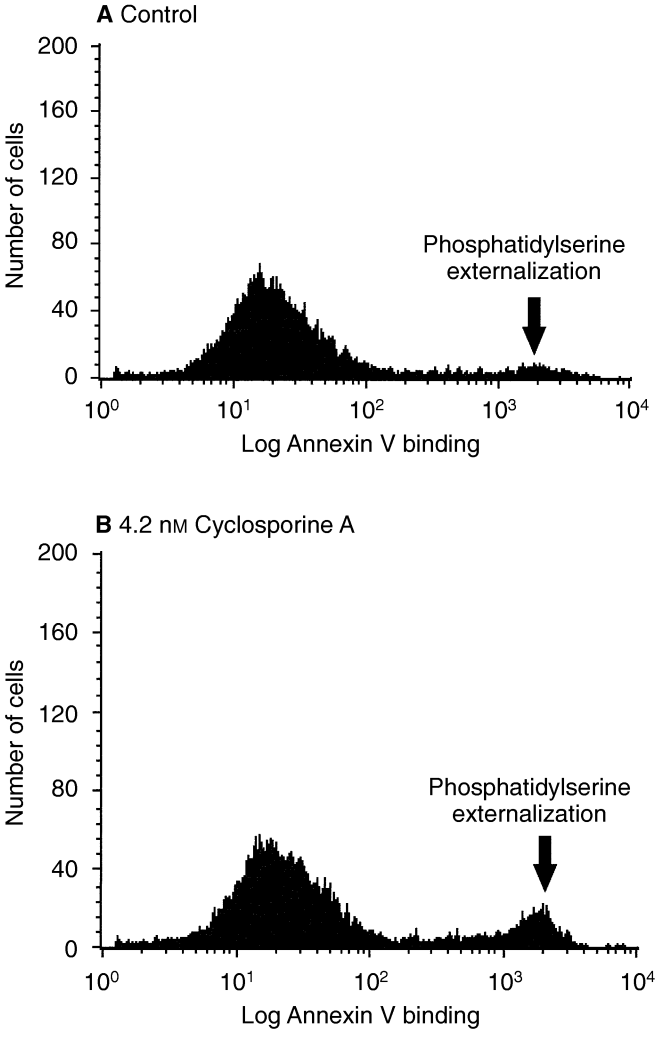


Fig. 6. FITC-annexin V binding in LLC-PK$_1$ cells following cyclosporine (CsA) exposure. Cells were treated for 24 hr with 4.2 nM CsA. The externalization of phosphatidylserine was assessed by measuring FITC-annexin V binding. The far right peak represents cells which have bound FITC-annexin V. One representative experiment out of 3 is shown.

24 hours induced an increase in the percentage of cells displaying annexin-V binding from 6% in control cells to 12% in treated cells. These cells displayed an intact plasma membrane as indicated by trypan blue exclusion.

Alterations in Fas expression

Alterations in Fas expression following CsA treatment are shown in Figure 7. Low dose CsA (4.2 nM) treatment for 24 hours was seen to cause a significant elevation in the expression of Fas in LLC-PK$_1$ cells (Fig. 7A, $N = 3$, $P < 0.05$), as evidenced by flow cytometric immunofluoresence techniques (Fig. 7B). This was supported by Western blot analysis where an increase in the expression of the 45 kD Fas protein was seen (Fig. 7C). In both cases, the appropriate isotype controls were included (results not shown).

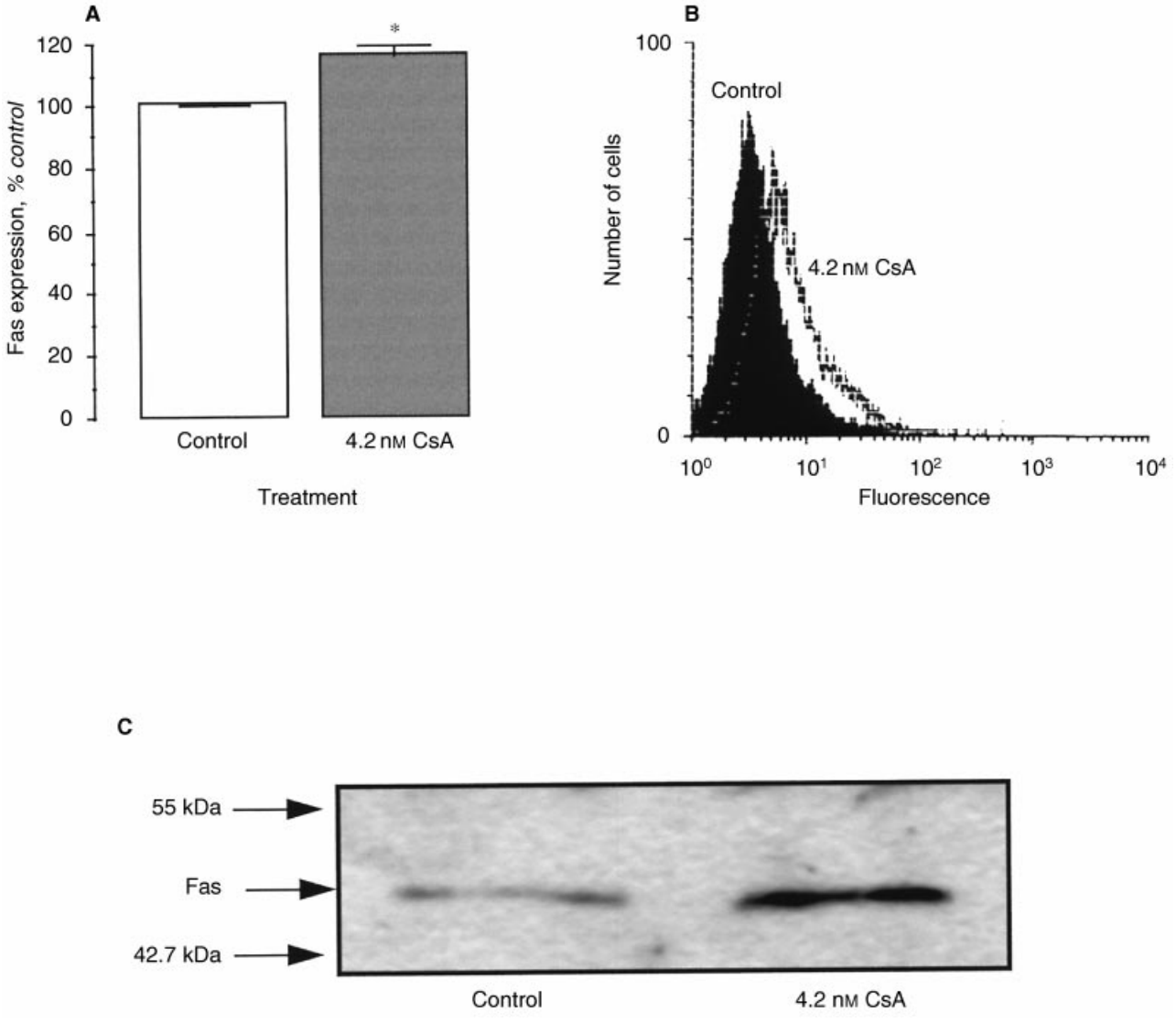


Fig. 7. Effect of cyclosporine (4.2 nM CsA) on the expression of Fas in LLC-PK$_1$ cells. Cells were treated for 24 hours with 4.2 nM CsA, and Fas expression was assessed using flow cytometric immunofluoresence and Western blotting technqiues as described in the Methods section. (*A*) Graphical representation of Fas expression as assessed by flow cytometric immunofluoresence. Results are expressed as the percentage of control cell Fas expression and represent the mean ± SEM of 3 independent experiments. $^*P < 0.05$. (*B*) Flow cytometric histogram illustrating the increase in Fas expression following CsA treatment. (*C*) Western blot analysis of Fas expression.

Effects of the Fas antibody in the presence and absence of CsA on the light scattering properties of LLC-PK$_1$ cells

The light scattering properties of cells incubated in the absence of primary anti-Fas antibody are shown in Figure 8A. Examination of the light scattering properties of both non-CsA treated (Fig. 8B) and CsA treated cells (Fig. 8C) that were both incubated with the Fas antibody demonstrated increases in the proportions of "apoptotic" cells in both cases in up to 32% and 70% of the total population, respectively. Therefore, this "apoptotic" population was much more evident in the CsA treated cells (Fig. 8C).

DISCUSSION

In this study, we have demonstrated that CsA has direct toxic effects on the renal proximal tubular LLC-PK$_1$ epithelial cell line. All doses of CsA used caused a blockade in the G_0/G_1 phase of the cell cycle. Low dose (4.2 nM) CsA induced apoptosis whereas high dose (21 μM or greater) CsA induced necrosis. Other investigators have reported that severe injury to tissues usually results in necrosis, but mild injury is capable of stimulating apoptosis [16, 17, 22].

The CsA concentrations (0.42 nM to 83 μM) chosen in this study were taken to reasonably approximate the concentration range reached *in vivo* in kidney tissue so as to

obtain a nephrotoxic profile *in vitro* that was relevant to the clinical situation [23, 24]. We have previously shown that short term exposure to low doses of CsA (less than 0.42 μM), while not causing any significant changes in cellular viability, were capable of inducing sustained elevations in intracellular calcium in LLC-PK_1 cells [5, 6]. Several lines of evidence suggest that in some cell types, alterations in cytosolic calcium concentrations can regulate apoptosis by stimulating endonuclease activity [25].

In the present study, it is evident that all doses of CsA caused very significant decreases in DNA synthesis, as indicated by reductions in ^{3}H-thymidine incorporation. This was supported by flow cytometric cell cycle analysis where dose dependent elevations in the proportions of cells in the G_0/G_1 phases of the cell cycle with concomitant decreases of those in the S and G_2/M phases were observed. Protein synthesis was also decreased, but was only observed using higher doses (21 μM or greater) of CsA. A CsA induced blockade in the G_0/G_1 phase of the cell cycle has been previously observed in a murine B cell line [26]. These reports and the present findings indicate that DNA is a vital target for CsA injury. The p53 tumor suppressor gene encodes a transcriptional activator whose targets may include genes that regulate genomic stability, cellular responses to DNA damage and cell cycle progression [27, 28]. It has been postulated that in some cell types following DNA damage, the gene product p53 is activated, which causes cells to stall in the G_0/G_1 phase to allow vital repairs to be carried out. If these repairs are not possible then cell death by apoptosis is triggered [29–31]. This indeed may be the case for the lower doses (in the nM range) of CsA, which in addition to causing the cell cycle blockade, induces changes in the cells indicative of apoptotic cell death.

In this study, flow cytometry was seen to be a useful tool for the discrimination between apoptotic and necrotic cell death induced following a toxic insult. Necrosis and apoptosis are two distinct modes of cell death that differ both in morphology and in mechanism [7, 8]. Apoptosis is characterized by cell shrinkage, chromatin condensation, compaction of organelles, systematic DNA cleavage and is genetically regulated. In contrast, in necrotic cell death the plasma membrane is the major site of damage leading to cell swelling and ultimate rupture of cells. Alterations in the light scattering properties of cells can be used to distinguish between apoptotic, necrotic and viable cells [32]. In this study, cells treated with high doses of CsA (μM range) displayed increases in forward light scatter (FSC) indicative of cell swelling that was paralleled by a decreased side light scatter (SSC), which indicates a decreased granularity probably due to rupturing of the cell and increased release of cellular organelles. Cells treated with these high doses also displayed alterations in membrane integrity as evidenced by a decreased ability to exclude trypan blue and release of LDH. However, cells treated with lower doses of CsA showed decreases in FSC and increases in SSC indicative of cell shrinkage and an increase in granularity, respectively. This was paralleled by the appearance of a "sub-G_0/G_1" peak on the DNA histogram that is taken to represent decreased DNA stainability due to DNA degradation probably mediated by endonuclease activity [33, 34]. This peak did not appear with cells treated with higher doses of CsA. Cells treated for longer time points of 48 hours demonstrated a further increase in the size of this "sub-G_0/G_1" peak (results not shown). Using the TUNEL assay [20] further evidence for the induction of DNA fragmentation and apoptosis by 4.2 nM CsA in LLC-PK_1 cells was provided.

When cell death occurs, phosphatidylserine is translocated to the external surface of the membrane. This occurs during the early stages of apoptosis when the cell membrane remains intact. In this study, phosphatidylserine externalization was assessed by measuring the extent of FITC-annexin-V binding. Annexin-V is a Ca^{2+} dependent binding protein that has a high affinity for phosphatidylserine [21]. Low dose CsA (4.2 nM) increased the level of annexin-V binding while not causing any alterations in membrane integrity, as assessed using trypan blue exclusion. Therefore, it can be seen from these results that flow cytometry including DNA analysis, cell size and granularity and annexin-IV detection, is a useful technique that can be used to distinguish both apoptotic and necrotic cells in heterogeneous populations. This would not be possible using, for example, DNA gel electrophoresis to assess cell death. In fact, other studies have shown that changes typical of apoptosis could be visualized earlier when drug treated cells were studied by DNA flow cytometry compared to those detected by DNA electrophoresis [35].

Apoptosis is a genetically regulated form of cell death. A number of gene products have been implicated in its regulation. These include c-fos, c-myc, p53, Bcl-2 and Fas [36]. Most of these are known mainly for their roles in proliferation control, and their involvement in apoptosis provides further evidence for the important link between cell cycle progression and apoptosis. The 45 kD cell surface receptor Fas (APO-1/CD95) and its ligand are important mediators of apoptosis and show several structural and functional similarities to the TNF receptor system [37]. Fas sensitive cells can be induced to undergo apoptosis either via activation of the Fas receptor by its natural ligand or with agonistic anti-Fas antibodies. The ability of Fas to induce death is dependent on the balance between the expression of death factors such as interleukin-1-converting enzyme (ICE) and Fas-associated death domain (FADD) and the expression of protective factors such as Bcl-2, Bcl-xL, Bag-1 and Fas associated phosphatase (FAP-1) [38]. In the present study, anti-Fas antibodies were used to examine the role of Fas in CsA-induced apoptotic cell death in LLC-PK_1 cells. The reactivity of the Fas antibody with porcine cells was confirmed by virtue of the fact that a band corresponding to the exact molecular weight of Fas

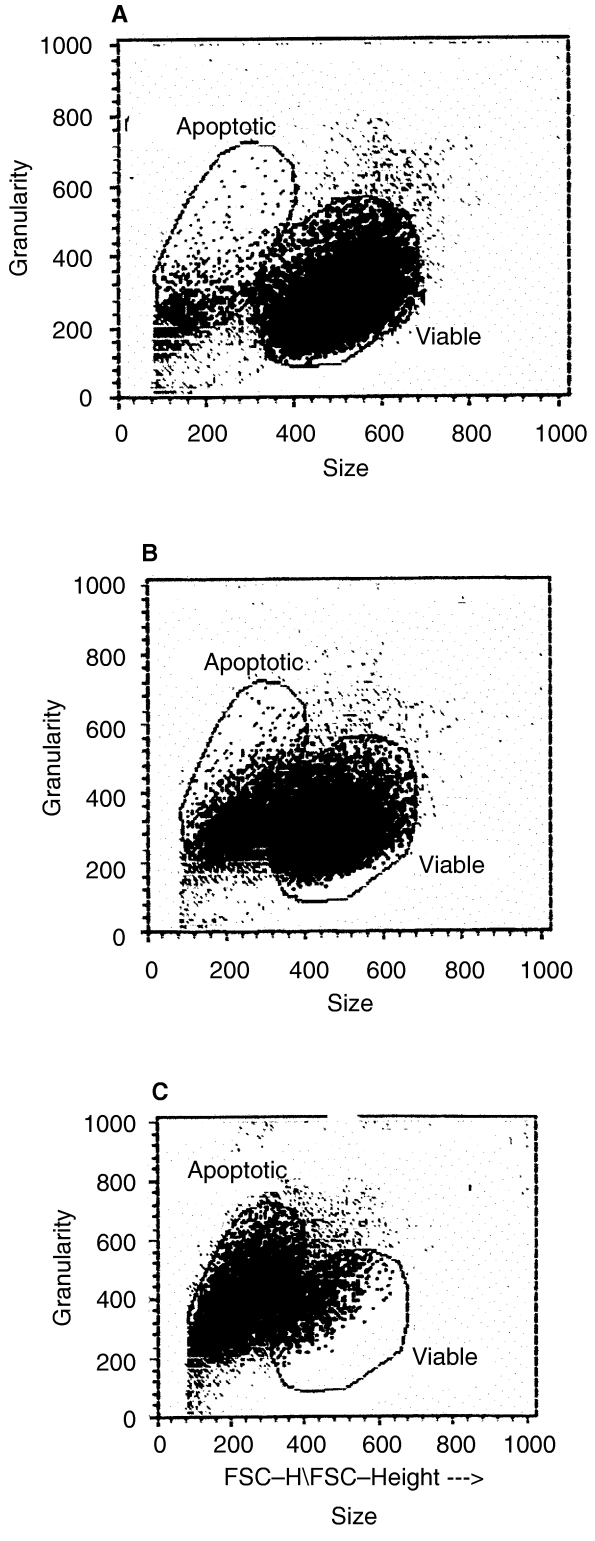


Fig. 8. Effect of the Fas antibody in the presence and absence of cyclosporine (CsA) on the light scattering properties of LLC-PK$_1$ cells. (*A*) Light scattering properties of control cells incubated in the absence of primary antibody (−/+). (*B*) Light scattering properties of control cells incubated with both primary and secondary antibody (+/+). (*C*) Light scattering properties of CsA treated cells incubated with both primary and secondary antibody.

was apparent in the LLC-PK$_1$ cellular extracts. Using flow cytometry and Western blot analysis, a low dose of CsA (4.2 nM) was seen to cause elevations in Fas expression. This alteration in Fas expression was paralleled by changes in the light scattering properties of the cells that displayed characteristics of apoptosis. In addition, the Fas antibody itself appeared to increase the proportion of cells with apoptotic light scattering characteristics, an observation that has previously been reported in breast epithelial cells [39]. The anti-Fas antibody alone also increased phosphatidylserine expression and induced alterations in morphology typical of apoptosis (results not shown). It has been speculated that agonistic antibodies may mimic the effect of the ligand for the Fas receptor and therefore induce apoptosis. Anti-Fas antibodies have been shown to induce apoptosis in the glomerular region of the nephron, both *in vivo* and in *vitro* [15, 40]. In addition murine proximal tubular epithelial cells expressed a Fas transcript in culture that could be up-regulated by lipopolysacchride (LPS) and pro-inflammatory cytokines [41]. The Fas system has also been implicated in a mouse model of ischemia-reperfusion induced apoptosis in tubular epithelial cells [42]. Hence, activation of the Fas receptor system could be an important mechanism in the mediation of CsA-induced apoptotic renal tubular epithelial cell death.

A recent study by Thomas and co-workers demonstrated that CsA induced nephropathy in a rat model was associated with a marked increase in apoptosis of tubular and interstitial cells [43]. Furthermore, the CsA-induced apoptosis correlated with interstitial fibrosis. In that *in vivo* study, evidence was presented that the CsA-induced apoptosis of tubular and interstitial cells was partially mediated by angiotensin II and nitric oxide inhibition, suggesting a role for renal ischemia in the process [43]. However, our present study now also provides evidence for a direct effect of CsA in inducing apoptosis in renal tubular cells.

In addition to our findings on a possible involvement of the Fas system in CsA mediated apoptosis, the fact that CsA was seen to induce sustained elevations in cytosolic calcium in our cellular system [5, 6] also suggests that calcium may be involved in activating calcium dependent endonucleases, which in turn mediate DNA fragmentation that gave rise to the "sub-G_0/G_1 peak" observed on the flow cytometric DNA histogram. The calcium and magnesium dependent nuclease involved in mediating glucocorticoid induced apoptosis in rat thymocytes NUC18 [44] has been shown to have sequence homology to the CsA binding proteins cyclophilins A, B and C. In addition, the recombinant cyclophilins A, B and C have been shown themselves to have an inherent nuclease activity [45, 46]. We have localized cyclophilin A within LLC-PK$_1$ cells [6] and cyclophilin C has been found to be present in high amounts in the proximal tubule [47, 48]. Therefore, it is also conceivable that CsA may mediate DNA damage within the kidney

via its cytosolic binding proteins the cyclophilins, which have inherent endonuclease activity.

In summary, the results from this study indicate that all doses of CsA induce a block in the G_0/G_1 phase of the cell cycle of LLC-PK_1 cells, a renal epithelial cell line of proximal tubular origin. The mechanism of cell death induced by CsA in these proximal tubular cells is dose dependent and Fas may be an important mediator of the CsA induced apoptosis.

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