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Effects of Cyclosporin A on Cultured Human Epidermal Keratinocytes

Growth and 5-Bromo-2'-deoxyuridine Incorporation

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We have studied the in vitro effect of CsA at various doses (0.5, 1, 5, 10 μg/ml) on the growth and 5-bromo-2'-deoxyuridine (BrdU) incorporation by cultured normal human epidermal keratinocytes (EK). CsA was found to reduce the growth of EK at all doses used after 24, 48 and 72 h in culture, but the difference with appropriate controls became statistically significant for the dose of 10 μg/ml after a 48- and 72-h culture. On the other hand, CsA-treated EK comprised a reduced fraction of BrdU+ (S-phase) cells, the difference being statistically significant for the dose of 10 μg/ml at 24, 48 and 72 h. Ultrastructural examination of CsA-treated EK, despite the presence of cytoplasmic vacuoles also observed in control EK did not show signs of severe cytoplasmic or nuclear damage. These results confirm the antiproliferative effect of CsA on cultured EK and suggest that at the concentration used, CsA acts through a cytostatic rather than a cytotoxic mechanism, most likely by blocking EK in an early phase (G0/G1) of the cell cycle. ***Key word: Proliferation.***

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Cyclosporin A (CsA) is a cyclic undecapeptide that causes suppression of primary immune stimulation in vivo and in vitro. It is used clinically for prophylaxis and treatment of allograft rejection (1–3), and has been reported to be effective in the treatment of patients with a wide variety of immune mediated disorders (4–6). Recent studies have shown that CsA is effective in the treatment of psoriasis (7–9) and suggested that it may be also beneficial in the treatment of ichthyosis vulgaris (10).

Although the beneficial effect of CsA in psoriasis has been claimed to be mediated through anti-lymphocyte activity (11), substantial evidence has been accumulating suggesting that CsA exerts a direct cytostatic effect on epidermal keratinocytes (EK). In vitro studies have shown the antiproliferative effect of CsA on cultures of rapidly proliferating EK (12–14). Whereas CsA has been shown to arrest in vitro sensitive T-lymphocytes at the G0/G1 phase of the cell-cycle, similar studies on EK are lacking.

The use of BrdU is an elegant way to study the fraction of S-phase cells in a proliferating cellular population. Recently we observed that normal human skin xenografts onto nude mice treated with CsA gave decreased numbers of 5-bromo-2'deoxyuridine-incorporating (BrdU+) EK as compared with control xenografts (15). The present study was undertaken in order to investigate the antiproliferative effect of CsA on rapidly growing human EK in culture and to correlate it with its effect on the fraction of cells in the S-phase of the cell cycle.

MATERIAL AND METHODS

Epidermal cell cultures

Human epidermal keratinocyte suspensions were prepared by standard trypsinization procedures from normal adult skin specimens removed during plastic surgery. Briefly, the specimens were incubated in a trypsin solution (trypsin 1/250, 0.25%, Difco, Detroit, Mich.) for 1 h at 37°C. The epidermis was then detached from the dermis with fine forceps and the epidermal cell suspension was obtained through vigorous pipetting. After centrifugation, cells were resuspended in complete medium: DMEM (Dulbecco's modified Eagle's medium, Gibco, Grand Island, USA) and Ham's F12 (Gibco) 3:1, supplemented with 10% fetal calf serum (FCS) (Boehringer Mannheim, Meylan, France), 100 U/ml penicillin/streptomycin (Gibco Laboratories), 5 μg/ml insulin (Sigma, St. Louise, USA), 0.4 μg/ml hydrocortisone (Sigma), 10^{-10} M cholera toxin (Sigma), 10 ng/ml epidermal growth factor (EGF) (Sochibo Boulogne, France) and seeded on 3T3 feeder cell layers *ad modum* Green et al. (16). The cultures were carried out at 37°C in a 5% CO_2 atmosphere.

At confluence, the EK were trypsinized with a 1:1 mixture

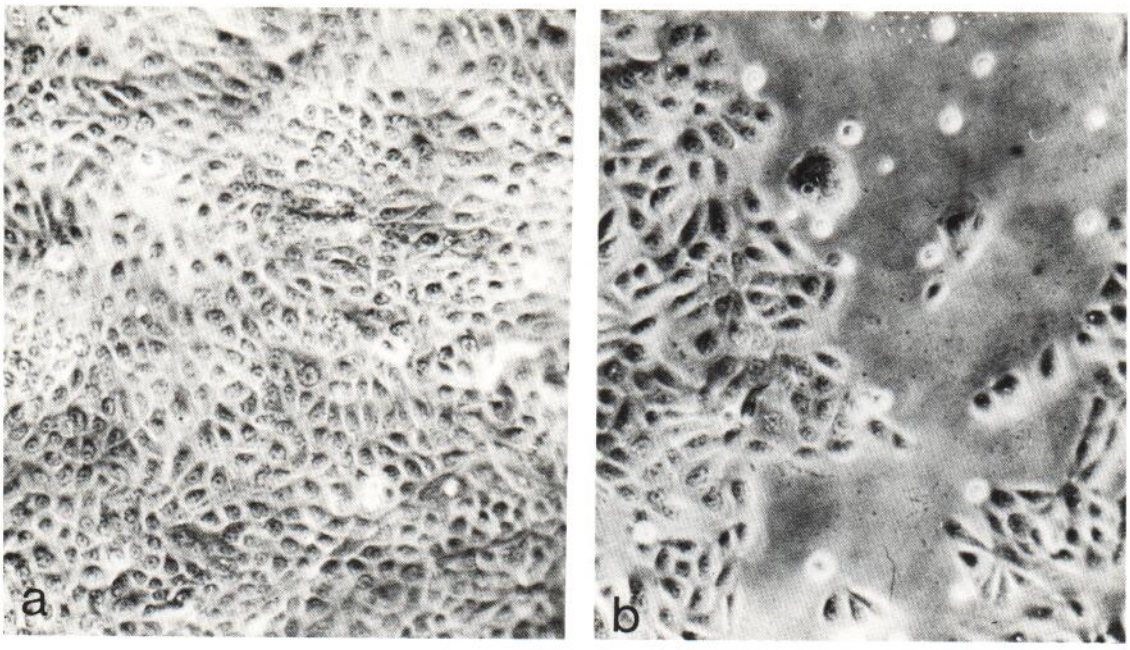

Fig. 1. EK cultured for 48 h in the presence of control medium (*a*) and Cyclosporin A (5 μg/ml) (*b*). (×250).

of trypsin-EDTA (Gibco). They were then plated at a low cell density (6×10^4 cells) on 35-mm plastic dishes in serum-free Medium MCDB 153 (Irvine Scientific, USA) supplemented with: 5 ng/ml EGF, 5 μg/ml insulin, 1.4 M (0.5 μg/ml) hydrocortisone, 0.1 mM ethanolamine (Sigma), 0.1 mM phosphoethanolamine (Sigma), 1% glutamine (Gibco) and 100 U/ml penicillin/streptomycin (Gibco), with 0.1 mM $CaCl_2$ concentration (17). After a 48-h culture, the culture medium was replaced by CsA-containing medium (see below) and EK further cultured for 24, 48 and 72 h.

All experiments were performed in triplicate with EK of the same donor.

Drug solutions

CsA (powder) was a generous gift from Sandoz (Paris). It was initially dissolved in 95% ethanol and subsequently further diluted in the culture medium to final concentrations of 0.5, 1, 5 and 10 μg/ml. Control EK cultures were incubated with MCDB 153 medium containing equivalent concentrations of ethanol (i.e. 0.0475, 0.095, 0.475 and 0.95%, respectively).

BrdU incorporation and immunolabelling (18)

After incubation of the EK cultures with CsA for 24, 48 and 72 hours, 5-bromo-2-deoxyuridine (BrdU, Sigma) was added to the culture medium at a concentration of 10 μM. After a 30-min incubation (37°C, 5% CO_2), EK were trypsinized and fixed with 70% ethanol in PBS (30 min, 4°C). The total number of EK in each well was counted under a light microscope using a Burker microchamber.

For revealing incorporated BrdU, cellular DNA was denatured with 2 N HCl (30 min, 20°C), and neutralized with a 0.1 M borax solution (pH 8.5, 5 min). The cells were then incubated for 45 min with an anti-BrdU monoclonal antibody (Becton Dickinson, Mountain View, USA) at a 1:10 dilution in PBS containing 0.5% Tween 20 and 10% bovine serum albumin (BSA). After washing in PBS–10% BSA, the intranuclear label was revealed by FITC-conjugated F(ab')2 goat antimouse IgG (dilution 1:20 in PBS–10% BSA). After two washes in PBS–10% BSA, fluorescent nuclei containing cells were counted under a Zeiss Orthoplan microscope.

The total number of cells per field was alternatively counted under visible light.

Statistical analysis

For comparing the total number of EC between CsA-treated and control EK cultures Student's *t*-test was used. The χ^2-test was used to compare the number of BrdU+ cells between CsA-treated and control cultures.

Electronmicroscopy

After trypsinization, EK were fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide dehydrated in a graded series of ethanol and embedded in Epon. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and examined under a Jeol 1200 EX electron-microscope (CMEABG, Lyon I University, Villeurbanne, France).

RESULTS

Counting of total EK numbers

Under inverted-microscope examination, it could be seen that control EK proliferated rapidly and reached

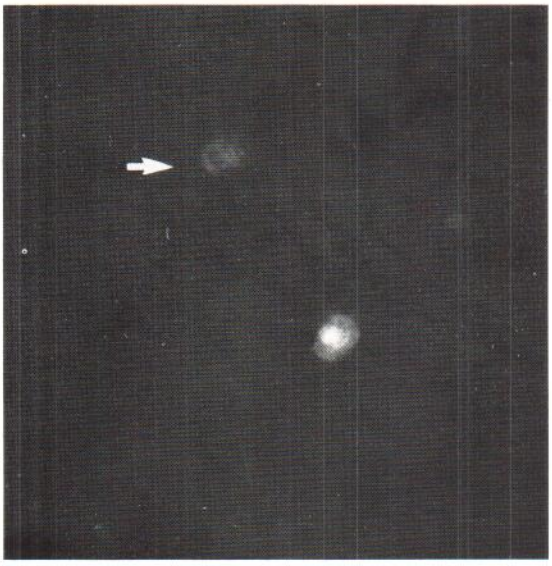

Fig. 2. Nuclear staining of a BrdU+ EK. Arrow points to a BrdU-negative cell. (×500).

Table I. *Total number of EK at the end of the culture (mean ± SD of 3 experiments)*

CsA (μg/ml)	Control (×10³)	CsA-treated (×10³)	Inhibition (%)
24 h			
0.5	312.5±51	270.8±29.5	13
1	218.75±54	145.8±18	33
5	260.4±18	208.3±48	20
10	208.3±18	166.7±48	20
48 h			
0.5	500.0±51	479.2±29.5	4
1	687.5±51	583.3±39	13
5	416.7±14.7	333.3±53	20
10	468.75±76.5	177.1±39*	62
72 h			
0.5	593.7±25.5	500.0±51	15
1	520.8±39	427.0±39	18
5	500.0±44.2	416.7±53	16
10	322.9±14.7	166.7±29.5*	48

* $p<0.01$.

confluence almost at the end of the 48h-culture. By contrast, CsA-treated EK never became confluent even after 72h-culture (Fig. 1 a–1 b).

The total EK number at the end of 24, 48 and 72 h culture with or without CsA at various concentrations can be seen in Table I. From this Table it can be seen that, at any day of culture and at any CsA concentration, the CsA-treated culture wells contained a decreased number of EK as compared to the control wells.

The growth inhibition reaches statistical significance for the dose of 10 μg/ml at 48 and 72 h of culture (p=0.009 and 0.003, respectively).

Numbers of BrdU+ (S-phase) EK (Fig. 2)

The numbers of BrdU+ (S-phase) EK out of the total number of EK per day of culture and CsA concentration can be seen in Table II. As can be noted from these figures, there is a statistically significant decrease ($p<0.001$) in the number of BrdU+ EK in CsA-treated vis-à-vis control EK for the concentration of CsA of 10 μg/ml at the end of 24, 48 and 72 h of culture.

Electronmicroscopy

Cells cultured for 72 h in MCDB 153/0.95% ethanol had a round shape and numerous villosities on their surface. Their nucleus was large and displayed marginated heterochromatin and voluminous nucleoli. The cytoplasm contained rather loose tonofilament bundles in a perinuclear pattern and dispersed mitochondria with an electron-dense matrix. The most noticeable finding was the presence, within the cytoplasm, of round, membrane-bound electron-lucent vacuoles, most likely of lysosomal origin, occasionally containing membranous material. Cells treated with CsA (10 μg/ml) showed, at the same time of culture (72 h), similar ultrastructural features; the only noticeable difference was the fact that their cytoplasmic vacuoles were slightly more abundant and more frequently contained membranous or granular material.

Table II. *Numbers of BrdU+ EK/total number of EK*

CsA (μg/ml)	Control (%)	CsA-treated (%)	Difference (%)
24 h			
5	105/166 (63)	99/155 (64)	1.6
10	121/210 (58)	37/193 (38)*	34.5
48 h			
5	101/170 (59)	82/160 (46)	22
10	90/180 (54)	60/150 (40)*	26
72 h			
5	90/173 (52)	59/141 (41)	21
10	58/136 (43)	32/129 (25)*	42

* $p<0.001$.

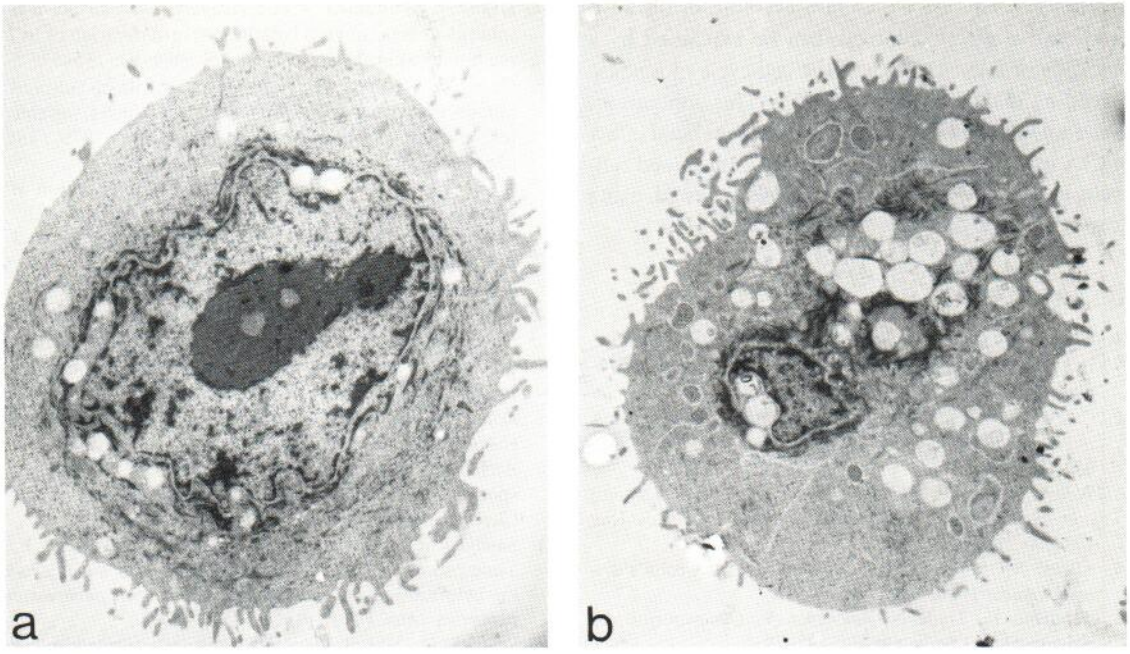

Fig. 3. EK Cultured for 72 h in the presence of control medium (*a*) and Cyclosporin A (10 μg/ml) (*b*). (Electron-microscopy, ×6000).

No signs of cellular or nuclear degeneration were noted (Fig. 3a–b).

DISCUSSION

CsA is a potent immunosuppressant inhibiting the proliferation of stimulated T-helper cells. However, several lines of evidence suggest that CsA may also affect in various ways the biology of other non-lymphoid cell-types, such as endothelial (19), renal (20) and pancreatic cells (21). CsA has a stimulatory effect on hair-follicle keratinocytes, resulting in hypertrichosis. This effect has been observed both on CsA-treated humans (22) and nude animals (23, 24). However, CsA exerts in vitro an antiproliferative effect on cultured (interfollicular) EK that is more easily evidenced when hyperproliferative, low-calcium serum-free culture media (25) are used.

Our results confirm the growth inhibition of cultured EK by CsA as evaluated by the total number of cells obtained at the end of the culture. The effect of CsA on EK proliferation was studied during 72 h of culture, since pilot experiments indicated that after the period of time under the culture conditions used in this work, EK become differentiated and detach from the wells. The antiproliferative effect of CsA on EK seems to be cytostatic rather than cytotoxic for the following reasons: *a*) ultrastructural examination of EK cultured for 72 h in the presence of the highest concentration of CsA (10 μg/ml) apart from the presence of cytoplasmic vacuoles slightly more abundant than in control cells did not reveal signs of severe cytoplasmic or nuclear degeneration; *b*) considering separately each dosage of CsA used, no decrease in the total number of viable EK in the course of the culture was observed, even at the dosage that caused complete growth inhibition (10 μg/ml).

The mechanism of cytostatic activity of CsA on EK is not known with certainty, although recent studies suggest that CsA may inhibit ornithine-decarboxylase induction and polyamine synthesis (21, 26, 27) and reduce the number of ^{125}I-EGF-bindning sites (28).

Flow-cytometry studies showed that CsA arrests in vitro sensitive T-lymphocytes in the G0/G1 phase of the cell cycle (29), before the acidification of the cytoplasm that precedes DNA synthesis.

Our previous results in vivo (15) and the results of the present work obtained through the study of BrdU incorporation suggest that CsA exerts a similar effect on EK: indeed, the finding of reduced numbers of EK

in S phase (BrdU+), which may account for the antiproliferative effect obtained, can be explained by a blockade or a lengthening of the cell-cycle in a phase preceding DNA synthesis (G0/G1). This contention, that could explain at least in part the beneficial effect of CsA in psoriasis, characterized by a shortened cell-cycle length of EK, is currently being tested using flow-cytometric analysis.

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