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The barrier function of organotypic non-melanoma skin cancer models

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ABSTRACT

Non-melanoma skin cancer (NMSC) is the most frequent human cancer with continuously rising incidences worldwide. Herein, we investigated the molecular basis for the impaired skin barrier function of organotypic NMSC models. We unraveled disturbed epidermal differentiation by reflectance confocal microscopy and histopathological evaluation. While the presence of claudin-4 and occludin were distinctly reduced, zonula occludens protein-1 was more wide-spread, and claudin-1 was heterogeneously distributed within the NMSC models compared with normal reconstructed human skin. Moreover, the cancer altered stratum corneum lipid packing and profile with decreased cholesterol content, increased phospholipid amount, and altered ceramide subclasses. These alterations contributed to increased surface pH and to 1.5 to 2.6-fold enhanced caffeine permeability of the NMSC models. Three topical applications of ingenol mebutate gel (0.015%) caused abundant epidermal cell necrosis, decreased Ki-67 indices, and increased lactate dehydrogenase activity. Taken together, our study provides new biological insights into the microenvironment of organotypic NMSC models, improves the understanding of the disease model by revealing causes for impaired skin barrier function in NMSC models at the molecular level, and fosters human cell-based approaches in preclinical drug evaluation.

1. Introduction

Non-melanoma skin cancer (NMSC) is the most frequent cancer in humans [1]. The carcinoma localization allows topical treatment, which is generally preferred to systemic drug administration due to (theoretically) higher drug doses at the target site and reduced systemic adverse effects. However, the lesional skin barrier remains a black box and may contribute to the frequent failure of NMSC treatment. Cutaneous squamous cell carcinoma (cSCC) is the most lethal tumor entity among NMSC with ten-year survival rates below 10%, if the carcinoma metastasizes to the brain and lungs [1]. Before metastasizing or dermal tumor invasion, the carcinoma grows within the epidermis, usually referred to as actinic keratosis (AK). This carcinoma *in situ* can be diagnosed clinically, by histological evaluation, or non-invasively by reflectance confocal microscopy (RCM) [2]. Frequent field-cancerization renders surgery a painful, disfiguring, and low effective treatment option, since a plethora of other malignant keratinocytes remain in the skin [3]. Ultimately, the topical treatment of multiple cSCC lesions would present a fascinating goal, if the drug proved at least similar efficacy compared with surgery.

First attempts to increase the efficacy of topical 5-fluorouracil by adding penetration enhancing agents failed to improve the low cure rate of AK [4]. Additionally, the few existing studies on NMSC skin barrier suggest an impaired barrier function [5–7] and question a high prevalence of AK lesions with hyperkeratosis [8,9]. Both pharmacodynamic and pharmacokinetic approaches are being developed to address the unmet clinical need of an effective and safe topical anti-cancer treatment. Ingenol mebutate offers a short-term treatment regimen, but causes rapid and unspecific necrosis [10]. Purine-analog phosphonates showed promising efficacy in monolayer cell cultures, but first attempts to use nanoparticles for enhanced drug delivery show marginal effects [11]. High-molecular-weight cationic membrane-active peptides [12] are theoretically excluded from topical treatment due to their physicochemical properties [13]. Thus, the detailed knowledge of lesional skin barrier is necessary for the development of new drug entities and novel drug delivery systems, respectively, but studies are currently lacking.

Currently NMSC is reproduced for preclinical drug evaluation by mouse models [14], organotypic skin models [11,15,16], or tumor specimens [17]. Each approach faces distinct limitations: *inter alia* murine

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skin morphology considerably differs from human actinic keratosis, organotypic skin models are based on cell lines and tumor specimens originate from invasive carcinomas. Since the tumor sample size is heavily limited, cell line-based approaches are still frequently used in cancer research despite their limited ability to represent tumor diversity.

Our previous study on the investigation of cutaneous penetration of dendritic core-multishell nanotransporters intrigued us to unravel the mechanisms of an impaired skin barrier function of organotypic NMSC models [18]. A detailed characterization of NMSC models has not been reported, but is urgently needed for the development of cancer targeted drug delivery. In this study, we systematically investigated the influence of carcinoma cells, co-cultured with reconstructed human skin (RHS), on the skin barrier function. In particular, we were interested in the impact on stratum corneum lipids and tight junction proteins.

2. Materials and methods

2.1. Materials

Collagen I was obtained from Biochrom (Berlin, Germany), cell-culture inserts (0.4 μm pore size) were purchased from Corning (Corning, NY, USA). Antibodies against the following proteins were purchased from Dianova (Hamburg, Germany): collagen-IV (1:200, C IV 22, cat-no. *T*-1201), *E*-cadherin (1:100, cat-no. PHA-70,112), involucrin (1:100, SY5, cat-no. DLN-07,100), Ki-67 (1:100, MIB1, cat-no. DIA-670), from Invitrogen (Darmstadt, Germany): Cldn1 (1:200, JAY.8, cat-no. 51–9000), Cldn4 (1:150, 3E2C1, cat-no. 18–7341), and ZO-1 (1:50, ZO1-1 A12, cat-no. 33–9100), from R&D Systems (Minneapolis, MN, USA) JAM-A (1:50, cat-no. AF1103), and from Santa Cruz Biotechnology (Dallas, TX, USA): occludin (1:300, N-19, cat-no. sc-8145). Radiolabeled [1-methyl^{14}C] caffeine (M_r 194, log$P$ − 0.08, PerkinElmer®) was bought from Waltham (MA, USA). Silica gel 60 high performance thin layer chromatography (HPTLC) plates (20 × 10 cm) were obtained from Merck (Darmstadt, Germany). Standards for HPTLC analysis (CER NS, AS, NP, AP, glucosylceramide, and sphingomyelin) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), cholesterol, fatty acid standards, L-α-phosphatidylcholine, and solvents from Sigma Aldrich (München, Germany). All consumables for surface pH measurements were obtained from PreSens Precision Sensing (Regensburg, Germany). Chemicals for the esterase assay were purchased from Sigma Aldrich. Picato® (150 μg ingenol mebutate per gram gel, 0.015%, Leo Pharma; Ballerup, DK) was bought in a local pharmacy. All ingredients for the vehicle control were purchased in pharmacopoeial quality from Caesar & Loretz (Hilden, Germany). The Cytotoxicity Detection Kit (LDH) was purchased from Roche Diagnostics (Mannheim, Germany).

2.2. NMSC models

We used $0.6 * 10^6$ normal human dermal fibroblasts, $3.0 * 10^6$ normal human keratinocytes, and $0.3–2.0 * 10^6$ SCC-12 cells per construct and constructs were cultivated for three weeks (Fig. 4A). Primary normal human keratinocytes and fibroblasts (passage 3, pooled from 3 donors) were from therapeutically indicated circumcisions (ethical approval EA1/081/13) after parents had signed the written informed consent. SCC-12 cells (passage 98–100, a generous gift from Howard Green, Dana-Farber Cancer Institute; Boston, MA, USA) were authenticated by single nucleotide polymorphism profiling (Multiplexion; Heidelberg, Germany). Cell culture was performed according to standard operating procedures and it referred to good cell culture practice. The dermal compartment was made at day 01 by pouring 1 mL collagen I into cell-culture inserts and by adding 3 mL collagen I with normal human dermal fibroblasts two hours later. Normal human keratinocytes were seeded onto the dermal compartment at day 08. SCC-12 cells were added at day 09; the ratio of SCC-12 cells to normal human keratinocytes determined the carcinoma stage: keratinocytes only for normal RHS, 10:100 for the cSCC-epidermis (cSCC-E) model and 50:100 for the cSCC-epidermis-dermis (cSCC-ED) model. In total $3 * 10^6$ cells were seeded onto each dermal compartment. Organotypic skin constructs were raised to the air-liquid interface at day 10. The culture medium [16] was changed three times a week.

2.3. Morphology and immunofluorescence

All models were analyzed at the end of the culture period by RCM, using a VivaScope® 3000 (MAVIG; München, Germany) and by immunofluorescence. Subsequent to RCM imaging, the constructs were snap frozen, sectioned into 5 μm slices (Leica CM 1510S; Wetzlar, Germany), and analyzed by hematoxylin-eosin and immunofluorescence staining. Tight junction proteins were analyzed from paraffin-embedded constructs as described [19]. Pictures were taken with a fluorescence microscope (BZ-8000, Keyence; Neu-Isenburg, Germany or Axiophot II, Zeiss; Göttingen, Germany), and analyzed with Openlab 2.0.9 or ImageJ software. Evaluation of at least six pictures per construct in one batch was done observer-blinded (CZ, MU, JMB, MSK).

2.4. SC lipid organization and profile

The stratum corneum (SC) was isolated and analyzed by infrared spectroscopy (IR) and high-performance thin layer chromatography (HPTLC) as previously described [20]. For standard curves, lipids were either purchased, or synthesized (CER NH and CER EOS); for ceramide (CER) nomenclature and standard curve ranges see Figure S2 and Table S3. The structure and the purity of the prepared CER NH and CER EOS were verified by nuclear magnetic resonance spectra (Varian; Palo Alto, CA, USA, Mercury-Vx BB 300 Instrument, operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C), infrared spectra (Nicolet 6700 Fourier transform infrared spectroscopy spectrophotometer; Thermo Scientific; Waltham, MA, USA), and mass spectrometry (Agilent 500 Ion Trap LC/MS; Santa Clara, *CA*, USA).

2.5. Surface pH

Fluorescence indicator foils using the pH indicator dye (fluorescein isothiocyanate) and of the reference dye (ruthenium(II)-tris(4,7-diphenyl-1,10-phenanthroline), were gently applied onto the surface of the organotypic constructs. The pseudo color images of VisiSens A2 were transformed into histogram data using VisiSens AnalytiCal 2 and ImageJ; for experimental details see [20].

2.6. Skin permeation

The permeation of radiolabeled caffeine was studied in the static setup (Franz-type diffusion cells, ∅ = 15 mm, V = 12 mL, Permegear; Bethlehem, PA, USA) by using the infinite dose approach (284.1 μg/cm^2, 2 mCi/L). Experiments were performed under standardized conditions [21] according to OECD test guideline 428 [22].

2.7. Esterase activity

Normal human dermal fibroblasts, normal human keratinocytes, and SCC-12 cells were seeded into black 96-well plates. On the following day, the cleavage of fluorescein diacetate into fluorescein was measured by fluorescence quantification; for experimental details see [23].

2.8. Ingenol mebutate effects

Ingenol mebutate gel or the vehicle control gel was topically applied to the constructs (10 μL/cm^2, finite dose approach) using a positive displacement pipette. We blinded the samples for analysis and set the sample-size to three biological replicates (three batches from different