Transplant Int (1992)5 [Suppl 1]: S87–S92

TRANSPLANT
International

In vitro FK506 kidney tubular cell toxicity

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Abstract. Nephrotoxicity is the most prominent side effect of the new immunosuppressive drug FK506. Some of the histopathological changes associated with cyclosporine (CyA) nephrotoxicity such as tubular vacuolization and glomerular thrombosis have also been reported with FK506 therapy. In this study we used kidney tubular cells in culture to address the issue of FK506- and CyA-induced tubular damage. Exposure of tubular cells to high concentrations of FK506 or CyA (10, 50 and 100 μM) induced a time- and dose-dependent cell injury in vitro characterized by a direct cytotoxic effect on tubular cells as expressed by release of ^{3}H-thymidine from prelabelled cells, *N*-acetyl-β-D-glucosaminidase (NAG) release and cell detachment. Ultrastructural changes (vacuolization, swelling and mitochondrial enlargement) and inhibition of the growth (DNA and RNA synthesis) of cultured tubular cells were also observed at high concentrations of FK506 and CyA. These concentrations are higher than those reached in clinical situations, but close to the concentrations that may be reached by FK506 or CyA in tissues. Low concentrations of FK506 and CyA (1, 0.1 and 0.01 μM) were not cytotoxic and induced only a minimal inhibitory effect on the growth of tubular cells in vitro. At the same concentration CyA induced more cell detachment, more NAG release and a stronger inhibitory effect on cell growth than FK506 ($P < 0.01$). Since an evident cytotoxic effect was observed only at high concentrations, we can speculate that tubular toxicity is due to the accumulation of drug in the cells inducing cell disruption and death.

Key words: FK506 – Cyclosporine – Kidney tubular cell – Drug toxicity

FK506 is a newly development immunosuppressive drug which has been used successfully in kidney [9] and liver transplantation [7]. Recently, a number of side effects have been described. Nephrotoxicity appears to be the major adverse effect of this valuable immunosuppressive drug [6]. Some of the features commonly associated with cyclosporine (CyA) nephrotoxicity, such as tubular vacuolization and glomerular thrombosis, have also been observed in patients treated with FK506 [8]. The mechanism by which FK506 and CyA exert their tubular toxicity is not clear, but it has been suggested that the accumulation of the drugs in the cells causes delayed regeneration, morpological changes, and cell death [8]. In the present study, we report an in vitro assessment of kidney tubular cell sensitivity to FK506 compared with CyA, morphological changes induced in tubular cells treated with FK506, and the comparative effect of both these drugs on the growth of tubular cells in vitro.

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Materials and methods

Preparation of tubular cells

The LLC-PK1 cell line was used in the present study. This porcine kidney tubular cell line has the characteristics of renal proximal tubular cells [5]. Tubular cells were cultured in medium M199 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., Utah. USA). Cell subcultures were obtained by incubating a washed confluent tubular cell culture with a solution of 0.25% trypsin and 0.02% EDTA (Gibco) for 5 min at 37°C.

Drug preparations

FK506 (Fujisawa, Osaka, Japan) was dissolved in absolute methanol. CyA (Sandoz, Basel, Switzerland) was dissolved in absolute ethanol before being added to the media.

Morphometric examination and electron microscopy

Tubular cells were scraped off the culture dish with a rubber spatula and centrifuged at 300 × *g* for 20 min. The cell pellets were fixed by immersion for 4 h at 4°C in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4). After washing three times in cacodylate buffer, the samples were postfixed for 90 min in 1% osmium at 4°C, dehydrated through ascending grades of alcohol, and embedded in Epon.

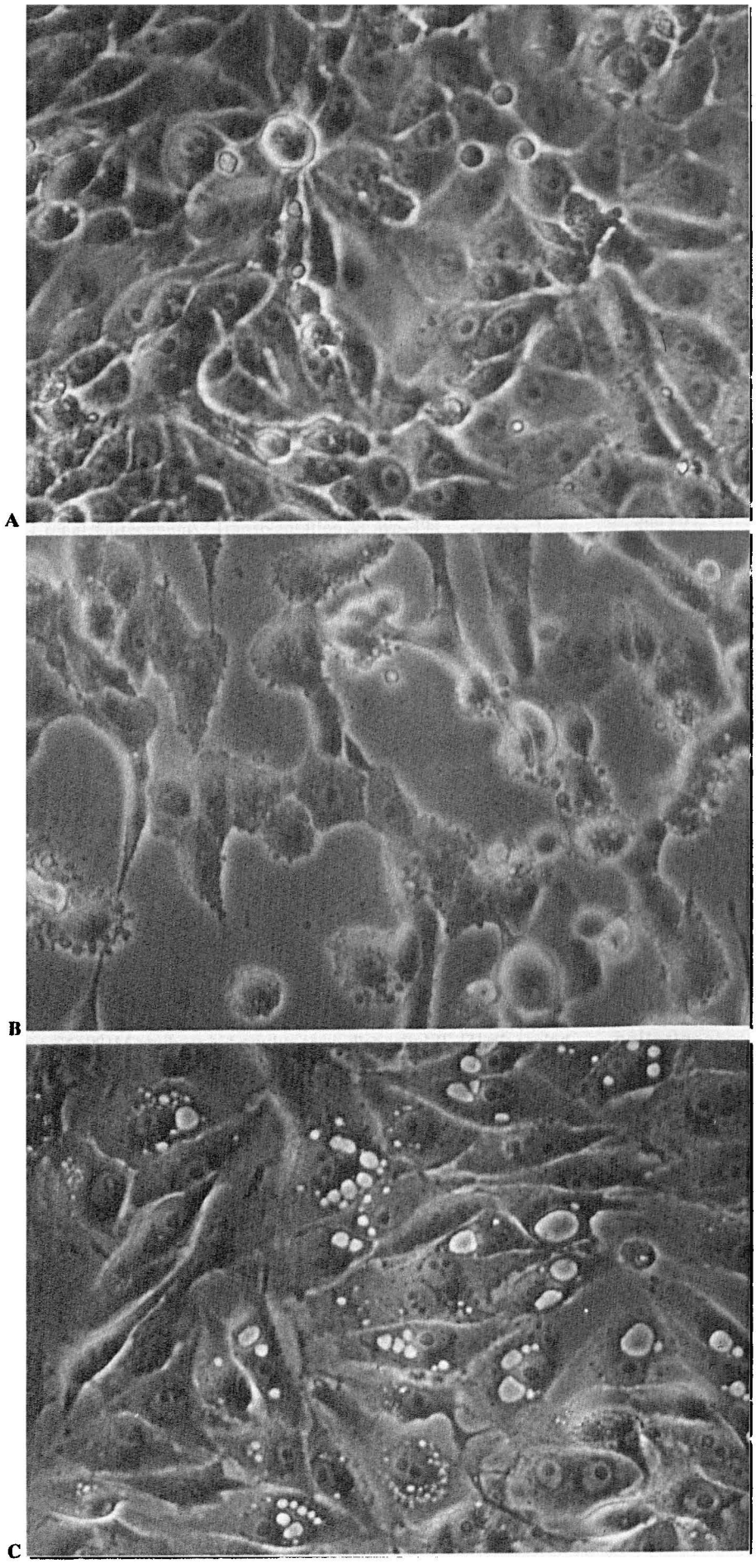

Fig. 1 A–C. FK506-induced cell detachment under light microscopy.
A cultured tubular cells were cultured with medium containing appropriate concentration of vehicle (methanol).
B Tubular cells were cultured with 50 μM FK506 for 5 h. Cell detachment is clearly visible.
C Tubular cells were cultured with 10 μM FK506 for 24 h. Many cells show vacuoles of different sizes

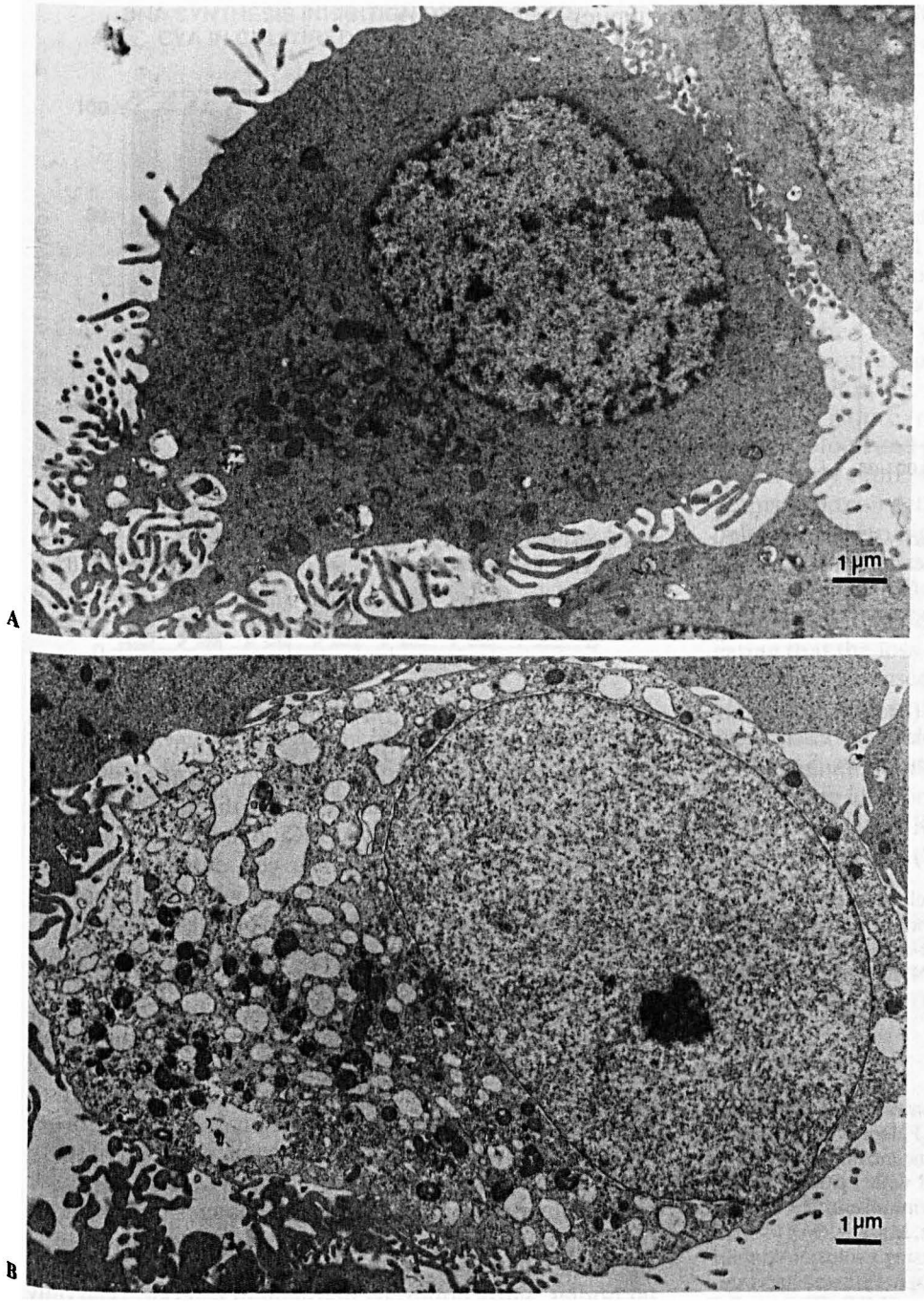

Fig. 2 A, B. Ultrastructural changes in tubular cells (LLC-PK1 cell line) exposed to FK506.
A cells incubated with the culture medium containing appropriate concentration of the vehicle.
B cells incubated with 10 µ*M* FK506 for 24 h. Swelling and vacuolization are clearly visible

Semithin (0.5 µm) sections were stained with 1% toluidine blue. Ultrathin (70–80 nm) sections were examined with uranyl acetate and lead citrate staining in a Hitachi H-7000 electron microscope.

Cell detachment assay

This was determined by direct counting of adherent cells as described previously [10]. Briefly, tubular cells (10^5 cells/cm^2) were plated in 35-mm diameter plastic multiwells in complete medium and were allowed to adhere until confluence was reached. Then the cells were incubated with different concentrations of FK506 or CyA for 4, 10 and 24 h. The vehicle alone served as control. Cell counts were determined by haemocytometer after harvesting the adherent cells with trypsin-EDTA. The data are expressed as percentage of detachment: (number of cells cultured in the presence of appropriate vehicle – number of cells cultured in the presence of test drugs) × 100/number of cells cultured in the presence of appropriate vehicle).

Drug cytotoxicity assay

This was performed as described previously [4]. Confluent monolayers of tubular cells grown in 24-well plates (Falcon, Oxnard, Calif., USA) were incubated with ^{3}H-thymidine (0.5 µCi/ml of ^{3}H-thymidine) (New England Nuclear, Boston, Mass., USA) in complete medium for 48 h. At the end of the incubation period, radioactive medium was discarded and radiolabelled cells were washed five times with medium. The cells were then incubated for 72 h with different concentrations of FK506 or CyA. ^{3}H-Thymidine-labelled tubular cells incubated with medium alone served as controls. Maximal release was assayed by lysing labelled cells with 0.5 *N* NaOH. After 72 h of incubation, radioactivity of the supernatants was

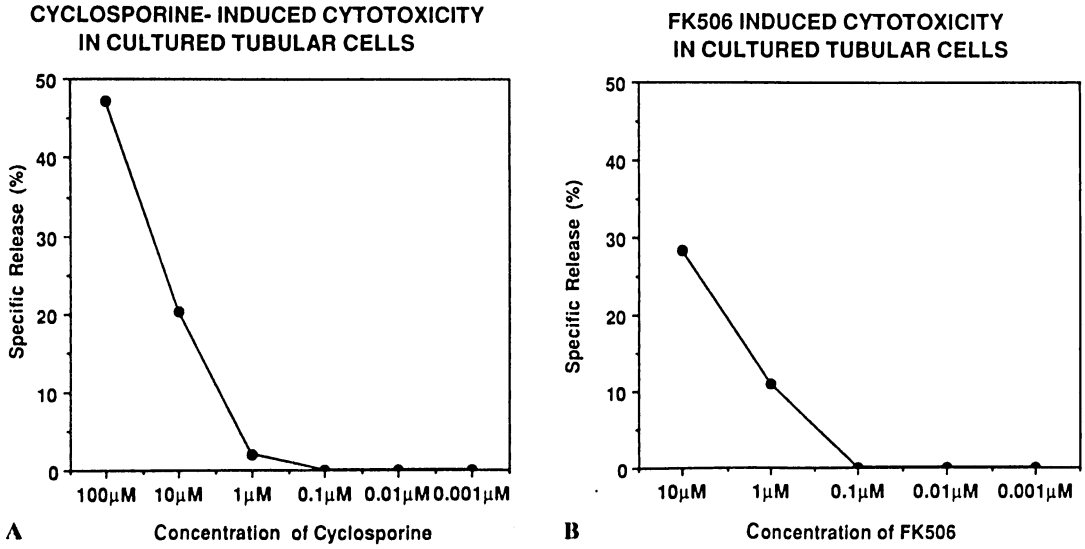

Fig. 3 A, B. FK506 and CyA induced cytotoxicity. Confluent tubular cells were labelled with ^{3}H-thymidine, thereafter cells were incubated for 72 h with different concentrations of FK506 or CyA. After the end of the period of incubation, radioactivity of the supernatants was measured. Spontaneous release was obtained from the vehicle alone. The maximal release was assayed by lysing cells with 0.5 *N* NaOH

measured using a liquid scintillation counter (Packard Instruments, Osaka, Japan). The percentage of specific release was calculated as (E-S) × 100/(M – S), where E is drug-induced release, S is spontaneous release and M is maximal release.

N-Acetyl-β-D-glucosaminidase (NAG) determination

The supernatants from monolayer tubular cells treated with different concentrations of FK506, CyA or appropriate vehicle for 10, 24 and 48 h were assayed for NAG using the *m*-cresolsuphonphthaeinyl-*N*-β-glucosaminidase method (Shionogi NAG assay kit, Shionogi, Osaka, Japan).

DNA and RNA synthesis

Solution (200 μl) containing cells at a concentration of 10^5 cells/ml were grown on a 96-well tissue culture plate (Falcon, Oxnard, Calif., USA) for 24 h in the presence of various concentrations of FK506, CyA or vehicle. The cells were then pulsed with 1 μCi of ^{3}H-thymidine or ^{3}H-uridine (specific activity 25 μCi/mmol and 27 Ci/mmol, respectively) (New England Nuclear, Boston, Mass., USA) for 16 h. Prior to harvesting, the cells were detached using a solution containing 0.05% trypsin in 0.5 mmol EDTA (Gibco) in PBS and harvested onto filter paper with a plate microharvester (LKB, Wallace Cell Harvester, Pharmacia). The filters were dried and the cells redissolved in 8 ml of scintillation liquid (LKB Scintillation Products, UK) and counted using a beta plate counter (LKB Wallace, Pharmacia, Finland). Results are the mean of three experiments. The data are expressed as percentage of inhibition = [1 – (cpm B/cpm A)] × 100, where cpm A represents the cpm of cells cultured in the presence of appropriate vehicle, and cpm B respresents the cpm of cells cultured in the presence of test drugs.

Results

Cell detachment and ultrastructural changes

Cell detachment was found to be an early marker of FK506- and CyA-mediated tubular toxicity. As early as 5 h after exposure to the highest concentrations of FK506 or CyA (100, 50 and 10 μ*M*), tubular cell detachment was clearly visible under the phase contrast microscope (Fig. 1 B). Table 1 shows the data obtained after 5, 10 and 24 h of incubation of tubular cells with three different concentrations of FK506 or CyA. FK506 or CyA at a concentration of 1 μ*M* did not induce cell detachment independently of the incubation time, but with FK506 or CyA at 10 μ*M* and 50 μ*M* the percentage of cells detached was higher and was increased by prolonging the incubation time. At the same concentration CyA seemed to induce more cell detachment than FK506 ($P < 0.01$).

The most prominent change observed by light microscopy after 24 h treatment with FK506 in non-detached cells was vacuolization. The vacuoles were of different sizes (Fig. 1 C). Profound morphological changes were detectable under the electron microscope after 24 h of tubular cell exposure to FK506 (Fig. 2). These changes included obvious signs of injury with changes in cell shape, membrane cellular irregularity, enlargement of the smooth endoplasmic reticulum, vacuolization and lamellar bodies. Mitochondrial enlargement was observed only at concentrations of 50 and 100 μ*M* of FK506 or CyA (data not shown).

FK506- and cyclosporine-induced cytotoxicity and NAG release

Release of ^{3}H-thymidine by prelabelled cells after exposure to FK506 or CyA was used as a marker of cell disruption. FK506 and CyA induced a dose-dependent release of ^{3}H-thymidine from tubular cells (Fig. 3). After 72 h, cells that were prelabelled with ^{3}H-thymidine and incubated with FK506 doses higher than 0.1 μ*M* released more thymidine than the control monolayer. The specific release induced by 1 μ*M* was 10%, and 10 μ*M*-induced specific release was around 30%. FK506 doses less than

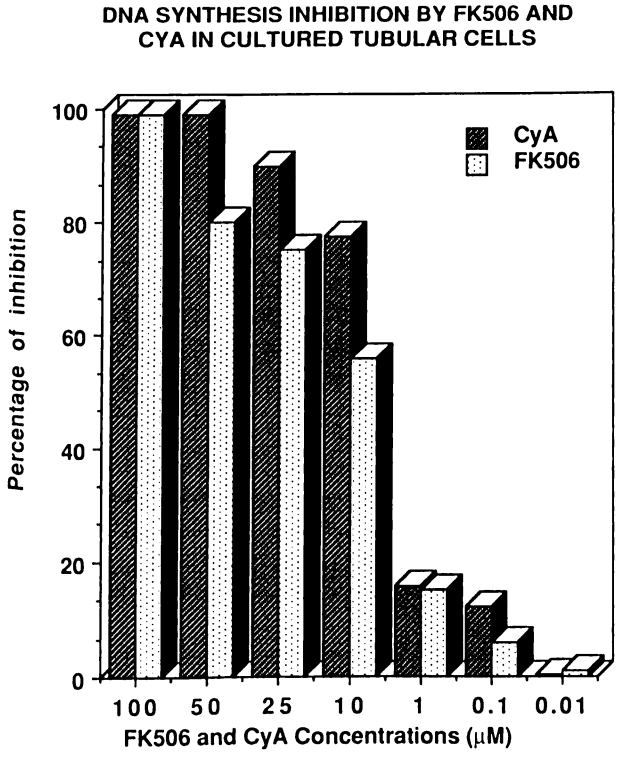

Fig. 4. DNA synthesis inhibition by FK506 and CyA in cultured tubular cells. Cells were incubated with FK506, CyA or vehicle for 24 h. At the end of incubation, the cells were pulsed with 1 μC of ^{3}H-thymidine for 16 h

0.1 μ*M* (0.01 and 0.001 μ*M*) were not cytotoxic. CyA at 1 μ*M* showed a specific release of 1–5%, and 10 μ*M*-induced specific release was about 20%. Doses of CyA less than 0.1 μ*M*) were not cytotoxic. The FK506- and CyA-induced cytotoxicity were not significantly different.

We used NAG release as a specific marker of tubular cell injury. NAG release from tubular cells incubated with FK506 at 10 μ*M* and 1 μ*M*, and CyA at 1 μ*M* for 10, 24 and 48 h was not different from the control (Table 2). In contrast high concentrations of FK506 and CyA (50 μ*M*) induced a significant release of NAG from treated tubular cells at 10, 24 and 48 h ($P < 0.01$), whereas CyA at 10 μ*M* significantly increased NAG release only after 48 h (Table 2). At the same concentration CyA seemed to induce more NAG release than FK506 ($P < 0.01$).

Effect of FK506 and cyclosporine on tubular cell growth

The uptake of ^{3}H-thymidine and ^{3}H-uridine by cultured tubular cells is a marker of cell growth in vitro. A significant dose-related inhibition of DNA synthesis was observed at FK506 or CyA concentrations higher than 1 μ*M*. FK506 and CyA concentrations below 1 μ*M* had only a minimal inhibitory effect (less than 25%) (Fig. 4).

FK506 and CyA produced a dose-dependent inhibition of RNA synthesis in tubular cells at concentrations higher than 1 μ*M*. Below this concentration the inhibition was modest (less than 20%) (Fig. 5). At the same concentration CyA had significantly more inhibitory effect on DNA and RNA synthesis than FK506 ($P < 0.01$).

Discussion

Our results show that FK506 and CyA at high doses exert a direct cytotoxic effect on tubular cells, induce ultrastructural changes and strongly inhibit cell growth in vitro. These doses are higher than those reached in plasma in clinical transplantation, but close to the tissue concentrations reached by CyA in the kidney [1]. There is no report available concerning the concentration of FK506 in the tissue but it is not unexpected that FK506, in view of its lipophilic nature, may reach concentrations higher than those in blood and plasma.

An early expression of FK506 and CyA cytotoxic effect seems to be cell detachment. In the first 5 h of exposure of cultured cells to high doses of FK506 or CyA, a high percentage of detached cells was observed. During the same incubation period, at high concentrations of FK506 or CyA, NAG release was very low (data not shown), indicating that the loss of adhesion properties of the cells was not simply a consequence of cell death. These results are consistent with those of Zoja et al. [11] who found that CyA mediated endothelial cell injury by inducing initially cell detachment and subsequently cell lysis. The electron microscope demonstrated non-specific cytoplasmic vacuolations with FK506 treatment. These ultrastructural

Table 1. Effect of FK506 and CyA on tubular cell detachment. After reaching confluency the cells were exposed to various concentrations of FK506 and CyA for 4, 10, and 24 h. The remaining cells were then detached with trypsin and counted using a haemocytometer

	cell detachment (%) Incubation time (hours)		
	5	10	24
FK506 50 μ*M*	25	47.5	70
FK506 10 μ*M*	1.2	0.8	20
FK506 1 μ*M*	0.5	0.6	1.0
CyA 50 μ*M*	57.5*	65*	80
CyA 10 μ*M*	0.1	7.5	27.4
CyA 1 μ*M*	0.2	0.0	0.4

* $P < 0.01$ CyA vs. FK506 at the same concentration

Table 2. Kinetics of NAG release from tubular cells treated with FK506 or CyA. Supernatants harvested from cultured tubular cells treated with different concentrations of FK506 or CyA for 10, 24 and 48 h were assayed for NAG

	NAG release Incubation time (h)		
	10	24	48
Control	1.704 ± 0.10	2.41 ± 0.02	4.640 ± 0.19
CyA 50 μ*M*	5.59 ± 0.34*	6.879 ± 0.27*	9.39 ± 0.81*
CyA 10 μ*M*	1.6 ± 0.038	2.403 ± 0.15	5.45 ± 0.09*
CyA 1 μ*M*	1.4 ± 0.002	2.308 ± 0.01	4.28 ± 0.01
FK506 50 μ*M*	4.31 ± 0.36*	4.7826 ± 0.03	6.059 ± 0.4*
FK506 10 μ*M*	1.68 ± 0.09	2.41 ± 0.13	4.27 ± 0.2
FK506 1 μ*M*	1.82 ± 0.02	2.59 ± 0.12	4.69 ± 0.3

* $P < 0.01$ vs. control

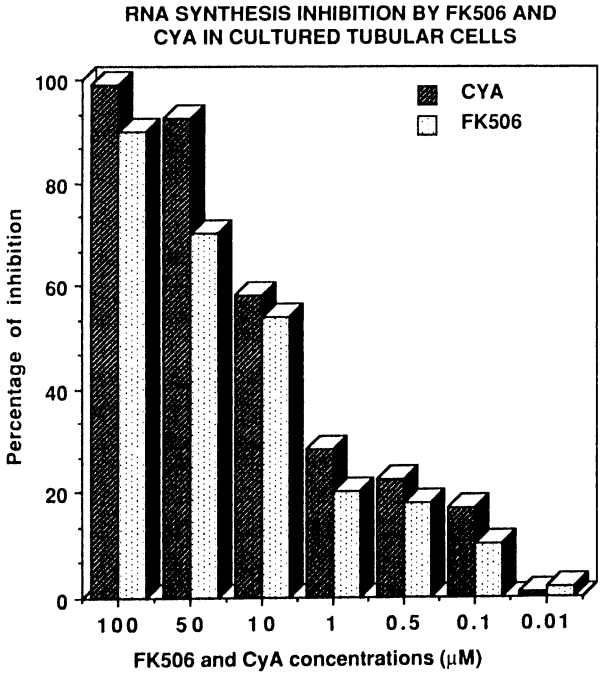

Fig. 5. RNA synthesis inhibition by FK506 and CyA in cultured tubular cells. Cells were incubated with FK506, CyA or vehicle for 24 h. At the end of incubation, the cells were pulsed with 1 μC of ^{3}H-uridine for 16 h

changes corresponded to the cytoplasmic vacuolations visible on light microscopy. The cytoplasmic vacuolization, mitochondrial enlargement and cell swelling induced by FK506 in tubular cells in vitro were similar to the effects reported for CyA treatment [8].

We have assessed the cytolytic effect of FK506 compared with CyA as expressed by ^{3}H-thymidine release from prelabelled cells and NAG release. It appeared that low concentrations of FK506 and CyA (1, 0.1 and 0.01 μ*M*) were without cytotoxic effect. In contrast, high concentrations of FK506 and CyA (100, 50 and 10 μ*M*) were cytotoxic. Zoja et al. [11] also reported that high concentrations of CyA (10 and 50 μ*M*) induced a significant release of ^{51}Cr and lactate dehydrogenase from cultured endothelial cells. The effect of 10 μ*M* and 50 μ*M* seems to be specific to tubular cells since we have observed that cell detachment and cell death in confluent culture of human mesangial cells can be seen only when the concentration of FK506 and CyA is increased to 100 μ*M* (unpublished data).

Our data agree with those of Cole et al. [2], who observed a significant inhibition of DNA and RNA synthesis in both tubular cells and mesangial cells with CyA concentrations higher than 1 μ*M*. The means by which FK506 or CyA exert tubular toxicity remain uncertain. Since significant cytolysis, morphological changes, and delayed growth were observed in vitro only at high concentrations of FK506, we can speculate as suggested by Mihatsch and Ryffel [8] in CyA toxicity, that at pharmacological doses (below 1 μ*M*) FK506 binds to its binding protein FKBP and inhibits specifically induced gene transcription in lymphoid cells. At these concentrations there is no general inhibition of tubular cell proliferation or protein synthesis. At higher concentrations, the FKBP might be saturated and FK506 may be bound to non-saturable membrane proteins, thus accumulating in the membrane and disrupting membrane function. This accumulation of FK506 may occur not only in the surface membrane but also in the endoplasmic reticulum, mitochondria and Golgi apparatus. Concomitantly, tubular cell proliferation and protein synthesis are inhibited and cellular changes are apparent.

Further studies are required to determine the subcellular basis of FK506 nephrotoxicity and to understand why the kidney is the main target of toxicity. We conclude that, at high concentrations, FK506 and CyA induce cytolysis and morphological changes, and inhibit cell growth. At established therapeutic levels, FK506 and CyA are not cytotoxic in vitro.

References

1. Atkinson K, Biggs JC, Britton K (1982) Distribution and persistence of cyclosporin in human tissues. Lancet II: 1165–1170
2. Cole E, Skorecki K, Cheung F, Wong PY, Fung LS, Levy GA (1988) Cyclosporine A in contrast to cyclosporine metabolite (OL-17) specifically inhibits growth of renal cells in culture. In: Kahan BD (ed) Cyclosporine: therapeutic use in clinical transplantation. Grune & Stratton, London, pp 732–737
3. Demetris AJ, Banner B, Fung J, Shapiro R, Jordan M, Starzl TE (1991) Histopathology of human renal allograft rejection under FK506. Transplant Proc 23: 944–946
4. Hruby ZW, Cybulsky AV, Lowry RP (1990) Effect of tumor necrosis factor on glomerular mesangial cells and epithelial cells. Nephron 56: 410–413
5. Hull RN, Cherry WR, Weaver GW (1976) The origins of a pig kidney cell strain. In Vitro 12: 670–673
6. Ishibashi M (for the Japanese study group) (1991) Japanese study of FK506 in kidney transplantation: Results of early phase II study. Transplant Proc (in press)
7. Jain AB, Fung J, Todo S, Alessiani M, Takaya S, Abu-Elmagd K, Tzakis A, Starzl TE (1991) Incidence and treatment of rejection episodes in primary orthotopic liver transplantation. Transplant Proc 23: 928–930
8. Mihatsch MJ, Ryffel B (1990) Evolution of cyclosporine nephrotoxicity. In: Hatano M (ed) Proceedings of the XIth International Congress of Nephrology. Springer Verlag, Tokyo Berlin Heidelberg New York London Paris Hong Kong Barcelona, pp 576–588
9. Shapiro R, Jordan M, Fung J, McCauly J, Johnston J, Iwaki Y, Tzakis A, Hakala T, Todo S, Starzl TE (1991) Kidney transplantation under FK506. Transplant Proc 23: 920–923
10. Wall RT, Harlan JM, Harker LA, Striker GE (1980) Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury. Thromb Res 18: 113–117
11. Zoja C, Furci L, Ghilardi F, Zilio P, Benigni A, Remuzzi G (1986) Cyclosporin-induced endothelial cell injury. Lab Invest 55: 455–462