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Letter to the Editor

Mitochondrial function evaluation in epidermal cells *ex vivo* after ultraviolet irradiation

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Abstract: Ultraviolet radiation (UVR) effects on skin have been extensively studied. However, mitochondrial dysfunction and superoxide ($O_2^{\cdot-}$) production have only been studied using cell cultures, which are useful models, but do not consider the crosstalk between tissues or cellular differentiation. We aimed to evaluate the usefulness of fluorescent dyes to study skin *ex vivo*. Mitochondrial alterations were evaluated in epidermal cells isolated from UVR-exposed mice. Furthermore, a combination of dyes and antibodies was tested to analyse specific skin cell types. UVR caused a decrease in the percentage of total cells with polarized mitochondria, but did not change the mitochondrial $O_2^{\cdot-}$ production. However, this production was increased significantly in $DiOC_6^+$ cells. Furthermore, it was possible to evaluate the cellular damage produced to basal keratinocytes and Langerhans cells. The results show that fluorescent dyes – alone or in combination with antibodies – are useful to analyse cellular events that take place in whole organs.

Key words: basal keratinocyte – Langerhans cells – mitochondrial polarization – superoxide production

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Background

Skin, the largest human body organ, plays an important role in health providing an interactive boundary between the organism and the environment (1). It is constantly exposed to sun-derived ultraviolet radiation (UVR), one of the main environmental carcinogens. UVR effects on skin have been extensively studied in different kinds of models, both *in vivo* and *in vitro* (2–5). However, some of these effects, such as mitochondrial dysfunction and reactive oxygen species (ROS) production, have been mostly studied in cell cultures. *In vitro* models are useful, but do not consider the crosstalk between keratinocytes and other tissues (such as the dermis), or the cellular differentiation within the epidermis, which involves cornification among other specialized events.

Mitochondrial function has been evaluated in different cellular types using cationic lipophilic dyes ($DiOC_6$, JC-1 and TMRE), which accumulate in the organelle according to the potential of the inner mitochondrial membrane ($\Delta\psi_m$) (6,7). This potential correlates directly with the metabolic activity of the cells (8). The production of different kinds of ROS has also been evaluated using fluorescent dyes (H_2DCF-DA, hydroethidine, H_2-rhodamine, MitoSOX Red). As all of these probes can only be applied to living (non-fixed) cells, they have been widely used in cell cultures (9,10).

Question addressed

The aim of the present work was to evaluate the usefulness of fluorescent dyes to study skin in *ex vivo* models. In this way, mitochondrial dysfunction and mitochondrial superoxide production were evaluated in epidermal cells isolated from UVR-exposed mice. Furthermore, the possibility of using a combination of dyes and antibodies was tested to analyse specific skin cell types.

Methods

SKH-1-*hr*BR hairless mice (Charles River Laboratories, Wilmington, MA, USA) were irradiated on their back as previously described (11) with UV light using an 8W UVM-28 Mid-Range Wave lamp (UVP, Upland, CA, USA; emission spectrum 280–370 nm, peak 302 nm; mainly UVB with a small amount of UVA). The animals were separated into three groups of five mice each. The first group was exposed to 400 mJ/cm^2 (two minimum erythema doses, MED), the second to 800 mJ/cm^2 (four MED) and the control group was mock irradiated. Twenty-four hours after the irradiation, the animals were killed using a CO_2 gas chamber. Skin samples (1 cm^2) were obtained from each mouse and were incubated with Dispase 25 mg/ml (Invitrogen, Carlsbad, CA, USA) in RPMI medium for 2 h at 37°C. Next, the epidermis was separated from the dermis and mechanically disrupted with a tissue homogenizer (Thomas Scientific, New Jersey, NJ, USA) in 1 ml of phosphate buffer supplemented with 10% of fetal bovine serum to obtain an epidermal cell suspension which was filtrated through a 50-μm nylon mesh. Cells were stained with $DiOC_6$ 30 nM (Sigma-Aldrich, St. Louis, MO, USA), MitoSOX Red 5 μM (Molecular Probes, Invitrogen), 0,2 μg CD49b (α2 Integrin) and/or 0,2 μg CD11c (BD Biosciences, San Jose, CA, USA). Data were acquired on a PAS III PARTEC flow cytometer (Partec, Görlitz, Germany) and analysed using Cyflogic software (CyFlo Ltd., Turku, Finland). Procedures were approved by the Review Board of Ethics of the *Instituto de Estudios de la Inmunidad Humoral* (IDEHU-CONICET). For a detailed description, see supporting information.

Results

The cellular population obtained was very heterogeneous (Fig. 1a), according to the different epidermal strata, with different size (Forward Scatter, FSC) and granularity (Side Scatter, SSC) features. As expected, UVR caused an important cellular damage leading to a decrease in the percentage of cells with polarized mitochondria (two and four MED-exposed mice) compared to non-irradiated control mice, which had almost 60% of their cells with depolarized mito-

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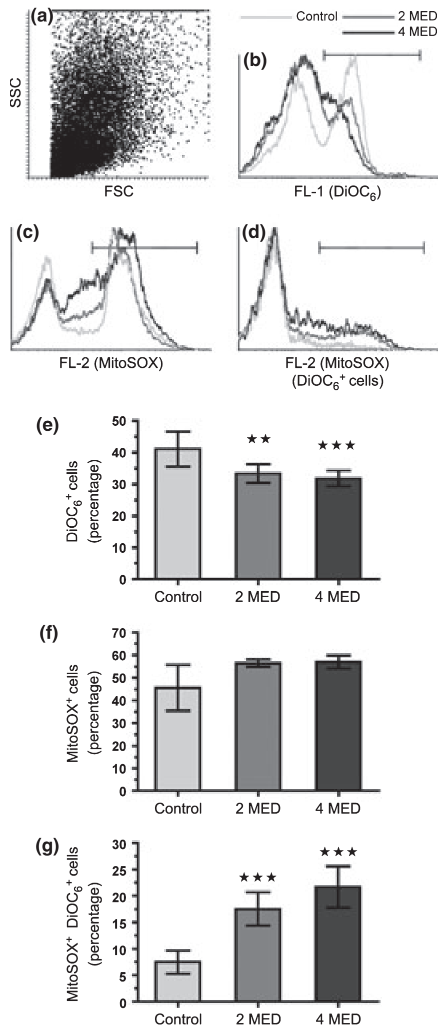

Figure 1. Mitochondrial depolarization and $O_2^{\cdot-}$ production in ultraviolet radiation (UVR)-exposed mice. (a) Side Scatter (SSC) versus Forward Scatter (FSC) of epidermal cell suspension obtained from a normal mouse. (b) Mitochondrial membrane potential detected by $DiOC_6$ staining. (c) $O_2^{\cdot-}$ production detected by MitoSOX red staining. (d) MitoSOX red staining of $DiOC_6^+$ epidermal cells. Marker indicates the region of cell population used for analysis in subsequent figures. Quantification of (e) mitochondrial depolarization, (f) mitochondrial $O_2^{\cdot-}$ production and (g) mitochondrial $O_2^{\cdot-}$ production in $DiOC_6^+$ cells in epidermal cells from control, two minimum erythema doses (MED) and four MED-exposed mice, $n = 5$. Bars represent mean ± SD. Data were analysed using One-way ANOVA, with a Student–Newman–Keuls post-test. $**P < 0.01$, $***P < 0.001$. Figures a, b, c and d represent one animal from each group.

chondria (Fig. 1b, e). Moreover, mitochondrial $O_2^{\cdot-}$ production was evaluated. Non-irradiated cells produced large amounts of $O_2^{\cdot-}$, as it was previously described for keratinocytes (12), and this percentage did not statistically change among groups (Fig. 1c, f). However, when only $DiOC_6^+$ metabolically active cells were analysed, the production of $O_2^{\cdot-}$ increased significantly in two and four MED-exposed mice (Fig. 1d, g).

Furthermore, it was possible to evaluate the cellular damage produced to different epidermal cell types in their natural environment. In particular, we studied mitochondrial dysfunction of basal stratum keratinocytes, which express α2 integrin ($CD49b^+$, Fig. 2a) (13), and Langerhans cells ($CD11c^+$, Fig. 2d). There was a slight increase in metabolic function of basal keratinocytes isolated

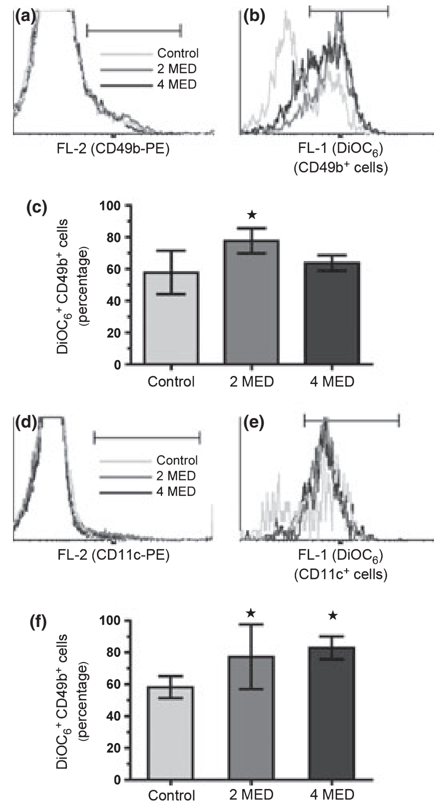

Figure 2. Mitochondrial depolarization in epidermal cells' subpopulations from ultraviolet radiation (UVR)-exposed mice. (a) CD49b-PE staining of epidermal cells. (b) $DiOC_6$ staining of $CD49b^+$ cells. (c) Quantification of mitochondrial depolarization in $CD49b^+$ cells from control, two minimum erythema doses (MED) and four MED-exposed mice, $n = 5$. (d) CD11c-PE staining of epidermal cells. (e) $DiOC_6$ staining of $CD11c^+$ cells. (f) Quantification of mitochondrial depolarization in $CD11c^+$ cells from control, two MED and four MED-exposed mice, $n = 5$. Bars represent mean ± SD. Data were analysed using One-way ANOVA, with a Student–Newman–Keuls post-test. $*P < 0.05$. Figures a, b, d and e represent one animal from each group.

from two MED-exposed mice. However, no differences were found between non-exposed mice and those exposed to four MED (Fig. 2b, c). Finally, there was an increment in Langerhans cells' mitochondrial activity after two and four MED exposure, demonstrating a higher metabolic function of these cells instead of cell damage (Fig. 2e, f).

Discussion

The presented results show that fluorescent dyes are useful to analyse cellular events that take place in whole organs, like the epidermis, as long as they are studied immediately after sampling. Besides, the epidermal response to UVR, extensively studied up to now, differs among the different types of cells analysed: total epidermal cells showed a decrease in mitochondrial polarization, whereas basal keratinocytes (from two MED-exposed mice) and Langerhans cells (from two and four MED-exposed mice) showed a higher percentage of $DiOC_6^+$ cells, which corresponds to cellular metabolism activation. The absence of such higher activity on basal keratinocytes from four MED-exposed mice may be owing to a larger damage produced by the higher UV dose. Unfortunately, keratinocytes from stratum granulosum and spinosum could not be differentiated by the expression of surface molecules,

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to identify the cell type responsible for the overall mitochondrial depolarization.

Previous *in vitro* studies performed with isolated Langerhans cells have analysed their susceptibility to low UVR doses, showing a decrease in costimulatory molecules expression and an increment in apoptotic cell death (14). Our results demonstrated that the cellular environment, abolished by *in vitro* models, plays a key role in the response of certain cell types. For example, a high percentage of normal epidermal cells with depolarized mitochondria (almost 60%) was observed in the control group, which is in accordance with normal keratinocyte differentiation (15), another important issue avoided by *in vitro* models, where depolarization in normal cells is negligible (16).

Cellular dyes in *ex vivo* studies on cutaneous cells from fresh skin biopsies could be a very useful tool to analyse the metabolic activity and the oxidative state of epidermal and dermal cells in many skin disorders, like psoriasis and atopic dermatitis (17–20).

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Conflict of interest

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

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