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Research paper

Increased permeability of reconstructed human epidermis from UVB-irradiated keratinocytes

Lilian Julia Löwenau [a], Christian Zoschke [a], Robert Brodwolf [b], Pierre Volz [b], Christian Hausmann [a], Suvara Wattanapitayakul [a,1], Alexander Boreham [b], Ulrike Alexiev [b], Monika Schäfer-Korting [a,*]

[a] Institute for Pharmacy, Freie Universität Berlin, Königin-Luise-Str. 2+4, 14195 Berlin, Germany
[b] Institute of Experimental Physics, Freie Universität Berlin, Arnimallee 14, 14195 Berlin, Germany

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ABSTRACT

Extrinsic (photo) aging accelerates chronologically aging in the skin due to cumulative UV irradiation. Despite recent insights into the molecular mechanisms of fibroblast aging, age-related changes of the skin barrier function have been understudied. In contrast, the constantly increasing subpopulation of aged patients causes a clinical need for effective and safe (dermatological) treatment.

Herein, we reconstructed human epidermis from UVB-irradiated keratinocytes (UVB-RHE). UVB-irradiated keratinocytes show higher activity of senescence associated β-galactosidase, less cell proliferation, and reduced viability. Higher amounts of β-galactosidase are also detectable in UVB-RHE. Moreover, UVB-RHE release more interleukin-1α and -8 into the culture medium and present altered differentiation with a thinner stratum corneum compared to normal RHE.

For the first time, the permeation of testosterone and caffeine through UVB-irradiated RHE indicate a clear influence of the UVB stress on the skin barrier function. Impaired barrier function was confirmed by the increased permeation of testosterone and caffeine as well as by the increased penetration of dendritic core-multishell nanocarriers into the constructs.

Taken together, UVB-RHE emulate hallmarks of skin aging and might contribute to an improved non-clinical development of medicinal or cosmetic products.

1. Introduction

Life expectancy and thus the subpopulation of 65 and over continuously grow. Since prevalences of skin diseases remain either unchanged (e.g. psoriasis [1]) or markedly rise (e.g. skin cancer [2]) in geriatric people compared to the average population, novel dermatics must consider the frequent comorbidity as well as other particularities of aged patients. Topical treatment generally reduces systemic pharmacological interactions as *inter alia* demonstrated for psoriasis [3] or osteoarthritis treatment [4]. The development of highly active topical dermatics for the aged population asks for a detailed insight into the aged skin barrier and thus for the development of reliable models for aged skin.

Aging is associated with the functional impairment of cells, tissues, and the entire organism. In the epidermis, the turnover rate and the stratum corneum transit time slow down with age [5]. The progression rates of aging show strong interindividual variation and are influenced by intrinsic (genetically driven, chronological) and extrinsic (induced, in particular by extensive exposure to UV radiation) factors. Cumulative effects of UV radiation increase the number of senescent, permanently non-dividing cells and thus accelerate intrinsic aging [6]. In fact, extrinsic aging is believed to account for 80% of facial aging [7]. Despite detailed insights into the molecular basis of fibroblast aging [8,9] and first attempts to describe morphological skin changes [10,11], the impact of age-related changes on the skin barrier function are not yet adequately understood. There is some evidence for reduced permeation of hydrophilic compounds through intrinsically aged skin [12]. However, extending the test panel to extrinsically aged skin, permeation of small molecules in UV-stressed murine skin increases compared to non-irradiated skin [13,14] and nanocarriers may get even access to the dermis [15].

* Corresponding author at: Freie Universität Berlin, Institute for Pharmacy (Pharmacology and Toxicology), Königin-Luise-Str. 2+4, 14195 Berlin, Germany.
E-mail address: monika.schaefer-korting@fu-berlin.de (M. Schäfer-Korting).
[1] Present address: Department of Pharmacology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand.

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Dendritic nanocarriers combine auspicious properties to improve dermatological treatment: superior stability over liposomes, tunable end group functionalities, defined architecture, and monodispersity [16]. Dendritic core-multi-shell (CMS) nanocarriers markedly enhance the penetration of their cargo compounds into the viable epidermis; thereby showing excellent local tolerability due to the outer polyglycerol shell [17–19]. Thus, CMS nanocarriers might be also promising for the topical treatment of e.g. chronic inflammatory skin diseases in geriatric patients.

To study the impact of extrinsic (photo) aging on the epidermal barrier function, we reconstructed human epidermis from UVB-stressed normal keratinocytes. The general prediction of skin absorption already uses (juvenile) reconstructed human skin (RHE), being internationally accepted by OECD guidelines [20,21]. Moreover, the evaluation of skin corrosion, skin irritation, and phototoxicity rely on RHE-based approaches [16]. Since RHE from juvenile human foreskin cells disregards age-related changes, mouse models have been frequently used to emulate (skin) aging (ICR-Foxn1nu mice [13,14], Tsumura Suzuki obese diabetic mouse [22]). Human cell-based models use either DNA-repair deficient cells [23] to emulate cumulative UV effects or investigate sunburn-like effects [24,25].

In this study, we aimed to reconstruct human epidermis from UVB-irradiated keratinocytes and to evaluate the impact of these senescent keratinocytes on the permeation of small molecules as well as on the penetration of CMS nanocarriers in UVB-RHE.

2. Materials & methods

2.1. Material

Keratinocyte growth medium was purchased from Lonza (Basel, Switzerland) and construct culture medium from CELLnTEC Advanced Cell Systems (CNT-PR-3D; Bern, Switzerland). Cell-culture inserts (0.4 μm pore size) were obtained from Merck Millipore (Darmstadt, Germany). Cell proliferation ELISA, BrdU (colorimetric) was purchased from Roche Diagnostics (Rotkreuz, Switzerland) and the senescence cells histochemical staining kit from Sigma Aldrich (München, Germany). Antibodies were from Cloud-Clone Corp. (Houston, TX, USA: β-gal; cat no. PAA196Hu01; 1:250) and from Cell Signaling Technology (Cambridge, UK, β-actin; cat no. 6883; 1:1000). Pierce bicinchoninic acid protein assay kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA) and nonfat dry milk powder sucofin from TSI (Zeven, Germany). Protein marker Western-C precision and protein marker (biotinylated, HRP-conjugated) were purchased from Bio-Rad (München, Germany), ammonium persulfate, polyvinylidene fluoride membrane Immobilon P, and Rotiphorese Gel 40 from Carl Roth (Karlsruhe, Germany). ECL reagent SignalFire was obtained from Cell Signaling Technology (Danvers, MA, USA). Indocarbocyanine-tagged CMS nanocarriers were generously provided by Prof. Dr. Haag (Freie Universität Berlin, Germany). All other chemicals were bought from Sigma Aldrich (München, Germany).

2.2. UVB-irradiated keratinocytes

Primary normal human keratinocytes (passage 3) were from therapeutically indicated circumcisions (ethical approval EA1/081/13); parents had signed the written informed consent for scientific use. Cell culture was performed according to standardized protocols and referred to good cell culture practice [26].

To induce UVB stress, we irradiated keratinocyte suspensions in keratinocyte growth medium in an UV irradiation chamber with 5×8 W tubes (Vilber-Lourmat; Eberhardzell, Germany). Cells were irradiated with UVB light (25 mJ/cm^2) at an energy rate of 4.28 mJ/s with the major spectrum peak at 312 nm.

72 h after UVB irradiation keratinocytes were analyzed for cell morphology and stained for senescence associated β-galactosidase (SAβ-gal) activity according to the manufacturer's instructions. SAβ-gal positive cells were counted and normalized to the total cell number.

Furthermore, cell proliferation and cell viability were analyzed by BrdU incorporation and formazan formation, respectively. BrdU detection was performed according to the manufacturer's instructions and the quantification of formazan formation was studied according to previously described protocols for the MTT dye-reduction assay [27]. SAβ-gal positive cells, BrdU incorporation, and formazan formation were displayed as % of non-irradiated keratinocytes.

2.3. Reconstructed human epidermis from UVB-irradiated keratinocytes

We used $3 * 10^5$ keratinocytes per construct and cultivated the constructs for two weeks according to the CELLnTEC Advanced Cell Systems protocol. Irradiated keratinocytes were used for UVB-stressed constructs (UVB-RHE); non-irradiated cells from the corresponding donor were used for normal reconstructed human epidermis (RHE). In brief, keratinocytes were seeded onto the insert membranes and cultivated submerged in the culture medium. 24 h after seeding, the keratinocytes were exposed to the air-liquid interface and cultivated for a total cultivation period of 14 days. The constructs were cultivated in an incubator at 37 °C and 5% CO_2; the culture medium of the constructs was changed three times a week.

Constructs were embedded in cryo medium (Leica; Wetzlar, Germany), snap frozen in liquid nitrogen, sectioned into 10 μm slices (Leica CM 1510S; Wetzlar, Germany), and analyzed by hematoxylin-eosin staining. Pictures were taken with a fluorescence microscope (BZ-8000, Keyence; Neu-Isenburg, Germany) and analyzed with ImageJ software. Evaluation of at least six pictures per construct in one batch was done observer-blinded (LL, MSK).

The detection of βgal protein content in UVB-RHE and RHE was performed according to [28]. In brief, constructs were dispersed in a mixture of pre-cooled RIPA basal buffer containing 10% (V/V) proteinase-phosphatase inhibitor cocktail, and lysed at 25 Hz for 2 min (Tissue Lyzer, Qiagen; Hildern, Germany). Supernatants were used to determine the total protein content of the samples and for western blotting.

Interleukin(IL)-1α and IL-8 levels in the culture medium were quantified by enzyme-linked immunosorbent assay according to the manufacturers' instructions (R&D Systems, Wiesbaden, Germany).

2.4. Permeation of radiolabeled testosterone and caffeine

The permeation of [1-methyl^{14}C]caffeine (M_r 194, log$P$ −0.08; Waltham, MA, USA) or 1,2,6,7-^{3}H]testosterone (M_r 288, log$P$ 3.47; Amersham Biosciences Europe, Freiburg, Germany) were studied in the static setup (cell culture inserts, Ø = 12 mm, V = 1.5 mL) by using the infinite dose approach (caffeine: 387.4 μg/cm^2, 2 mCi/L; testosterone: 15.4 μg/cm^2, 2 mCi/L). Experiments were performed according to a validated protocol [21], referring to OECD test guideline 428 [20].