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Stabilization of microtubules restores barrier function after cytokine-induced defects in reconstructed human epidermis

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ABSTRACT

Background: A variety of human skin disorders is characterized by defects in the epidermal barrier, leading to dehydration, itchiness, and rashes. Previously published literature suggests that microtubule stabilization at the cortex of differentiating keratinocytes is necessary for the formation of the epidermal barrier.
Objectives: We tested whether stabilization of microtubules with paclitaxel or epothilone B can repair barrier defects that were experimentally induced in three-dimensional culture models of epidermis.
Methods: We established two models of defective epidermis *in vitro*, using three-dimensional cultures of primary human keratinocytes on filter supports: immature reconstructed human epidermis (RHE), and RHE that was compromised by treatment with inflammatory cytokines, the latter mimicking defects seen in atopic dermatitis.
Results: Both paclitaxel and epothilone B promoted keratinocyte differentiation, accumulation of junctional proteins at the cell cortex, and the early appearance of lamellar bodies in immature RHE, whereas destabilization of microtubules by nocodazole had the reverse effect. Moreover, stabilization of microtubules rescued the barrier after cytokine treatment. The rescued barrier function correlated with the restoration of filaggrin and loricrin protein levels, the cortical accumulation of junctional proteins (E-cadherin, β-catenin, and claudin-1), and with the secretion of lamellar bodies.
Conclusions: Our data suggest that the microtubule network is important for the formation of the epidermis, and that stabilization of microtubules promotes barrier formation. Microtubule stabilization may support regeneration of damaged skin, by restoring or improving the barrier.

1. Introduction

At the interface between the organism and the environment, the skin provides mechanical protection and represents an important barrier against aggressions from the outside and against uncontrolled loss of body fluids. The barrier function is mainly attributed to the epidermis, a stratified epithelium with a basal layer of proliferative cells and multiple suprabasal layers of differentiated keratinocytes. Keratinocytes assemble massive amounts of cytoskeletal filaments that are in part anchored to intercellular junctions, such as desmosomes, tight junctions, and adherens junctions. These elements contribute to the mechanical stability and optimal elasticity of the epithelium. Furthermore, differentiated keratinocytes secrete the content of lamellar bodies – lysosome-related vesicles with a unique lipid and protein composition. This process increases insulation, by providing a hydrophobic barrier.

Because several events during epidermal barrier formation are likely to involve microtubules, we wanted to test whether stabilization of the microtubule network can attenuate or repair epidermal defects as seen in experimental models of atopic dermatitis (AD). This idea was fueled by reports on the

Abbreviations: AD, atopic dermatitis; RHE, reconstructed human epidermis; epoB, epothilone B; H&E, hematoxylin/eosin; TEWL, transepidermal water loss.

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reorganization and stabilization of microtubules at the keratinocyte cortex, and on the involvement of microtubule-dependent motor proteins as a prerequisite for the assembly of intercellular junctions such as desmosomes, tight, and adherens junctions [1–4]. Because cortical anchorage of multiple microtubule-organizing proteins (ninein, Ndel, and Lis1) depends on the desmosomal protein desmoplakin, and because loss of Lis1 causes defects in desmosome assembly and in the barrier of mouse skin [1,2], we strongly suspect that there is a mutual dependence between cell junctions and microtubule reorganization, and that both are needed to ensure the proper formation and function of the epidermal barrier. In agreement with this, interference with components of intercellular junctions in mouse models provokes skin abnormalities, inflammatory lesions and loss of homeostasis [5–10]. Also, several comparative transcriptomic analyses of normal versus AD skin, lesional and non-lesional, have shown that the expression of many genes encoding tubulins and microtubule-associated proteins is altered in the diseased skin [11–14]. Finally, a global proteomic analysis of lamellar body-enriched fractions has identified several proteins involved in microtubule-dependent motility [15], suggesting that lamellar bodies are transported along microtubules.

Our present study indicates a role of microtubules in the skin barrier. In a culture model of immature epidermis, microtubule stabilization increased the expression of epidermal differentiation markers, whereas destabilization had the reverse effect. In reconstructed human epidermis (RHE), we demonstrated that stabilization of microtubules repairs defects induced by Th2 cytokine treatment, such as reduced thickness of the cornified layer, reduced amounts of filaggrin expression, reduction of lamellar body secretion, reduced cortical localization of intercellular junction proteins, and increased transepidermal water loss (TEWL).

2. Materials and methods

2.1. Generation of RHEs

RHEs were generated as described [16,17]. For immature RHEs, 3-day old cultures were treated for 2 days with 10 nM of paclitaxel (Sigma-Aldrich, St. Louis, MO), epoB (Merck Millipore, Darmstadt, Germany), or 1 μM nocodazole (Calbiochem, San Diego, CA), diluted from a 100x stock in DMSO. For compromised RHEs, 6-day old cultures were treated with cytokines (CellSystems, Troisdorf, Germany), including 30 ng/ml IL-4, 30 ng/ml IL-13, 15 ng/ml IL-31, and 3.5 ng/ml TNF-α. Fresh culture medium with cytokine cocktail was added on days 8 and 10. At day 10, cytokine-treated RHEs were cultured in the presence or absence of paclitaxel or epoB. To control the effect of microtubule destabilization on mature RHE, 1 μM nocodazole was administered to RHE at day 10, without prior cytokine treatment. All RHE cultures were harvested on day 12.

2.2. Histological analysis, immunofluorescence, and antibodies

In RHEs, microtubules were immunostained after fixation for 1 h at 37 °C with 1% glutaraldehyde, 4% paraformaldehyde, in 50 mM PIPES, pH 6.8, 50 mM NaCl, 2 mM $MgCl_2$, 0.4 mM $CaCl_2$, 0.1% Triton X-100, followed by 30 min agitation in 0.1% sodium borohydride in PBS, extensive rinses with PBS, embedding in 5% agarose, and cutting of slices (40 μm) with a Leica VT1000 S vibratome (Wetzlar, Germany). Paraffin sections of RHEs were prepared and stained as described [16]. The following antibodies were used: anti-filaggrin (AHF3; [18]), anti-loricrin (AF62, Convance, Princeton, NJ), anti-Ki-67 (ThermoFisher, Waltham, MA), anti-caspase 3 active form (R&D, Minneapolis, MN), anti-actin MAB1501 clone C4 (Merck Millipore), anti-claudin 1 clone1C5-D9, anti-α-tubulin T5168, anti-acetylated tubulin 6-11B-1 (Sigma-Aldrich), anti-corneodesmosin (F28–27; [19]), anti-E-cadherin clone36, anti-β-catenin (BD Biosciences, Le Pont de Claix, France). Images for Figs. 1d, 3d, 4b, S3, S5a were acquired with Deltavision RT equipment, using objectives 40x/1.3NA or 60x/1.42NA (Applied Precision, Issaquah, WA). Images for Figs. 2b,c, 3c,f, S4b,c were acquired in "resonant" mode with a Leica TCS SP8 confocal microscope (Wetzlar, Germany), using a 40x/1.3NA objective. Projections of image stacks are presented in Figs. 2b, 3c, S4b. Images for Figs. 1c, 2a, 3b, S1a, b, and S4a were acquired using a Nikon Eclipse 80i wide field microscope (Champigny, France), using objectives 20x/0.5NA or 40x/1.3NA, and a Nikon DXM1200 camera.

2.3. Transmission electron microscopy analysis

RHE tissues were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, for 24 h at 4 °C, and post-fixed at 4 °C with 1% OsO_4 and 1.5% $K_3Fe(CN)_6$ in the same buffer. Tissues were treated for 1 h with 1% aqueous uranyl acetate, dehydrated in a graded acetone series, and embedded in EMbed 812 resin (EMS, Hatfield, PA). After 48 h of polymerization at 60 °C, ultrathin sections (80 nm) were mounted on 75 mesh Formvar/carbon-coated copper grids. Sections were stained with uranyl acetate and lead citrate. Grids were examined with a TEM (Jeol JEM-1400, Peabody, MA) at 80 kV. Images were acquired using a Gatan Orius digital camera (Pleasanton, CA).

2.4. Cell viability assay

Cell viability was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay (Sigma-Aldrich, St. Louis, MO). RHE samples were incubated at 37 °C in 500 μl of 0.5 mg/ml MTT in Epilife culture medium. The resulting formazan crystals were dissolved by adding 0.5 ml isopropanol with gentle agitation. Optical densities were measured at 550 nm, and cell viability was expressed as% relative to the absorbance of control RHEs.

2.5. Western blotting

At day 12, RHE tissues were lysed in 100 μl of Laemmli buffer. Equal volumes of protein were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies. Proteins were detected using the Odyssey imaging system (LI-COR, Lincoln, NE), with IRDye 800CW and 680CW-conjugated secondary antibodies (Invitrogen, Carlsbad, CA).

2.6. Determination of TEWL

Measurements of TEWL from RHE cultures were performed as described [20]. A TEWAMETER TM300 (Courage & Khazaka, Cologne, Germany) was used according to the manufacturer's instructions.

2.7. Reverse transcription-quantitative PCR

Total RNA extraction and reverse transcription was performed as described [16,20], using TATA box binding protein (TBP) expression for normalization. Primers: filaggrin: 5′-GCAAGGTCAAGTCCAGGAGAA-3′, 5′-CCCTCGGTTTCCACTGTCTC-3′; loricrin: 5′-CGAAGGAGTTGGAGGTGTTT-3′, 5′-ACTGGGGTTGGGAGGTAGTT-3′; TBP: 5′-TCAAACCCAGAATTGTTCTCCTTAT-3′, 5′-CCTGAATCCCTTTAGAATAGGGTAGA-3′.

2.8. Statistical analysis

Statistical analysis was done using Prism 5 software (GraphPad, La Jolla, CA). Two-tailed, unpaired *t*-tests were performed to compare experimental groups. The results are reported in the

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