

CELLULAR AND MOLECULAR CHANGES OF PSORIATIC KERATINOCYTES IN RESPONSE TO UVA *IN VITRO* TREATMENT

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Abstract. In psoriasis, disturbed differentiation, hyperproliferation, and inflammation are hallmarks that are clinically represented by scaling, plaque thickening, and erythema, respectively. The present study describes the response to UVA irradiation of cultured human keratinocytes isolated from lesional and symptomless skin biopsies obtained from a male patient in different stages of disease. The recurrence and remission phenomena are accompanied by keratinocyte alterations that suffer modification towards parental type – control keratinocytes from symptomless psoriatic skin (KCc) and keratinocytes from periphery of psoriatic plaque (KCps) respectively – without reaching the values of basis parameters: viability, proliferation and apoptosis. Keratinocyte UVA irradiation results in morphological changes, decrease of cell viability and proliferation and an increase in level of apoptosis. These parameters' changes suggest the presence of genetic alterations in psoriasis. KCps appear to be more sensitive to UVA irradiation than KCc.

Key words: psoriasis, keratinocytes, UVA irradiation, morphology, viability, DNA content, apoptosis, remission, recurrence.

INTRODUCTION

Psoriasis is a chronic, noncontagious skin disease which can begin at any age, although epidemiological studies demonstrate that it most commonly appears for the first time between the ages of 15 and 25 years [6]. It is a lifelong inflammatory disease with spontaneous remissions and exacerbations. While the cause of psoriasis remains unknown, it appears to result from a combination of genetic and environmental factors.

In the plaque stage, lesions are characterized as being symmetrically distributed, and well demarcated from adjacent symptomless skin, with erythema topped by white-silvery scale. Microscopically, plaque-stage lesions reveal significantly thickened skin with confluent parakeratotic scale, loss of the granular cell layer, and increased number of epidermal cell layers with mitotic figures in

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basal-layer keratinocytes. The inflammatory cell infiltrate may contain neutrophil collections in the epidermis, but a more consistent finding is the presence of T cells in the dermis and epidermis accompanied by increased numbers of dermal dendritic cells, macrophages and mast cells [11].

From a histopathologic point of view, characteristics of psoriasis are hyperproliferation and abnormal differentiation of keratinocytes and infiltration of inflammatory elements. Multiple stages are proposed for trafficking patterns of immunocytes, involving signals in which symptomless skin is converted into a psoriatic plaque. Symptomless skin is endowed with a confederacy of bone marrow-derived cells, and continuous leukocyte migration between skin and lymph nodes provides immunological vigilance to monitor invading pathogenic organisms. Following a stimulus, an acute psoriatic lesion forms in which dendritic cells and T cells become activated. No consistent antigen has been identified. Stimuli may include a dangerous signal, either an extrinsic, pathogen-associated signal or an intrinsic signal derived from within the body (e.g. heat shock proteins that bind to receptors, HIV-1, and ingested medications such as lithium or β -blockers). Once dendritic antigen presenting cells (APCs), such as Langerhans cells, and T cells become activated, they release cytokines, chemokines, and growth factors that trigger keratinocyte proliferation, altered differentiation, and an angiogenic tissue response. A vicious cycle of continuous T cell and dendritic cell activation can be envisioned within the chronic psoriatic plaque. Production of cytokines derived from APCs includes tumor necrosis factor- α (TNF- α) [10] and IL-23 [1, 8]. T cells are the likely source for interferon- γ (IFN- γ), IL-15 [14, 19] and IL-17 [2], whereas keratinocytes can produce IL-1, IL-6, and IL-8, as well as IL-18 [12] and IL-20 [3]. Besides these cytokines, numerous chemokines and chemokine receptors are present in psoriatic plaques which conspire with resident and recruited cells to create and sustain psoriatic plaques. Key inflammatory events include intra-epidermal trafficking by CD8+ T cells and neutrophils [11].

In general, activated CD4+ T cells are primarily located in dermis and CD8+ T cells in psoriatic epidermis, accompanied by tangled collections of dendritic APCs predominantly located in the dermis.

While this theory explains the maintenance of psoriatic plaques *via* establishment of a vicious cycle, precisely how T cells are activated in the genesis of psoriatic lesions is unknown. It is also unknown whether the inciting antigen is self-derived (thereby qualifying psoriasis as an autoimmune disease) or is of non-self origin.

There exists a variety of treatments for psoriasis, but no cure. Available psoriasis therapies, including topical treatments, phototherapy, and systemic treatments (methotrexate, cyclosporine, acitretin) are less than optimal because they fail to clear disease or are too inconvenient or too toxic.

Phototherapy comprises all forms of therapy which use the ultraviolet radiation (UVR) spectrum for therapeutic purposes. Both UVB (290–320 nm) and UVA (320–400 nm) have substantial photodynamic effects, although the mode of action of UVR therapy has not yet been fully understood. UVB light inhibits Langerhans cell function and is cytotoxic for keratinocytes. If the Langerhans cells are indeed causing the activation of CD 4+ T lymphocytes, it makes sense that this type of therapy would have a beneficial effect. UVA therapy is usually done in conjunction with psoralen (PUVA), a photosensitiser which increases the skin reactivity to UVR. A proposed theory for the efficacy of this treatment states that there is an intercalation of psoralen into DNA forming cross-links between strands that interfere with DNA synthesis and block cell proliferation. This form of therapy is known to have a suppressive effect on cell-mediated immune responses in the skin, which is an important point because CD8+ T lymphocytes play an important role in pathogenesis of this disease.

PUVA requires an intensive treatment regimen (about three times per week for many months), thereby reducing patients' compliance. Additionally, PUVA has been associated with skin cancers including malignant melanoma. The remission is observed after repeated treatments, and most patients do not achieve prolonged treatment-free remissions.

In our study, we investigated some aspects concerning keratinocyte alterations after UVA irradiation, using primary cultures of keratinocytes isolated from lesional and symptomless psoriatic skin biopsies obtained from a male patient in different stages of disease. The specific aims were to investigate the changes of (i) cell morphology, and (ii) cell viability, proliferation and apoptosis.

MATERIALS AND METHODS

Patients and skin biopsies. The biopsies from lesional and symptomless skin were obtained, under local anaesthesia, from a male patient, age 44, with psoriatic plaques on the limb and trunk. Then, he was treated with PUVA thrice weekly for five weeks. After treatment, remission of skin lesions was observed, and treatment was no longer necessary. At this time, new skin biopsies were taken. After four weeks, recurrence of the disease with appearance of skin lesions was observed. Other skin biopsies were taken.

Tissue selection and keratinocyte culture. The following primary cultures of keratinocytes were obtained from skin biopsies: a) psoriatic keratinocytes (KCps) – from periphery of psoriatic plaque; b) control keratinocytes (KCc) – from symptomless psoriatic skin; c) keratinocytes in remission (KCreM) – from post-lesional skin, and d) keratinocytes in recurrence (KCrec) – from relapsing psoriatic plaque.

Keratinocytes were isolated from biopsies by serial disaggregation using enzymatic and mechanical techniques as previously reported [4]. All procedures were performed aseptically, and all materials were sterile. The biopsies were washed in isotonic HEPES buffered saline (HBS) solution with antibiotics, cut into strips 2–3 mm wide and incubated with 2.4 U/ml Dispase II (Sigma Chemical Co.) in HBS for 30–120 min. Epidermis was mechanically separated from dermis. The separated epidermal sheet was treated with 1–2 ml of 0.25% trypsin – 0.1% EDTA solution per cm² of epidermal tissue at 37°C for 30 minutes. The keratinocytes were disaggregated by vigorous pipetting and trypsin was inactivated with selective medium for keratinocyte – KMK-2 (Sigma Chemical Co.) containing 10% fetal bovine serum (FBS). The cell suspension was centrifuged at 250g for 10 minutes at 4°C, and the pellet was resuspended into a small volume of KMK-2 medium. The keratinocytes were plated at a density 1.1x10⁴ cells/cm² into 25cm² flasks containing KMK-2 medium, and incubated at 37°C in a humidified atmosphere of 5% CO₂. Medium was refreshed at 2 days intervals. Our experiments were performed on confluent keratinocytes (third passage). Cell viability was checked by the standard trypan blue exclusion test.

Keratinocyte UVA irradiation. Prior to irradiation, the keratinocytes were plated at 1.1x10⁴ cells/cm² into Petri dishes and incubated at 37°C in a humidified atmosphere of 5% CO₂. 24 hours before irradiation, the culture medium was removed and refreshed. Confluent cells were rinsed twice with HBS solution, suspended in HBS and subjected to UVA irradiation for 3 hours. The UVA source was a Mineralight Lamp UVGL-58, Multiband UV, with a wavelength selector, 365 nm (Ultra-Violet Products, Cambridge, UK). The UVA dose 3.8 mW/cm², effectively received by the cells through the plastic cover was evaluated with a radiometer. Non-irradiated cells (controls) were similarly treated and kept in the dark at 20°C during the same time as the irradiated cells. After irradiation, cells were rinsed twice with PBS, replaced in fresh medium and placed in an incubator for 18 hours.

The irradiated cells were designated: UVA treated control keratinocytes (KCC^{uv}) and UVA treated psoriatic keratinocytes (KCps^{uv}).

Phase contrast microscopy. Keratinocyte morphological changes induced by UVA treatment were examined with an inverted phase-contrast microscope Nikon Eclipse TS 100 at different stages of experiment.

Trypan blue exclusion method. After trypsinization, a 100 µl aliquot of the treated cells was transferred to an Eppendorf tube and used for the viability assay. These cells were diluted (1:10) in PBS and stained with an equal volume of a 0.08% trypan blue solution for 2 minutes. The cells were counted by using a Thoma-Zeiss chamber. The percentage of viable cells was calculated as viable cells (unstained)/ total cells (stained plus unstained) × 100.

Quantification of DNA content. DNA content was determined by a method that is based on the binding of Hoechst 33258 dye to DNA [15]. The cells were seeded at 20 000 cells/well into 12 well plates. Trypsin-EDTA (500 μ l) was used to trypsinize the cells. 500 μ l of 0.01% Triton X-100 in TNE (10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 7.0) was added to lyse the cells. A 500 μ l aliquot of 1 mg/ml Hoechst dye in TNE was combined with the cell lysate. Fluorescence of the samples was recorded using an excitation wavelength of 356 nm and an emission wavelength of 458 nm in a spectrofluorometer TECAN reader with Soft Magellan 3.0. The results are expressed in fluorescence units.

Apoptosis detection. DNA fragmentation in keratinocyte exposed to UVA radiation (3.8 mW/cm²) was measured using a commercially available kit “Cell Death detection ELISA Plus” from Roche Diagnostics (Cat. No. 1544675). This assay is performed on streptavidin-coated microtiter plate and detects the appearance of the DNA-histone complex in the cytosol of apoptotic cells. After transfer of cell lysate to the microtiter wells, a mixture of anti-histone biotin-antibodies and peroxidase-coupled anti-DNA antibodies was added. The anti-histone antibodies recognize the protein fraction of the nucleosomes and immobilize it via streptavidin-biotin interaction on the microtiter plate. The DNA coiled around histones is recognized by the peroxidase-coupled anti-DNA antibodies. A chromogenic peroxidase substrate was added and the absorption was measured with TECAN reader. Results were expressed as relative absorbance at 405 nm, which increases as the amount of cytoplasmic nucleosomes rises. As recommended by Roche, the incubation buffer was used as blank. Data were corrected by subtracting the background value linked to the incubation buffer.

RESULTS AND DISCUSSION

PUVA therapy, also called photochemotherapy, involves ingestion of oral psoralen followed by irradiation with UVA, and is one of the most potent treatment regimens for a variety of skin diseases, including psoriasis, an inflammatory skin disorder. The epidermis is a self-renewing structure that requires a balance between epithelial cell proliferation and terminal differentiation for its formation and maintenance. The disruption of this balance is a predominant feature of epidermal hyperproliferation in psoriasis [5]. PUVA treatment of psoriasis leads to a reduced proliferation of epidermis [18] and appears to restore the balance between proliferating and differentiating keratinocytes.

PUVA treatment has successfully been used for more than two decades, but the underlying molecular mechanisms, which restore the epidermal homeostasis, are poorly understood.

In our study, we examined keratinocytes cultures (third passage) isolated from a male patient in different stages of disease. The studies were performed in

the same conditions for all six types of cultures. Changes of keratinocyte size and shape could be observed by phase-contrast microscopy.

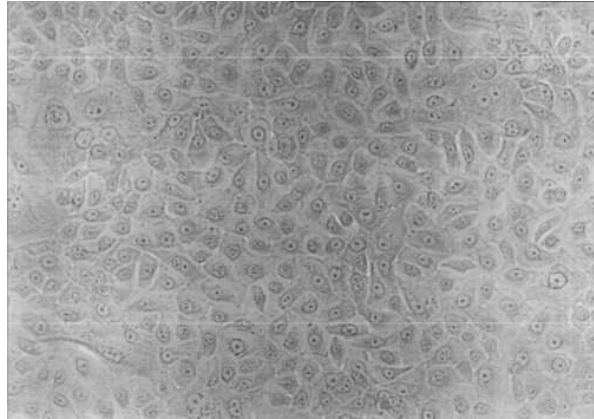


Fig. 1. Appearance of human control keratinocytes in culture (third passage) as viewed by phase contrast microscopy (x 100).

Cultured keratinocytes displayed a typical morphology for epidermal cells, with a polygonal shape, a large nucleus and 1–2 prominent nucleoli, despite their provenience and treatment (Fig. 1–6). The keratinocytes KCc (Fig. 1) exhibited a normal monolayer culture type, which had the characteristics of contact inhibition when cells started to become fully confluent, and because the medium used in this study had a low calcium concentration (0,09mM).

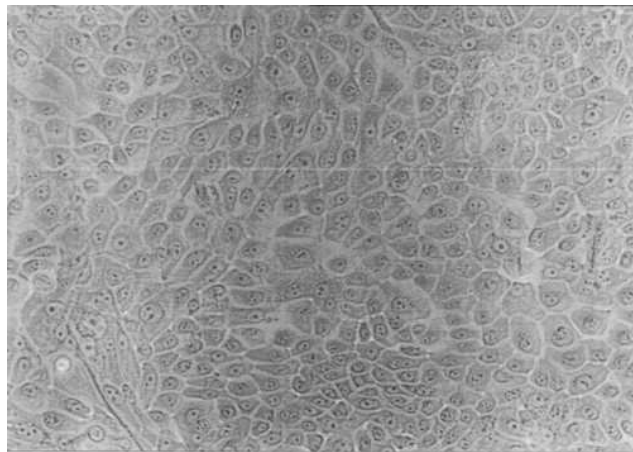


Fig. 2. Appearance of keratinocytes from psoriatic plaque (third passage) as viewed by phase contrast microscopy (x 100).

In the case of KCps isolated from periphery of the psoriatic plaques (Fig. 2), an increase in cell size, and a tendency to rich confluence was observed. The cytoplasm is vacuolated, and nuclei contain dense chromatin granules.

Keratinocytes isolated from post-lesional skin (Fig. 3) presented the same morphology with KCc, but cells were more compact and hypertrophied.

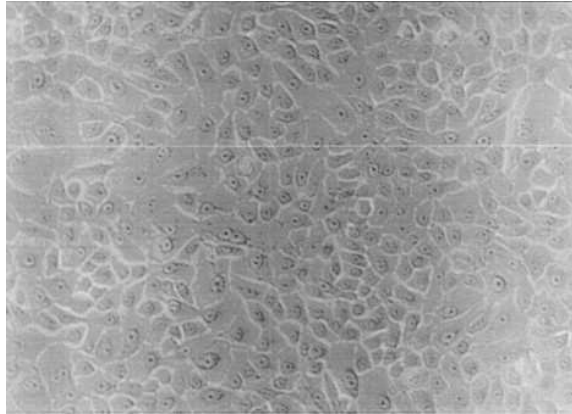


Fig. 3. Morphological appearance in phase-contrast microscopy of keratinocytes in remission (x100).

The keratinocytes obtained from relapsing psoriatic skin (Fig. 4) were very dense, but a pericellular refractile halo was observed, indicating extracellular matrix breakdown and lost attachment to the support. SACHSENMEYER et al. [16] considered that a decrease of cell-substrate or cell-cell interactions is a stimulus which may initiate the apoptotic cell signalling pathways.

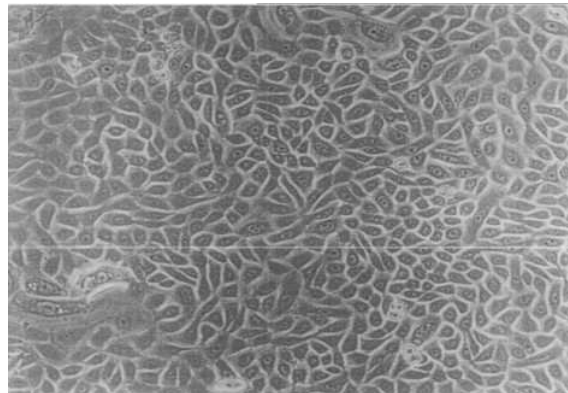


Fig. 4. Morphological appearance in phase-contrast microscopy of keratinocytes in recurrence (x100).

UVA irradiated control keratinocyte cultures (Fig. 5) presented a disorganized cell monolayer which contained cells with different sizes, hypertrophic cells having tendency to form colonies. There were many dead cells and cellular debris floating in the medium.

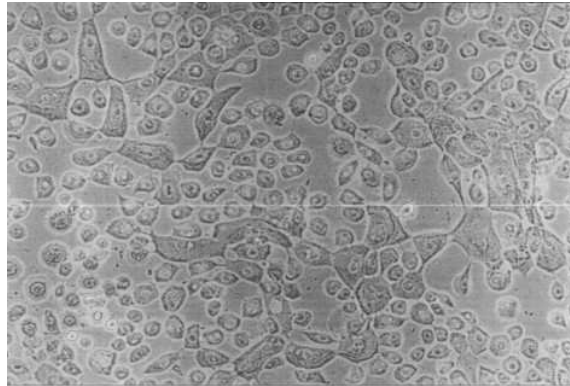


Fig. 5. Morphological appearance in phase-contrast microscopy of control keratinocytes UVA irradiated (x100).

The morphological changes observed in KCc^{UV} cells were more pronounced in the case of keratinocytes isolated from the periphery of psoriatic plaque and UVA irradiated (Fig. 6). Many degenerative effects, with a halo around the nucleolus and many vacuoles in cytoplasm were observed. The cell density was lower than that displayed by the KCc^{UV} cells. Some cells became round following disruption to their matrix substrate.

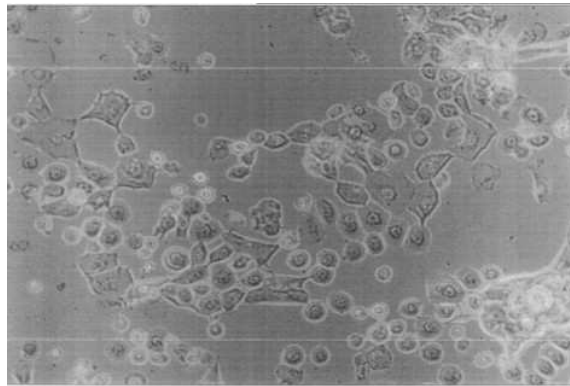


Fig. 6. Morphological appearance in phase-contrast microscopy of psoriatic keratinocytes UVA irradiated (x100).

Our microscopic studies showed the most dramatic morphological changes in UVA treated keratinocytes. It is known that UVA irradiation induces lipid peroxidation and depletion of antioxidant defence mechanisms in human keratinocyte cultures [13].

Although highly effective for psoriasis, PUVA treatment has been associated with squamous cell carcinomas, and, in at least one study, with malignant melanomas [17]. LARKO [7] published a study about the clinical aspects of phototherapy of psoriasis, with helpfully information about the recurrence and remission of the disease in UVB, UVB plus dithranol, and PUVA treated patients, signalling that UVA radiation is carcinogenic. In PUVA treatment, the recurrence and remission could be reached after 12 and 25 weeks, respectively.

The studies on keratinocyte cultures isolated from psoriatic plaque must be carefully interpreted because they can't reproduce: keratinocyte differentiation program; epidermal conditions, the absence of mesenchymal influence *in vivo* when the homeostasis and epidermal differentiation program are regulated by soluble factors derived from mesenchym, adjacent cells or circulatory system and the relations with inflammatory cells.

Cell viability of all six types of keratinocyte cultures was assessed by trypan blue method (Table 1). We observed that the viability of keratinocytes was statistically significant affected by UVA irradiation. After UVA irradiation, the control keratinocyte viability decreased with 51.5% and the viability of cells isolated from the periphery of psoriatic plaque decreased with 67%. Therefore, the psoriatic keratinocytes are more sensitive to UVA irradiation besides normal keratinocytes. This may have significance for efficacy of PUVA treatment and may suggest genetic alteration.

Table 1

The viability of keratinocytes isolated from epidermis of patient with psoriasis vulgaris

Keratinocytes	Viability (%)
KCc	97 ± 2.3
KCps	94 ± 4.6
KCrem	82 ± 11.2
KCrec	87 ± 14.6
KCc ^{UV}	47 ± 4.9
KCps ^{UV}	32 ± 2.5

Determination of the DNA content per cell, that is a measure of cell proliferation, proved that in comparison with KCc, the DNA content increased sevenfold in keratinocytes isolated from the periphery of psoriatic plaque, 1.6 fold

in post-lesional keratinocytes (remission), and 5.8 fold in keratinocytes from recurrent psoriatic plaque (Table 2).

The keratinocyte proliferation after UVA irradiation has decreased to a higher degree in the case of $KCps^{UV}$ than KCc^{UV} , probably because of DNA damage induced by reactive oxygen species generated by irradiation.

Table 2

DNA content of keratinocytes isolated from epidermis of patient with psoriasis vulgaris

Keratinocytes	Fluorescence units
KCc	148 ± 2.3
KCps	1043 ± 58
KCrem	233 ± 27
KCrec	854 ± 81
KCc^{UV}	98 ± 9
$KCps^{UV}$	115 ± 12

PUVA treatment of skin clearly induces the type of DNA damage that triggers apoptotic biochemical pathways. Apoptosis is characterized by changes of cell morphology and serial cleavage of molecules along the biochemical apoptotic pathway that lead to cell death. Although PUVA treatment is very potent in inducing apoptosis in lymphocytes [9], this PUVA effect alone may not be sufficient to explain long-term remissions of psoriasis.

To assess DNA damage and apoptosis in cultured keratinocytes exposed or not to UVA irradiation we evaluated apoptosis using an ELISA method to detect the appearance of the DNA-histone complex in the cytosol of apoptotic cells. Control cell absorbance was considered 100%, and in the others DNA fragmentation was calculated as percents from control (Table 3).

Keratinocytes separated from the periphery of the psoriatic plaque exhibited a decreased level of apoptosis with 41% comparatively with KCc. Remission of psoriasis is characterized by an increased level of keratinocyte apoptosis without reaching the control value, and the disease recurrence is accompanied by a decrease of apoptosis level. UVA irradiation results in an increase of keratinocytes apoptosis, with 71% in the case of KCc^{UV} , and with 53% in $KCps^{UV}$ in comparison with KCc. However, taking into account that KCps displayed a lower level of apoptosis than KCc we conclude that UVA irradiation induces a higher degree of cell death in the KCps than KCc. This may have significance for efficiency of PUVA treatment in psoriasis.

Table 3

The percent of keratinocytes undergoing DNA fragmentation

Keratinocytes	OD _{405 nm}	Apoptotic cells (%)
KCc	0.159	100
KCps	0.092	59
KCrem	0.128	81
KCrec	0.102	64
KCc ^{UV}	0.272	171
KCps ^{UV}	0.244	153

CONCLUSION

The recurrence and remission phenomena are accompanied by keratinocyte changes that suffer modification towards parental type – KCc and KCps respectively – without reaching the values of basis parameters: viability, proliferation and apoptosis.

Keratinocyte UVA irradiation results in morphological changes, a decrease of cell viability and proliferation, and an increase in level of apoptosis. Measure of changes of these parameters suggests genetic alterations in psoriasis. KCps appear to be more sensitive to UVA irradiation than KCc.

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