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Cyclosporin-A affects the organization of cytoskeleton of normal human keratinocytes in culture

F. Prignano[1], L. Domenici-Lombardo[2], G. Gerlini[1], N. Pimpinelli[1] and P. Romagnoli[2]

[1]Second Dermatology Clinic and [2]Department of Human Anatomy and Histology, University of Florence, Florence, Italy

Summary. Cyclosporin-A (CsA) is a potent immunoregulatory molecule which has been widely used in many immunomediated and inflammatory skin diseases. It inhibits the proliferation of keratinocytes, but its possible effects(s) on cell differentiation are poorly known. To address this issue, we have studied the influence of CsA on the assembly of intermediate filaments by normal human keratinocytes in culture. Control keratinocytes were flat; the cells which had not reached confluence stained intensely for vimentin and weakly for cytokeratins; confluent cells stained with intermediate intensity for both types of proteins and the cells adhering on the top of others, interpreted as the best differentiated ones, stained for cytokeratins but not for vimentin. CsA (1.6 μg/ml for 10 days) inhibited the growth of keratinocytes, which never reached confluence; most cells appeared small and roundish, only some stained for cytokeratins and few for vimentin. By electron microscopy, a well organized meshwork of tonofibrils was recognized in many control keratinocytes, but never in CsA-treated keratinocytes. We propose that the cytoskeleton could be a target of CsA and that its alteration mediates other effects of CsA on keratinocytes, including those on cell growth.

Key words: Cytokeratin, Electron microscopy, Immunohistochemistry, Intermediate filaments, Vimentin

Introduction

Epidermal keratinocytes undergo dramatic and strictly regulated changes during their progression from the basal layer to shedding. Only the cells of the deepest, basal layer can synthesize DNA and proliferate. Some, yet undefined, cellular events transform a part of newly formed keratinocytes into growth-arrested cells that become committed to terminal differentiation. Both basal and the more superficial prickle cells synthesize cytokeratins, which aggregate into filaments and bundles of filaments (tonofibrils; Nelson and Sun, 1983; Eichner et al., 1986). The cells of the granular layer produce other differentiation-dependent proteins, different from cytokeratins (Fuchs, 1990), which are implicated in the final step of keratinocyte differentiation into cornified, metabolically inactive cells.

Keratinocytes are unable to undergo spontaneous terminal differentiation in vitro; in this condition, moreover, they change the expression of intermediate filament proteins, from cytokeratins to vimentin (Assalineau et al., 1986). The addition of substances like calcium, retinoids and some growth factors are able to induce a differentiation program, with some differences depending on the substance applied (Fuchs, 1990). One of the criteria to establish the differentiation degree of human keratinocytes in culture is to identify whether and which intermediate filament antigens they do express (Roop et al., 1987).

Cyclosporin-A (CsA; Shevach, 1985) affects the immune reactions within skin and inner organs and has been widely used in the treatment of immune-mediated systemic and skin diseases. Interference with the synthesis of cytokines (Quesniaux, 1993) and with the function of dendritic cells and lymphocytes (Dupuy et al., 1991; Furue and Katz, 1988) seems to play a relevant role for the effects of CsA on the skin. It is known (Ansellem et al., 1992) that keratinocytes from both normal and diseased (psoriatic or ichthyotic) skin, as well as transformed keratinocytes, become smaller and proliferate less intensely if cultured in the presence of CsA; however, the question whether CsA affects the differentiation of keratinocytes remains open. To address this issue, we have investigated on normal human keratinocytes cultured for some time in standard conditions and either with or without CsA. To estimate differentiation, we have checked the expression of intermediate filament proteins and the spatial arrangement of these filaments.

Materials and methods

Reagents and disposables

D-MEM, ethylen-diamino-tetraacetic acid (EDTA),

Offprint requests to: Dr. Francesca Prignano, Department of Human Anatomy and Histology, Section «E. Allara» - Viale Pieraccini, 6, Firenze, Italy

fetal calf serum (FCS), Ham's F12, Hank's balanced salt solution (calcium and magnesium free: HBSS-), Hepes, $NaHCO_3$, and trypan blue were purchased from Seromed (Berlin, Germany); 3,3',5-triiodothyronine, adenine, cholera toxin, epidermal growth factor, glutamine, insulin, mitomycin-C, penicillin-streptomycin, transferrin and trypsin were from Sigma (Milan, Italy); dispase (grade II) was from Boehringer (Mannheim, Germany), hydrocortisone from Calbiochem (Milan, Italy); and CsA was generously provided by Sandoz (Milan, Italy). Cell strainers were from Becton-Dickinson (Franklin Lakes, New Jersey), centrifuge tubes and culture flasks from Costar (Concorezzo, Italy), chamber-slides from Nunc (Nepperville), and sterile filters, with 0.22 μm-pores, from Schleicher and Schuell (Dassel, Germany).

Culture of normal human keratinocytes

Human keratinocytes were isolated from specimens obtained at plastic surgery from skin unexposed to sunshine. The epidermis was separated from the dermis with dispase II, cut into small pieces and incubated for 15 min at 37 ºC, with 0.25% trypsin and 0.02% EDTA. The cells were filtered through a sterile gauze and a cell-strainer, and then centrifuged at 1100 rotations per minute (approximately 200g) for 10 minutes in a Universal 30 RF centrifuge (Hettich, Tuttlingen, Federal Republic of Germany). After further washing with keratinocyte medium (see below), they were seeded on a feeder layer of 3T3-J2 cells which had been previously treated with mitomycin-C (4 μg/ml for 2 hours at 37 ºC) and were left to adhere again for 2 hours before seeding the keratinocytes. All cultures were maintained at 37 ºC in an atmosphere of air and 5% CO_2. The keratinocytes culture medium was composed of D-MEM (60%), Ham F12 (30%), FCS (10%), glutamine (4 mmol/l), penicillin (1000 UI/ml), streptomycin (100 μg/ml), adenine (0.18 mmol/l), insulin (5μg/ml), transferrin (5 μg/ml), hydrocortisone (0.4 μg/ml), 3,3',5-triidothyronine (2 mmol/l), and cholera toxin (0.1 mmol/l), EGF (10 ng/ml); EGF was added to the cells after 48 hours culture. Some cultures were incubated with CsA (1.6 μg/ml), starting after 48 hours culture without CsA; control cultures were incubated without CsA for the whole experiment. Some cultures were grown in flasks, some others directly on chamber slides. After 12 days for the cultures grown in flasks, and 8 days for those grown on chamber slides, the cells were treated with 0.1% glucose and 0.02% EDTA to remove possibly surviving fibroblasts, and keratinocytes were prepared for immunofluorescence or electron microscopy.

Immunofluorescence

Upon removal of fibroblasts, human keratinocytes grown on chamber slides were fixed in methanol/aceton (1:1). The cells were rehydrated, blocked with 1% bovine serum albumin or 5% normal calf serum, rinsed three times with 138 mmol/l NaCl, 2.5 mmol/l KCl and 10 mmol/l phosphate buffer (PBS) and incubated with the mouse monoclonal antibodies listed in Table 1, for 1.5 hours at 37 ºC, followed by a fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma, Milan, Italy) for 1 hour at 37 ºC. The slides were mounted with gel mount (Biomeda, Foster City, USA) and photographed in a Zeiss Axioskop microscope (Oberkochen, Federal Republic of Germany) equipped for epifluorescence.

Table 1. Monolonal antibodies.

ANTIBODIES	SOURCE	SPECIFICITY
Anti-cytokeratin 18	Boehringer	Human cytokeratin 18
CAM 5.2	Becton-Dickinson	Cytokeratins No 8 and 18
AE1	Ortho	Cytokeratins in basal cells
Anti-pancytokeratin	Sigma	Simple, cornifying and non-cornifying squamous epithelia
Anti-vimentin	Boehringer	Vimentin

Electron microscopy

Upon removal of fibroblasts, human keratinocytes grown in flasks were washed with FCS additioned medium, followed by PBS, and incubated with 0.25% trypsin and 0.02% EDTA, for 15 min at 37 ºC. The cells were fixed with Karnovsky's mixture in 0.1 mol/l cacodylate buffer, pH 7.4, osmicated and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate followed by bismuth tartrate or lead citrate and observed in a Siemens (Munich, Germany) 102 electron microscope, at 80 kV.

Results

The keratinocytes grown in presence of CsA-additioned medium progressively became less numerous (from the 4th to the 12th days of culture) and changed their shape, appearing more roundish and losing contact with each other.

Immunohistochemistry

The cells grown in a CsA-free medium were wide and flat. Three main subpopulations could be recognized, which differed from each other in morphology and immune reactivity: (a) cells which had not reached confluence; (b) cells which had reached confluence; and (c) cells adhering on the top of others. The cells which had not reached confluence (Fig. 1a) stained intensely for vimentin (Fig. 1b), and weakly for cytokeratins. Confluent cells stained with intermediate intensity for both types of proteins and the best differentiated cells, i.e. those adhering on the top of others (Fig. 2a), stained with all the anti-cytokeratin antibodies tested (Fig. 2b), but not for vimentin. The

cells grown in CsA-additioned medium never reached confluence. They were fewer and smaller than in control cultures and were usually roundish, or at most oval in shape. Some of them, those which appeared larger and more flat, stained for cytokeratins (Fig. 3a) and very few stained for vimentin (Fig. 3b).

Electron microscopy

Keratinocytes of control cultures contained

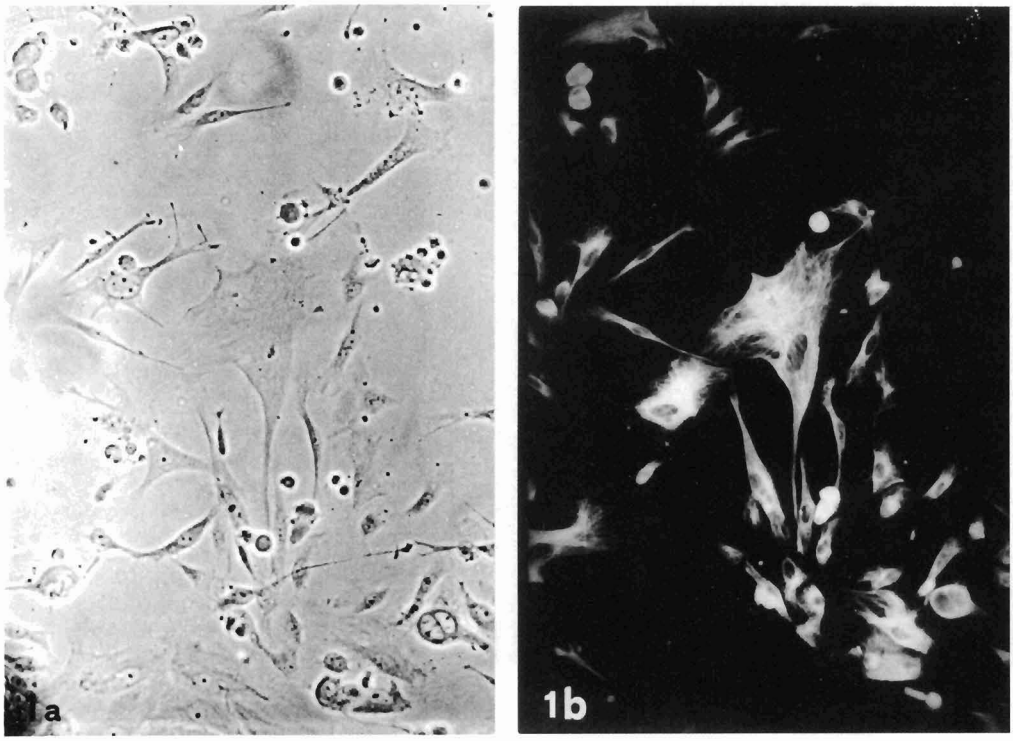

Fig. 1. Keratinocytes cultured without CsA after 8 days culture. **A.** Phase contrast: the cells have not reached confluence. **B.** Fluorescence microscopy: most non-confluent cells express vimentin. x 300

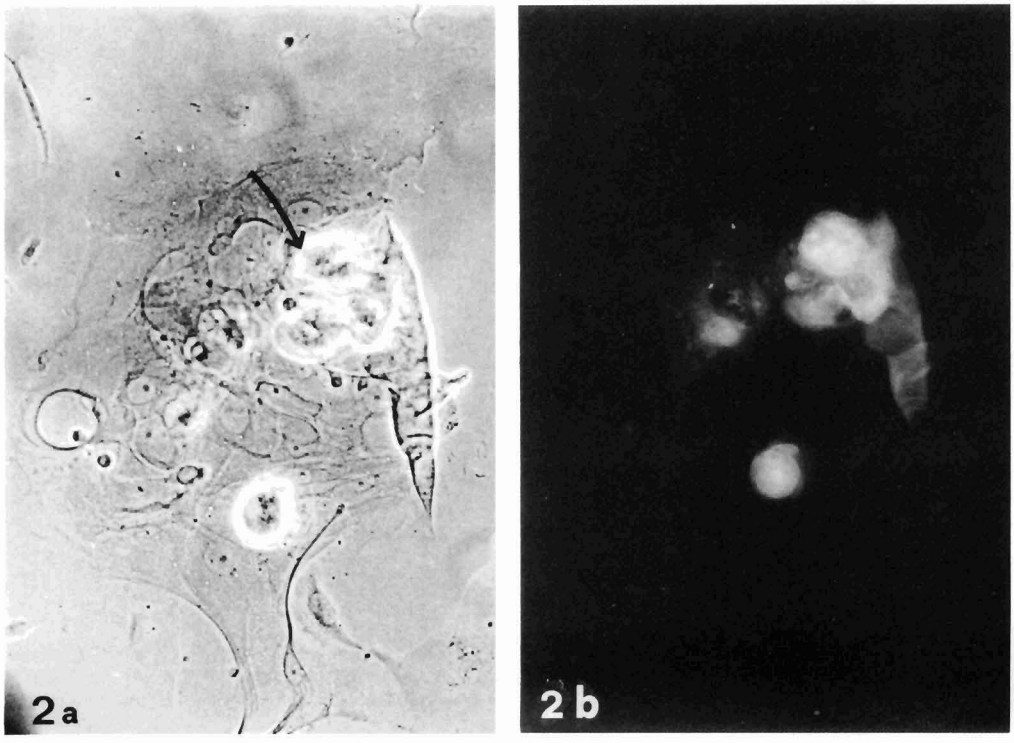

Fig. 2. Keratinocytes cultured without CsA after 12 days culture. **A.** Phase contrast: the cells reach confluence and some adhere on the top of others (arrow). **B.** The cells on the top express cytokeratins (CAM 5.2). x 300

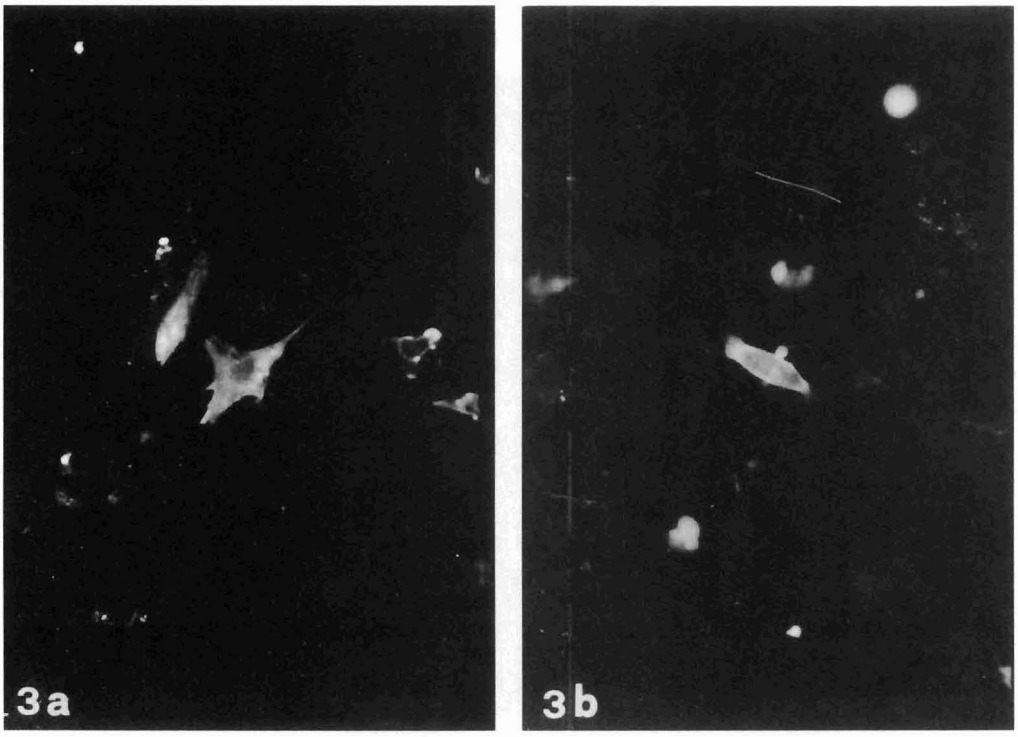

Fig. 3. Keratinocytes cultured with CsA (1.6 μg/ml for 8 days). Dramatic reduction of cell density; the cells are fewer, smaller and with a smoother profile than controls. Fluorescence microscopy. **A.** Some cells express vimentin. **B.** Other cells express cytokeratins (anti-pancytokeratin). x 150

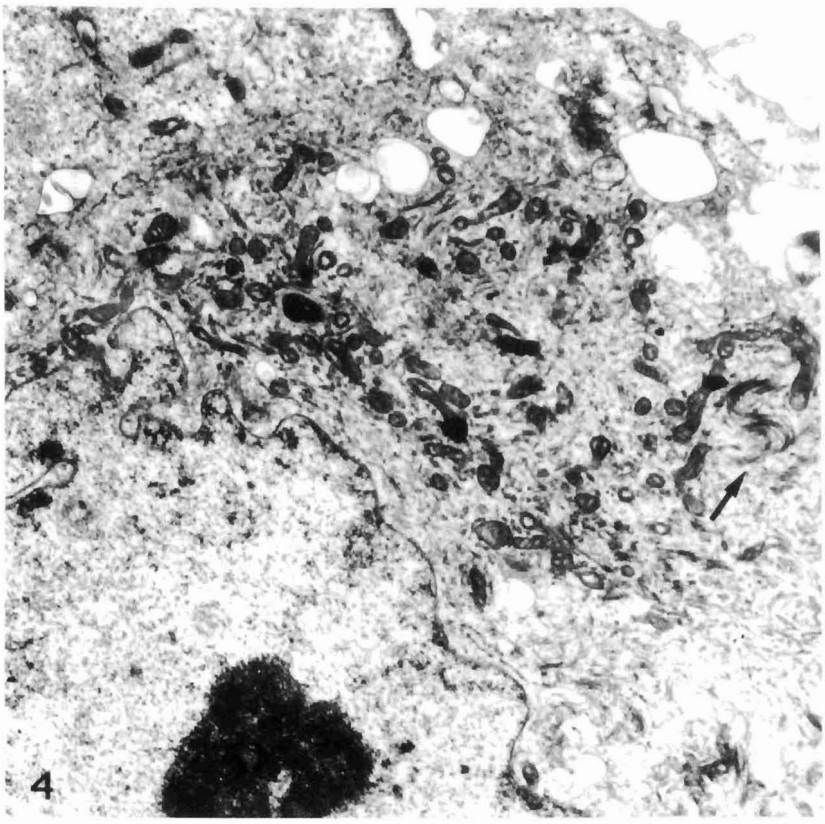

Fig. 4. Electron microscopy. Many keratinocytes cultured without CsA contain a mesh of fibrils, made up of intermediate filaments (arrows). The peripheral vacuoles are presumably endosomes (arrowheads). x 8,000

intermediate filaments arranged into fibrils. These filaments occupied the central, perinuclear part of the cells, whereas a peripheral rim of cytoplasm appeared devoid of organelles (Fig. 4). The number of fibrils varied greatly among cells, from virtually none to many, arranged into a mesh. Many vacuoles, presumably endosomes, were also present. Keratinocytes grown in CsA-additioned medium appeared rich in organelles and contained many lysosomes, but no fibrils of intermediate filaments (Fig. 5). The organelles were normal in shape, size and electron density, except for the Golgi apparatus, which was often much better developed than in control conditions.

Discussion

The results show that CsA at a relatively low concentration (1.6 μg/ml) affects the cytoskeleton of keratinocytes, so that these cells do not succeed in forming tonofibrils and remain roundish or oval in shape, although they can synthesize cytokeratins and vimentin. Besides, CsA induces modifications of the cell shape and inhibits growth, so that cells do not reach confluence. Whereas this latter result could be forecast on the basis of the literature (Dykes et al., 1990; Ramírez-Bosca et al., 1990), the former requires some comment. The alterations of the cytoskeleton were seen in cells which appeared normal, or even hypertrophic, for all other cytoplasmic structures; therefore, we propose that the effect of CsA on the cytoskeleton be interpreted as a specific one, and not simply as part of unspecific cell damage. Lysosomes were found in keratinocytes cultured in CsA-aumented medium; however this finding, in the absence of signs of degeneration of lysosome-containing cells, suggests that the lysosomes have formed as a consequence of phagocytosis of cell debris, presumably following the death of some cells because of CsA at time points earlier than those when cells were harvested for microscopy. In fact, the cells became progressively fewer from the sixth day of culture in CsA-aumented medium and on day 12 many dead cells could be recognized in the culture medium. We cannot at present answer the question as to whether CsA leads to disorganization of the cytoskeleton in cells surviving in culture in the presence of this drug, or if cells with a more simple, perhaps immature cytoskeleton survive CsA, whereas the others die. Future studies will address this point.

CsA is reported to affect the proliferation of many cell types, by inhibiting calmodulin-dependent calcium-regulated processes, altering intracellular pH (Rosoff and Terres, 1986; Daniel and Ives, 1987) and interacting with cyclophilins (Takahashi et al., 1989).

Several recent reports point to the cytoskeleton as a structure involved in the transduction of signals from the cell surface to the cytoplasm and nucleus; in this perspective, modifications of the cytoskeleton can be implicated in the regulation of gene expression,

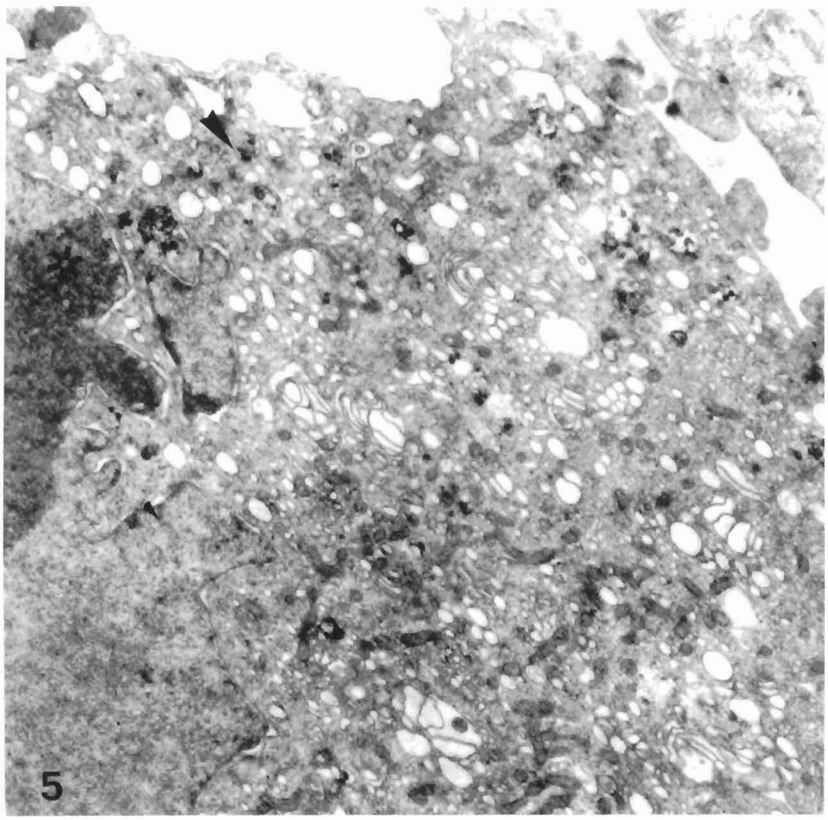

Fig. 5. Electron microscopy. Keratinocytes cultured with CsA (1.6 μg/ml, for ten days) do not contain fibrils of intermediate filaments. These cells contain electron dense, inhomogeneous bodies, presumably lysosomes (arrowheads). Other organelles appear normal and the nuclei contain prominent nucleoli (asterisk). x 7,500

including the regulation of cell proliferation and programmed death (Traub and Shoeman, 1994). Therefore, we should like to propose that CsA affects primarily the cytoskeleton of cultured keratinocytes, as a step of its action on these cells.

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