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Damage at the root of cell renewal—UV sensitivity of human epidermal stem cells

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ABSTRACT

Background: The epidermis harbors adult stem cells that reside in the basal layer and ensure the continuous maintenance of tissue homeostasis. Various studies imply that stem cells generally possess specific defense mechanisms against several forms of exogenous stress factors. As sun exposition is the most prevalent impact on human skin, this feature would be of particular importance in terms of sensitivity to UV-induced DNA damage.
Objective: To investigate whether human epidermal stem cells are susceptible to UV-induced DNA damage and subsequent functional impairment.
Methods: A method to isolate human epidermal stem cells from suction blister epidermis was established and validated. Volunteers were treated with solar-simulated irradiation on test areas of the forearm and stem cells were isolated from suction blister material of this region. DNA damage was analyzed by staining for cyclobutane thymidine dimers. The functional consequences of UV-induced damages were assessed by colony forming efficiency assays and gene expression analyses.
Results: Compared to an unirradiated control, stem cells isolated from areas that were exposed to solar-simulated radiation showed significantly more DNA lesions. Although the number of stem cells was not reduced by this treatment, a functional impairment of stem cells could be shown by reduced colony forming efficiency and altered gene expression of stem cell markers.
Conclusions: Despite their essential role in skin maintenance, epidermal stem cells are sensitive to physiological doses of UV irradiation *in vivo*.

1. Introduction

The integrity of the human epidermis depends on a tightly regulated balance between the loss of cornified cells at the skin surface and their replenishment by proliferating cells in the basal layer [1]. Whereas the majority of keratinocytes follows a specific differentiation pathway that is accompanied by a gradual migration towards the *stratum corneum*, epidermal stem cells constantly reside within the proliferative compartment and thereby ensure steady tissue homeostasis [2]. Accordingly, these adult stem cells are maintained a whole lifetime whereas their progeny only stays in the tissue for about 39 days [3]. With respect to any cellular damage that is induced by exogenous stress, a normal keratinocyte that has acquired a defect would no longer remain in the tissue than for the epidermal turnover time. In contrast, defects in epidermal stem cells would, if not repaired, persist in the basal layer and potentially be passed on to all succeeding daughter cells.

Regarding this special importance of stem cell function it is not surprising that several specific protection mechanisms have been detected in different stem cell types. For example, a higher resistance of embryonic and adult stem cells against reactive oxygen species (ROS), DNA-reactive drugs, gamma- or UV-irradiation have been reported [4–7]. As these data predominantly emerge from studies that are conducted to uncover principles of chemoresistance, considerably less is known about stem cell defense mechanisms against physiologically occurring stress factors. Since several areas of the human skin are frequently sun-exposed, UV irradiation is the most prevalent of these physiological impacts. Excessive sun exposure leads to a phenomenon that is often referred to as "photoaging" [8] and that is mainly described by alterations in the connective tissue architecture [9] and a disturbed epidermal turnover [10]. Whether this phenotype can be ascribed to defects in skin stem cells is currently unknown. A recent study in mice shows that a subpopulation of basal keratinocytes, characterized by prolonged retention of DNA label, accumulates cyclobutane pyrimidine dimers (CPD) DNA lesions

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after repetitive irradiation *in vivo* [11]. Although it is not clear if this accumulation occurs because slowly proliferating stem cells do not dilute the damage during DNA replication or if it rather represents a difference in UV-sensitivity, these data strongly imply that epidermal stem cells do not show a general UV-resistance. To clarify whether UV-sensitivity of epidermal stem cells can also be observed in human skin, we developed a strategy to quantify DNA damage after UV irradiation *in vivo*. The results show that human epidermal stem cells are susceptible to UV-induced damages and suggest that the resulting defects in epidermal stem cells may contribute to the manifestation of the phenotypical characteristics of photoaging.

2. Materials and methods

2.1. Antibodies and real-time PCR probes

Immunofluorescent stainings of suction blister epidermis section and isolated stem cells were performed with antibodies against CD29 (Chemicon, [TDM29]), CD104 (AbD SeroTec, [MCA1456F], FITC conjugated), keratin 15 (Chemicon [LHK15]), p63 (Sigma–Aldrich, [4A4]) and thymidine dimers (Kamiya Biomedical, [KTM53]). Real-time RT PCR was performed with predesigned TaqMan© assays (Applied Biosystems) for keratin 5 (Hs00361185_m1), keratin 14 (Hs00265033_m1), keratin 1(Hs00196158_m1), keratin 10 (Hs00166289_m1), keratin 15 (Hs00267035_m1), integrin β_1 (Hs00559595_m1), integrin β_4 (Hs00236216_m1), integrin α_6 (Hs00173952_m1), β-catenin (Hs00170025_m1), delta-1 (Hs00194509_m1), MCSP (Hs00426981_m1), hTERT (Hs00162669_m1) and a previously described costum-made assay for the ΔN form of p63 [12]. All chemicals were purchased from Sigma–Aldrich unless not otherwise specified.

2.2. Isolation of suction blister epidermis (SBE)

Suction blisters were obtained from ten healthy female volunteers (45 ± 2.2 years of age, median, standard deviation) who provided written informed consent. The study was conducted according to the recommendations of the current version of the Declaration of Helsinki and the guideline of the International Conference on Harmonization Good Clinical Practice (ICH GCP). The proceeding was approved and cleared by the institutional ethics review board (Beiersdorf AG, Hamburg, Germany). Only individuals who could exclude any relevant previous sun exposure of the test areas were integrated into the study. Each individual minimal erythema dose (MED) was determined 24 h after the irradiation of six test areas on the inner forearm with increasing intensities of solar-simulated radiation (SRR) using the SU 5000 Solar Sun Simulator (MUT). Two additional test areas were then chosen for the generation of three suction blisters, respectively. One area was subsequently irradiated with two individual MED of SSR. Suction blister epidermis was carefully prepared and either subjected to fixation for immunofluorescent staining, RNA extraction or isolation of epidermal stem cells.

2.3. Immunohistochemical analysis of suction blister epidermis

Suction blister epidermis was embedded in Tissue Tek (Sakura) and cooled to −80 °C. 5 μm Cryosections were prepared using a Leica CM3050 cryostat. Sections were placed on microscopic slides, fixed in methanol/acetone (1:1, −20 °C, 5 min) and blocked with 1% BSA/PBS (4 °C, o/n). Primary antibodies were incubated at recommended dilutions for 1 h, replaced by the secondary antibody (1:500, 30 min) and mounted with DAPI/Mowiol (1:1000). Acquisition of immunofluorescent pictures was performed with an IX71 fluorescence microscope (Olympus).

2.4. Isolation of epidermal stem cells

Epidermal stem cells were isolated using the collagen IV adhesion assay [13]. Briefly, suction blister material was disinfected with 70% ethanol and washed in PBS. The tissue was dissociated by incubation in 0,25% Trypsin/EDTA (PAA, 5 min 37 °C) and subsequent repetitive resuspension using sterile filter tips. Singularized cells were resuspended in KGM-2 medium (Lonza), automatically counted (CasyCounter, Roche Innovatis) and immediately plated on collagen IV coated dishes (BD Biosciences). After 10 min adherence time, the supernatant was transferred to another collagen IV coated dish. Rapidly adhering stem cells were either manually detached (Cell Lifter, Corning), counted and subjected to quantification of DNA damage or cultured in KGM-2 for three days and used for colony forming efficiency assays and gene expression analysis.

2.5. Colony forming efficiency assay

After a short growth period of 72 h, rapidly adhering stem cells were detached from the collagen IV coated dish by trypsinization and counted. 1000 cells were seeded into 25 cm^2 cell culture flasks (Nalge Nunc) and grown in KGM-2 for ten days. Colonies were visualized by staining with 1% neutral red (30 min, 37 °C).

2.6. Real-time RT PCR

Total RNA extraction from rapidly adhering stem cells was performed using the High Pure RNA Isolation Kit (Roche Applied Sciences) according to the manufacturer's instructions. For gene expression analysis of suction blister epidermis, the samples were homogenized using a Precellys 24 system (Bertin technologies) and total RNA was isolated by usage of the RNeasy Mini Kit (Qiagen). Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and real-time RT PCR was conducted with a TaqMan 7900 RT PCR System (Applied Biosystems). Amplification data were analyzed with the ΔC_T-method [14] using the SDS 2.3 and RQ manager 1.2 software (Applied Biosystems). All semi-quantitative gene expression levels are displayed as relative values referring to the expression level of 18S rRNA. Referring to the suppliers data analysis recommendations, genes that exhibited C_T-values larger than 35 were regarded as not expressed.

2.7. Immunocytochemical quantification of thymidine dimers

Rapidly adhering stem cells were resuspended at 1×10^6 cells/ml in PBS and 20 μl of this suspension were placed on poly-L-lysine coated microscopic slides (Menzel) and air-dried. Cells were fixed by 5 min incubation in methanol/acetone (5 min, −20 °C) and rehydrated with PBS. DNA was exposed by protein digestion with 0.5% pepsin/0.05 N HCl (30 min, 37 °C). Thymidine dimers were stained by 1 h incubation with the respective antibody (1:100 in 0.1% BSA) followed by 30 min incubation of FITC-conjugated secondary antibody (1:500). Samples were mounted with Mowiol containing DAPI (1:1000) and immunofluorescent pictures were acquired using an Olympus IX71 fluorescence microscope system. ImageJ software was used for CPD intensity value extraction.

2.8. Statistics

For the analysis of CPD intensity data (Fig. 2B) a two-tailed, unpaired *t*-test was used. Comparisons of CFE and gene expression values (Fig. 3D and E) were performed using a two-tailed, paired *t*-test. In both cases one asterisk indicates p-values < 0.05 and two asterisks represent p-values < 0.01.