J Periodont Res 2006; 41; 297–302

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00873.x

The effect of cyclosporin on cell division and apoptosis in human oral keratinocytes

J. Birraux[1], J. A. Kirby[2], J. M. Thomason[1], J. J. Taylor[1]
[1]Oral Microbiology and Host Responses Group, School of Dental Sciences, and [2]Transplantation Immunobiology Group, School of Surgical and Reproductive Sciences, Faculty of Medical Sciences, University of Newcastle upon Tyne, UK

Birraux J, Kirby JA, Thomason JM, Taylor JJ. The effect of cyclosporin on cell division and apoptosis in human oral keratinocytes. J Periodont Res 2006; 41: 297–302. © 2006 The Authors. Issue compilation © 2006 Blackwell Munksgaard

Background and Objective: Gingival overgrowth (GO) is a side-effect of cyclosporin A (CsA) therapy and is characterised by enlargement of the gingiva with epithelial thickening and overproduction of extracellular matrix components. The pathogenesis of the epithelial thickening in GO remains obscure. The objective of the present study was to investigate the effects of CsA on the growth of oral epithelial cells *in vitro* and to test the hypothesis that CsA influences apoptosis in these cells.

Material and Methods: Cyclosporin was cocultured with an immortalized normal human oral keratinocyte cell line (HOK-16B), an epitheloid cervical carcinoma cell line (HeLa) and primary oral keratinocytes. Cell division was quantified using a CyQUANT™ kit. Apoptosis was induced using tumour necrosis factor-α (TNF-α) and assayed by analysis of caspase-3 activity. Expression of the anti-apoptotic protein, Bcl-2, was measured by western blotting.

Results: CsA exhibited a dose- and time-dependent inhibition of cell division in all three keratinocyte cell cultures. Significantly, HOK-16B cells treated with high doses of CsA (10 μg/ml) did not recover their proliferative capacity 3 d after withdrawal of CsA, indicating that CsA-induced inhibition of growth is not temporary. Concentrations of CsA that inhibited cell division (1 μg/ml) did not have any effect on constitutive or TNF-α -induced apoptosis or Bcl-2 expression in HOK-16B cells.

Conclusion: CsA inhibits oral epithelial cell division and this effect is not associated with changes in apoptosis in these cells. The action of CsA on oral epithelial cells may be associated with a long-lasting stress signal, which might account for some of the pathological effects of this drug.

Dr John J. Taylor, Oral Microbiology and Host Responses Group, Oral Biology, School of Dental Sciences, University of Newcastle upon Tyne, NE2 4BW, UK
Tel: +44 191 2228694
Fax: +44 191 2226137
e-mail: j.j.taylor@ncl.ac.uk

Key words: apoptosis; cyclosporin; gingival overgrowth; keratinocyte

Accepted for publication November 14, 2005

Cyclosporin A (CsA) is a widely used immunosuppressive agent in organ transplant patients (1). CsA acts through the inhibition of T-lymphocyte activation and proliferation after antigen recognition (2). The downregulation of nuclear factor of activated T cells (NFAT)-activated interleukin-2 secretion by T cells is a key element of this action (2,3). However, cells outside the immune system may be targets for the action of CsA and this may have pathological consequences (4,5). The response of particular tissues to CsA is dependent on both the cell type and their structural relationships within the tissue (4). The proliferation of epidermal keratinocytes, hair epithelial cells, renal tubule epithelial cells and lung epithelial cells suggests that these tissues are particularly sensitive to the action of CsA (5–7). CsA inhibits cell proliferation in renal tubule epithelial cell lines through an effect on apoptosis (4,5,8).

The gingiva is an important component of the periodontium and comprises epithelial tissues and underlying connective tissue. The main cell type of the gingival epithelia is the keratinocyte and these cells have properties in common with other keratinocytes of stratified epithelial tissues, such as the

epidermis (9). Gingival overgrowth (GO) is an unwanted effect of CsA therapy (10). It is characterized by the enlargement of the gingiva with epithelial thickening, a large number of proliferating fibroblasts and overproduction of extracellular matrix components (11,12). The pathogenesis of the condition remains unclear (12). The majority of work has focused on factors affecting the gingival fibroblasts (13,14), but the gingival epithelium may also be important. The gingival epithelium is juxtaposed to dental plaque, which has been identified as a risk factor for the condition (10). Plaque may also act as a reservoir for CsA (15,16) and there is evidence for accumulation of CsA on the surface of the gingival epithelium (15). Although the pathological effects of CsA on the gingival epithelium have been described in detail (16–19), the mechanisms underlying these observations remain unknown. To our knowledge, there are no published reports on the direct effects of CsA on oral epithelial cells in culture.

It has been suggested that fibroblast accumulation in drug-induced GO is caused by the inhibition of apoptosis (20); we hypothesized that this may also be the biological basis for the observed effects of CsA on the gingival epithelium. We therefore aimed to study the effect of CsA on the proliferation and apoptosis of oral epithelial cells. We compared the effects of CsA on an immortalized normal human oral keratinocyte cell line (HOK-16B) (21), an epitheloid cervical carcinoma cell line (HeLa) and primary oral keratinocytes. We found that CsA inhibited cell division in all these cells. Although CsA seemed to deliver a long-lasting inhibitory signal to HOK-16B cells, we found no evidence to suggest that CsA influenced apoptosis of these cells.

Material and methods

Cell culture

Ethical approval for this study was obtained from the Joint Ethics Committee of Newcastle University and Newcastle Health Care Trust. KB cells (ECACC 94050408) were serially cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, Poole, UK). The KB cell line was originally derived from human oral epidermoid carcinoma. Genetic analysis confirmed that the KB cell line is derived from a HeLa contaminant and is now described more accurately as a human cervix epitheloid cell line (22). The human papilloma virus (HPV)-immortalized human oral keratinocyte HOK-16B cell line (21) was cultured at 37°C, in 5% CO_2, in the serum-free medium, KBM® (BioWhittaker UK, Wokingham, UK) supplemented with 0.1 ng/ml epidermal growth factor (EGF), 5 μg/ml insulin, 30 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamycin and 50 μg/ml amphotericin (BulletKit®; BioWhittaker UK). Biopsies of human gingiva were obtained from patients undergoing gingival surgery or from healthy volunteers in the Departments of Periodontology and Oral Surgery, Newcastle Dental Hospital. Primary cultures of oral keratinocytes were established from gingival biopsies using the mouse 3T3 fibroblasts as a feeder layer (23). Before reaching confluence, keratinocytes were harvested and subcultured in serum-free medium KBM with BulletKit® (BioWhittaker UK).

Cyclosporin preparation

Solutions of CsA were prepared in ethanol and optimal immunosuppressive concentrations were determined by measurement of the inhibition of T-cell proliferation. Peripheral blood lymphocytes were stimulated in 96-well plates, precoated with CD3 (orthoclone OKT3), in the presence of varying concentrations of CsA. After 72 h, each culture was labelled with 1 μCi of [^{3}H]-thymidine (TRA61; Amersham, International, Chalfont, UK); the cells were harvested onto fibreglass filters, after a further 6 h, for beta-scintillation counting (LKB microbeta). It was found that CsA was inhibitory at concentrations between 0.1 and 10 μg/ml (data not shown), and these concentrations were used in subsequent cell-culture experiments.

Cell proliferation assay

Cells were seeded in 96-well plates (Greiner Bio-one, Stonehouse, UK) at densities of 1000–2000 cells per well. Various concentrations of vehicle (ethanol) or CsA solutions were added, 24 h after seeding, to three or five replicate cultures, and incubation was continued for different periods of time. After treatment, the medium was removed by gentle suction and plates were stored at −20°C until use. Cell proliferation was assessed using the CyQUANT™ nucleic acid fluorescence assay kit, according to the manufacturer's instructions (Molecular Probes, Leiden, the Netherlands). Calibration curves for each cell line showed the fluorescence reading to be linearly related to the cell number (data not shown). In order to determine whether the inhibitory effects of CsA on keratinocyte proliferation were permanent or temporary, the ability of HOK-16B cells to recover from CsA treatment was analysed. Triplicate wells of HOK-16B cells were incubated with CsA concentrations (0.01–10 μg/ml) for 72 h, after which the medium was removed and replaced with nontreated culture medium for 48 h. Control cultures, containing equivalent volumes of ethanol, were grown in parallel. Cell numbers were determined, before and after the 48-h recovery period, in parallel plates.

Caspase-3 activity assay

The effect of CsA on apoptosis in cell culture was determined by assays for the intracellular enzyme, caspase-3, which initiates the DNA degradation associated with this process. Cells were seeded in T25 tissue culture flasks (Greiner Bio-one) and cocultured for 24 h with 1 μg/ml CsA and tumour necrosis factor-α (TNF-α) (Peprotek, TOTAM Biologicals, Peterborough, UK) at concentrations of 500 U/ml for HOK cells and 1000 U/ml for KB cells. Cyclohexamide (30 μg/ml for HOK-16B cells and 2 μg/ml for KB cells) was used to sensitize the cells to TNF-α.

After incubation, supernatants (containing dead cells) were retained and adherent cells were harvested with 0.05% Trypsin–EDTA. Supernatants and detached cells were then centrifuged (400 *g*, 5 min, 4°C), and the pellets were resuspended in culture medium and transferred to centrifuge tubes to be pelleted by centrifugation (4000 *g*, 5 min, 4°C). Pellets were treated with lysis buffer [10 mM HEPES pH 4.0, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol (DTT), 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF)] and frozen/thawed four times in liquid nitrogen and a 37°C water bath. Lysates were then centrifuged at 5000 *g* for 5–10 min, and the supernatants retained for measurement of caspase-3 activity using a FluorAce™ Apopain Assay Kit, according to the manufacturer's instructions (Bio-Rad, Hemel Hempstead, UK). Briefly, 10 μl of supernatant was loaded in triplicate into black 96-well plates (Greiner Bio-one) and mixed with reaction buffer containing a fluorescently labelled peptide substrate for caspase-3 (AC-DEVD-AFC, final concentration 40 μg/ml). Release of fluorescence by cleaved AFC was monitored in a multiwell plate fluorescence spectrophotometer (Bio Tek FL600, excitation 390–400 nm and emission 510–550 nm, Fisher Scientific, Loughborough, UK) with regular measurements taken during a 3-h period. Caspase-3 activity was calculated by comparing the sample Δfluorescence with a calibrated AFC standard curve. Protein concentration in cell lysates was measured using a bicinchoninic acid (BCA) Protein assay (Pierce & Warriner, Chester, UK) and the specific activity of caspase-3 per unit of protein was thus determined (unit of activity/min/g of protein).

Western blot for Bcl-2 expression

HOK-16B cells, grown to 60–70% confluency in six-well plates (Greiner Bio-one), were incubated for 72 h with 1.5 mM $CaCl_2$ (Sigma) and/or 1 μg/ml CsA. After incubation, the cell layers were washed with ice-cold phosphate-buffered saline (PBS) and lysed with 100 μl of ice-cold 1× sodium dodecyl sulphate (SDS) sample buffer [50 mM HEPES, 150 mM NaCl, 0.2 mM Na_2CO_3, 1 mM $MgCl_2$, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 1 : 50 protease inhibitor cocktail; Sigma]. Extracts were centrifuged (14,000 *g* for 5 min at 4°C), diluted in H_2O (1 : 5) and equivalent amounts of protein (BCA assay) mixed with 2× sample buffer [125 mM Tris-HCl, pH 6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 1% (v/v) β-mercaptoethanol and 1 : 50 protease inhibitor cocktail], heated for 5 min at 95°C and separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using 15% gels. Separated proteins were transferred to a nitrocellulose membrane (Optitran BA-S83; Schleicher & Schuell, London, UK) using a semidry electro-blotter (BDH, NELS, Newton Aycliffe, UK). A prestained protein marker (Bio-Rad) was used to monitor the molecular weight of the separated proteins. Ponceau's staining was performed to assess the quality of the transfer and to confirm equal protein loading. The membrane was blocked for 1 h at room temperature in a buffer of 137 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl, pH 7.4, 0.1% Tween 20 (TBS/Tween), containing 5% (w/v) dried skimmed milk. Primary antibodies were diluted in TBS/Tween containing 5% (w/v) bovine serum albumin (BSA) and incubated with the blots for 1 h. Dilutions (1 : 100) of monoclonal mouse anti-human Bcl-2 (Dako, Glostrup, Denmark) and anti-human actin (Oncogene, Nottingham, UK) were used. Horseradish peroxidase (HRP)-linked goat anti-mouse immunoglobulin G (IgG) (Cell Signalling Technologies, Danvers, MA, USA) was used as a secondary antibody at a 1 : 2000 dilution.

Hybridizing proteins were detected using an enhanced chemiluminescence (ECL) reaction, which was performed following the manufacturer's instructions (ECL + plus western blotting detection system; Amersham) and blots were developed by exposure to X-ray film (Super Rx Medical X-ray film; Fujifilm, London, UK). Extracts from the T-cell leukaemia line, MOLT-16, were used as a positive control for Bcl-2 expression.

Statistics

The statistical differences between groups were determined by analysis of variance (ANOVA), with a one-way classification, using MINITAB software. Significant F ratios were tested using Tukey's test. Statistical significance was assumed at the 5% level.

Results

CsA inhibits epithelial cell growth

Exposure to CsA concentrations of ≤ 0.1 μg/ml did not influence the total cell number compared with control cultures (Fig. 1). However, at higher concentrations, CsA had a dose-dependent inhibitory effect on cell division. CsA concentrations of 1 μg/ml inhibited cell proliferation in all three cell types, and the co-incubation of HOK-16B cells with a CsA concentration of 10 μg/ml almost completely inhibited cell proliferation (Fig. 1).

HOK-16B cells do not recover from the effects of CsA on cell proliferation

CsA concentrations of 1 and 10 μg/ml inhibited HOK-16B cell division (Fig. 2A). The cells were able to recover from the inhibitory effect of 1 μg/ml CsA, whereas pre-incubation of the cells with 10 μg/ml CsA had an inhibitory effect that persisted, even after the 48-h recovery incubation (Fig. 2B).

CsA has no effect on TNF-α-induced apoptosis in HeLa and HOK-16B cells

Background levels of caspase-3 activity in both HeLa and HOK-16B cell lines were not affected by treatment with 1 μg/ml CsA for 24 h (Fig. 3). To investigate a potential effect of CsA on apoptosis induced by environmental factors, we used TNF-α, a cytokine produced during primary immune responses to infections and known to induce apoptosis in target cells.

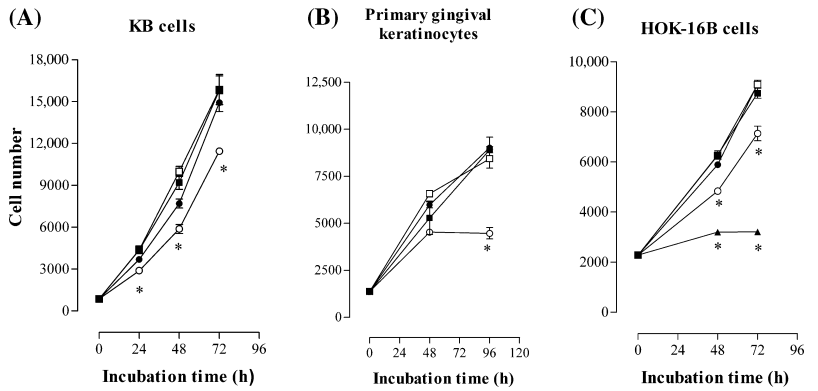

Fig. 1. Epithelial cell proliferation is inhibited by cyclosporin A (CsA) treatment in a dose-dependent manner. Data from experiments with KB cells (Fig. 1A), primary oral keratinocytes (Fig. 1B) and HOK-16B cells (Fig. 1C) are illustrated. Cells were initially seeded and preincubated for 24 h and then cultured for up to 96 h with ethanol control (■) or CsA at the following final concentrations: 0.01 μg/ml (□), 0.1 μg/ml (●), 1 μg/ml (○) or 10 μg/ml (▲). Cell proliferation was measured using the CyQUANT™ nucleic acid fluorescence assay kit, and mean (± standard deviation) data from three independent cultures are presented. $*p < 0.05$.

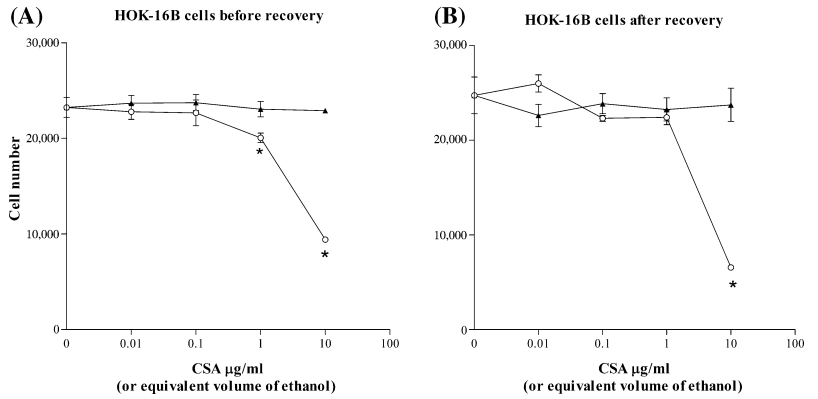

Fig. 2. HOK-16B cells fail to recover from inhibition by high concentrations of cyclosporin A (CsA). HOK-16B cells were grown in medium containing different concentrations of CsA or the equivalent volume of ethanol vehicle for 72 h (▲, vehicle;○, CsA). Cell numbers were determined immediately after CsA treatment (Fig. 2A) or after a 48-h recovery period in CsA-free medium (Fig. 2B). Data represent the mean data (± standard deviation) from three independent cultures. $*p < 0.05$.

Treatment with TNF-α for 24 h induced a substantial increase in caspase-3 activity in both KB and HOK-16B cells (Fig. 3). However, co-incubation with 1 μg/ml CsA had no effect on TNF-α-induced apoptosis levels in either cell line (Fig. 3).

CsA has no effect on Bcl-2 expression in HOK-16B cells

Western blot experiments revealed that HOK-16B cells constitutively expressed only very low levels on Bcl-2 protein (Fig. 4). CsA (1 μg/ml), alone or in combination with Ca^{2+} (a known keratinocyte-differentiation factor), had no effect on Bcl-2 protein expression in HOK-16B cells (Fig. 4).

Discussion

The antiproliferative effect of CsA on epithelial cells is shared by some minimally immunosuppressive or non-immunosuppressive cyclosporin analogues, suggesting that the relevant molecular mechanisms differ from those responsible for immunosuppression (24,25). In contrast, CsA stimulates the growth of hair follicle keratinocytes, causing hypertrichosis (19); thus, the effects of CsA are not consistent in all epithelial cell types. In the present study, we showed that CsA exhibited a dose- and time-dependent inhibition of cell division in an immortalized normal human oral keratinocyte cell line (HOK-16B) and in primary oral keratinocytes in culture. The effect was maintained for at least 48 h after removing CsA, although it would be interesting to investigate whether this effect was more long lasting. It is not clear how the observed inhibition of cell growth relates to the epithelial thickening observed in CsA-induced GO. Moreover, Nurmenniem *et al.* demonstrated an increase of epithelial mitotic activity in the gingiva of patients undergoing therapy with CsA or nifedepine compared with similar sections from healthy controls (26). However, no correlation between mitotic activity and thickness of the oral epithelium was found (26). In contrast, others have found that CsA has little or no effect on markers of epithelial cell proliferation in sections of GO tissue (15,27). These results suggest that epithelial hyperplasia is not caused by an increased keratinocyte-proliferation rate, but by an enhanced keratinocyte life span, or an alteration in the ability of the cells to undergo apoptosis in a balanced manner. It is difficult, however, to relate the results of our *in vitro* experiments to the *in vivo* observations on tissue sections. Interactions between different cell types (e.g. fibroblasts and keratinocytes) may be important in the pathogenesis of GO (12). Also, the local immune response to dental plaque may modify tissue responses to CsA and other drugs (11,12).

The aberrant regulation of growth factors, such as EGF and keratinocyte growth factor (KGF), may be a pathogenic mechanism relevant to CsA-induced GO (28–30). CsA induces transforming growth factor-β (TGF-β) synthesis and secretion by fibroblasts, a mechanism that is thought to be important in CsA-induced tissue fibrosis and GO (13,14,28). TGF-β-induced growth arrest in epidermal keratinocytes is associated with

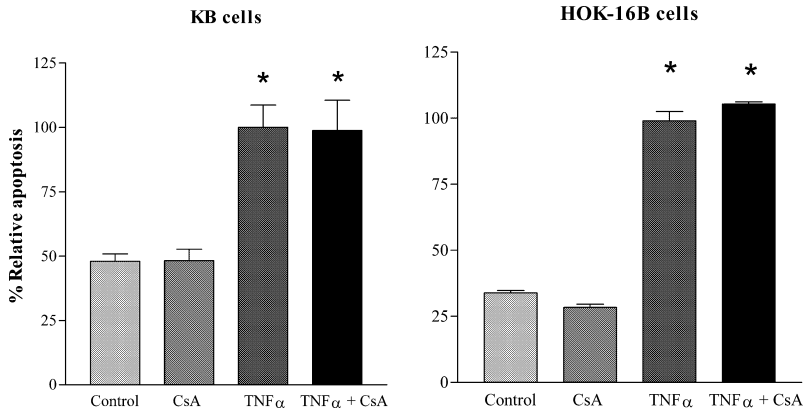

Fig. 3. Cyclosporin A (CsA) has no effect on tumour necrosis factor-α (TNF-α)-induced apoptosis in KB and HOK-16B cells. Apoptosis was measured by analysis of caspase-3 activity in control cells and cells co-incubated with TNF-α (1000 U/ml for KB cells or 500 U/ml for HOK-16B cells), CsA (1 μg/ml) or CsA + TNF-α. Levels of apoptosis are presented as a percentage of the values obtained with TNF-α alone and represent the mean data (± standard deviation) from three independent cultures. $*p < 0.05$.

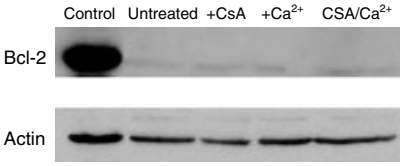

Fig. 4. Intracellular Bcl-2 in HOK-16B cells is not influenced by cyclosporin A (CsA). Bcl-2 was determined in HOK-16B cell extracts using western blotting. HOK-16B cells were incubated with medium alone (untreated), with 1.5 mM $CaCl_2$ and/or 1 μg/ml CsA for 72 h. Equal loading was confirmed by analysis of actin expression. Extracts from the T-cell leukaemia line, MOLT-16, were used as a positive control for Bcl-2 expression.

resistance to apoptosis (29). Furthermore, inhibition of apoptosis has been proposed to explain fibroblast accumulation in drug-induced GO (20). The pathway of apoptosis mediated by TNF-α is relevant to keratinocyte homeostasis, and CsA-induced apoptosis in epithelial cells is mediated by caspase-3 (30,31). Furthermore, CsA inhibits renal epithelial cell growth by the induction of apoptosis (4,5,30,32). In hepatoma cells, CsA opposes TNF-α-induced apoptosis *in vitro*, and this effect is regulated by CsA action on the calcineurin pathway involved in T-cell activation, but also thought to regulate cytotoxicity induced by TNF-α (33). In contrast, we found that TNF-α-induced apoptosis in both KB and HOK-16B cells was not influenced by CsA. Similarly, no differences were observed in keratinocyte apoptosis in the gingiva of patients with CsA-induced apoptosis compared to similar tissues from systemically healthy individuals (34). Thus, the effects of CsA on apoptotic pathways may be cell-type specific.

We showed that the epithelial cells could not recover from exposure to high-dose CsA. CsA may induce a major stress on the epithelial cells, influencing a number of biochemical pathways (2). This may result in the activation of cytoprotective mechanisms aimed at maintaining cell survival, perhaps including altered growth factor synthesis, and which may be enhanced by localized connective tissue reactions and inflammatory processes.

CsA may influence cell survival through effects on intracellular mediators involved in cell stress signalling, such as Bcl-2 or heat shock proteins (35,36). For example, CsA upregulates Bcl-2 in endothelial cells, promoting cell survival during stressful conditions (37). However, we were not able to demonstrate any effect of CsA on the intracellular levels of Bcl-2 in oral keratinocytes. The nuclear factor-κB (NF-κB) pathway protects keratinocytes from premature apoptosis during the process of upward migration and differentiation (29,31). The sensitivity or resistance to apoptosis in keratinocytes and other cells may be determined by the steady-state levels of NF-κB and other molecules that influence cell survival.

In conclusion, we have observed that CsA has a significant and long-lasting inhibitory effect on the cell division of both primary oral keratinocytes and an oral keratinocyte cell line. This effect seems to be independent of both basal and TNF-α-induced apoptosis and is not associated with any changes in Bcl-2. Further detailed experiments, investigating the effects of CsA on intracellular pathways and the influence of other cells relevant to gingival physiology, will be required to delineate the mechanism underlying these observations and their relationship to the pathogenesis of GO.

Acknowledgements

We thank Professor No-Hee Park (UCLA School of Dentistry) for supplying HOK-16B cells. CsA was a kind gift of Sandoz.

References

1. Allison AC. Immunosuppressive drugs: the first 50 years and a glance forward. *Immunopharmacology* 2000;**47:**63–83.
2. Matsuda S, Koyasu S. Mechanisms of action of cyclosporine. *Immunopharmacology* 2000;**47:**119–125.
3. Nabel GJ. A transformed view of cyclosporine. *Nature* 1999;**397:**471–472.
4. Esposito C, Fornoni A, Cornacchia F *et al.* Cyclosporine induces different responses in human epithelial, endothelial and fibroblast cell cultures. *Kidney Int* 2000;**58:**123–130.
5. Healy E, Dempsey M, Lally C, Ryan MP. Apoptosis and necrosis: mechanisms of cell death induced by cyclosporine A in a renal proximal tubular cell line. *Kidney Int* 1998;**54:**1955–1966.
6. Reynolds NJ, Al-Daraji WI. Calcineurin inhibitors and sirolimus: mechanisms of action and applications in dermatology. *Clin Exp Dermatol* 2002;**27:**555–561.
7. Takahashi T, Kamimura A. Cyclosporin a promotes hair epithelial cell proliferation and modulates protein kinase C expression and translocation in hair epithelial cells. *J Invest Dermatol* 2001;**117:**605–611.
8. Justo P, Lorz C, Sanz A, Egido J, Ortiz A. Intracellular mechanisms of cyclosporin

A-induced tubular cell apoptosis. *J Am Soc Nephrol* 2003;**14:**3072–3080.
9. Suchett-Kaye G, Morrier JJ, Barsotti O. Interactions between non-immune host cells and the immune system during periodontal disease: role of the gingival keratinocyte. *Crit Rev Oral Biol Med* 1998;**9:**292–305.
10. Thomason JM, Seymour RA, Ellis JS *et al*. Determinants of gingival overgrowth severity in organ transplant patients. An examination of the role of HLA phenotype. *J Clin Periodontol* 1996;**23:**628–634.
11. Boltchi FE, Rees TD, Iacopino AM. Cyclosporine A-induced gingival overgrowth: a comprehensive review. *Quintessence Int* 1999;**30:**775–783.
12. Seymour RA, Thomason JM, Ellis JS. The pathogenesis of drug-induced gingival overgrowth. *J Clin Periodontol* 1996;**23:**165–175.
13. James JA, Irwin CR, Linden GJ. Gingival fibroblast response to cyclosporin A and transforming growth factor beta 1. *J Periodont Res* 1998;**33:**40–48.
14. Wright HJ, Chapple IL, Matthews JB. TGF-beta isoforms and TGF-beta receptors in drug-induced and hereditary gingival overgrowth. *J Oral Pathol Med* 2001;**30:**281–289.
15. Niimi A, Tohnai I, Kaneda T, Takeuchi M, Nagura H. Immunohistochemical analysis of effects of cyclosporin A on gingival epithelium. *J Oral Pathol Med* 1990;**19:**397–403.
16. Pisanty S, Rahamim E, Ben-Ezra D, Shoshan S. Prolonged systemic administration of cyclosporin A affects gingival epithelium. *J Periodontol* 1990;**61:**138–141.
17. Rateitschak-Pluss EM, Hefti A, Lortscher R, Thiel G. Initial observation that cyclosporin-A induces gingival enlargement in man. *J Clin Periodontol* 1983;**10:**237–246.
18. Tyldesley WR, Rotter E. Gingival hyperplasia induced by cyclosporin-A. *Br Dent J* 1984;**157:**305–309.
19. Wysocki GP, Gretzinger HA, Laupacis A, Ulan RA, Stiller CR. Fibrous hyperplasia of the gingiva: a side effect of cyclosporin A therapy. *Oral Surg Oral Med Oral Pathol* 1983;**55:**274–278.
20. Nishikawa S, Nagata T, Morisaki I, Oka T, Ishida H. Pathogenesis of drug-induced gingival overgrowth. A review of studies in the rat model. *J Periodontol* 1996;**67:**463–471.
21. Park NH, Min BM, Li SL, Huang MZ, Cherick HM, Doniger J. Immortalization of normal human oral keratinocytes with type 16 human papillomavirus. *Carcinogenesis* 1991;**12:**1627–1631.
22. Masters JR, Thomson JA, Daly-Burns B *et al*. Short tandem repeat profiling provides an international reference standard for human cell lines. *Proc Natl Acad Sci USA* 2001;**98:**8012–8017.
23. Rheinwald JG, Green H. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. *Cell* 1975;**6:**317–330.
24. Furue M, Gaspari AA, Katz SI. The effect of cyclosporin A on epidermal cells. II. Cyclosporin A inhibits proliferation of normal and transformed keratinocytes. *J Invest Dermatol* 1988;**90:**796–800.
25. Kanitakis J, Thivolet J. Cyclosporine. An immunosuppressant affecting epithelial cell proliferation. *Arch Dermatol* 1990;**126:**369–375.
26. Nurmenniemi PK, Pernu HE, Knuuttila ML. Mitotic activity of keratinocytes in nifedipine- and immunosuppressive medication-induced gingival overgrowth. *J Periodontol* 2001;**72:**167–173.
27. Bulut OE, Sokmensuer LK, Bulut S, Tasman F, Muftuoglu S. Immunohistochemical study of cyclosporin-induced gingival overgrowth in renal transplant recipients. *J Periodontol* 2004;**75:**1655–1662.
28. Ellis JS, Morgan CL, Kirby JA, Taylor JJ, Thomason JM. Plasma TGF-beta1 as a risk factor for gingival overgrowth. *J Clin Periodontol* 2004;**31:**863–868.
29. Chaturvedi V, Qin JZ, Denning MF, Choubey D, Diaz MO, Nickoloff BJ. Apoptosis in proliferating, senescent, and immortalized keratinocytes. *J Biol Chem* 1999;**274:**23358–23367.
30. Ortiz A, Lorz C, Catalan M, Coca S, Egido J. Cyclosporine A induces apoptosis in murine tubular epithelial cells: role of caspases. *Kidney Int Suppl* 1998;**68:**S25–S29.
31. Weisfelner ME, Gottlieb AB. The role of apoptosis in human epidermal keratinocytes. *J Drugs Dermatol* 2003;**2:**385–391.
32. Hortelano S, Castilla M, Torres AM, Tejedor A, Bosca L. Potentiation by nitric oxide of cyclosporin A and FK506-induced apoptosis in renal proximal tubule cells. *J Am Soc Nephrol* 2000;**11:**2315–2323.
33. Kantrow SP, Gierman JL, Jaligam VR *et al*. Regulation of tumor necrosis factor cytotoxicity by calcineurin. *FEBS Lett* 2000;**483:**119–124.
34. Alaaddinoglu EE, Karabay G, Bulut S *et al*. Apoptosis in cyclosporin A-induced gingival overgrowth: a histological study. *J Periodontol* 2005;**76:**166–170.
35. Burlacu A. Regulation of apoptosis by Bcl-2 family proteins. *J Cell Mol Med* 2003;**7:**249–257.
36. Nollen EA, Morimoto RI. Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins. *J Cell Sci* 2002;**115:**2809–2816.
37. Longoni B, Boschi E, Demontis GC, Ratto GM, Mosca F. Apoptosis and adaptive responses to oxidative stress in human endothelial cells exposed to cyclosporin A correlate with BCL-2 expression levels. *Faseb J* 2001;**15:**731–740.