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Research paper

Tight junctions form a barrier in porcine hair follicles

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A B S T R A C T

Follicular penetration has gained increasing interest regarding (i) safety concerns about (environmentally born) xenobiotics available to the hair follicle (HF), e.g. nanomaterials or allergens which should not enter the skin, and (ii) the possibility for non-invasive follicular drug and antigen delivery. However, not much is known about barriers in the HF which have to be surpassed upon uptake and/or penetration into surrounding tissue. Thus, aim of this work was a detailed investigation of this follicular barrier function, as well as particle uptake into the HF of porcine skin which is often used as a model system for human skin for such purposes. We show that follicular tight junctions (TJs) form a continuous barrier from the infundibulum down to the suprabulbar region, complementary to the *stratum corneum* in the most exposed upper follicular region, but remaining as the only barrier in the less accessible lower follicular regions. In the bulbar region of the HF no TJ barrier was found, demonstrating the importance of freely supplying this hair-forming part with e.g. nutrients or hormones from the dermal microenvironment. Moreover, the dynamic character of the follicular TJ barrier was shown by modulating its permeability using EDTA. After applying polymeric model-nanoparticles (154 nm) to the skin, transmission electron microscopy revealed that the majority of the particles were localized in the upper part of the HF where the double-barrier is present. Only few penetrated deeper, reaching regions where TJs act as the only barrier, and no particles were observed in the bulbar, barrier-less region. Lastly, the equivalent expression and distribution of TJ proteins in human and porcine HF further supports the suitability of porcine skin as a predictive model to study the follicular penetration and further biological effects of dermally applied nanomaterials in humans.

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1. Introduction

The hair follicle (HF) is a putative entry route for topically available substances or substances present in the environment (Nasir et al., 2012; Zakrzewski, 2002). This may be on purpose, e.g. for the delivery of drugs or antigens, or accidentally, as for e.g. environmentally born xenobiotics, allergens, toxins, or nanomaterials. In either case, knowledge about the biological barriers present in the HF is crucial.

In the interfollicular epidermis the *stratum corneum* (SC) has been identified as the first invasion barrier (Elias, 1983). Tight junctions (TJs), which are barrier-forming, paracellular junctions (Farquhar and Palade, 1963) composed of various TJ transmembrane and plaque proteins (Aijaz et al., 2006), are found in the cell layer beyond the SC, in the *stratum granulosum* i.e. granular cell

Abbreviations: Biotin-SH, EZ-Link™-Sulfo-NHS-LC-Biotin; CL, companion cell layer; CCL, central cell layers; Cldn, claudin; EDTA, ethylenediaminetetraacetic acid; EM, electron microscopy; He*, differentiated Henle cells of inner root sheath; HF, hair follicle; IRS, inner root sheath; NP, nanoparticle; Ocln, occludin; ORS, outer root sheath; PLGA, poly(lactic-co-glycolic acid); SC, stratum corneum; TJ, tight junction; ZO, zonula-occludens.

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layer (Brandner et al., 2002; Furuse et al., 2002; Morita et al., 1998; Pummi et al., 2001). Their function as a barrier has been characterized in detail over the years in simple epithelia and endothelia; however, only in the last decade their involvement in skin barrier function was revealed (see also discussion; for reviews see: Brandner et al., 2015; Kubo et al., 2012; Niessen, 2007; O'Neill and Garrod, 2011; Tsukita and Furuse, 2002). Due to their localization beneath the well-known SC barrier, TJs are likely to act as a second line of defense for the outside-in passage of substances, especially when the SC barrier is impaired. However, they can also actively influence the SC (Sugawara et al., 2013; Yuki et al., 2013; Kirschner et al., 2013) and are therefore likely to be involved in the regulation of SC barrier function.

The HF represents an invagination in which the SC barrier is interrupted, meaning that the SC is only present in the upper part (Rancan et al., 2013). Our group and others demonstrated expression and localization of various TJ proteins throughout human HFs – including areas without SC – (Brandner et al., 2003; Langbein et al., 2002a), as well as the presence of follicular TJ proteins in murine skin (Morita et al., 1998; Tatari et al., 2014; Troy et al., 2005) several years ago. However, functionality of TJs has up to now not been shown in any mammalian HF. Thus, the question arose whether barrier forming TJs are present in the HF that may compensate for the missing SC in deeper follicular regions. The correct expression, distribution, and functionality of follicular TJs is of further interest, because claudin-1 (Cldn-1) knock-out mice, patients with NISCH syndrome (deficiency of Cldn-1 in human), as well as Cldn-6 over-expressing mice all show a peculiar hair phenotype (Hadj-Rabia et al., 2004; Paganelli et al., 2011; Troy et al., 2005), pointing at the importance of TJs for normal hair growth. In this study we therefore started by investigating TJ protein expression, distribution, and barrier function in porcine HFs. We used porcine ear skin, as this model has been well accepted as a good surrogate for human skin, and more importantly as the "gold standard" for follicular uptake studies in the field of drug delivery (Lademann et al., 2010). In order to be able to compare our results with previously published data on humans (Brandner et al., 2003; Kirschner et al., 2013; Langbein et al., 2002b) we focused on TJ proteins that have been localized in human HFs and are known to play important roles in TJ barrier function of human keratinocytes: Cldn-1, -4, occludin (Ocln), and zonula-occludens protein-1 (ZO-1) (Igawa et al., 2011; Kirschner et al., 2010, 2013; Yuki et al., 2011b). For a comparison with mouse data we also investigated Cldn-3. Indeed, we could show that expression and distribution of TJ proteins is similar between human and pig. Further, we were successful in identifying areas with and without barrier-forming TJs in porcine HFs. Finally, we could demonstrate the possibility of modulating this barrier using EDTA. To correlate these TJ barriers with the uptake of xenobiotics into the HF we used model nanoparticles (NPs) and applied those to porcine ear skin. We observed that most NPs were found in the upper part of the HF where the SC and a TJ barrier are present, and some were found in deeper areas where TJs are the only barrier. Finally, areas where no barrier is present were not at all accessible to the used NPs.

2. Materials and methods

2.1. Materials

Oleic acid-coated primary magnetite particles were kindly provided by the Leibniz Institute for New Materials, Saarbruecken. Poly-D,L-lactide-co-glycolide (Resomer® RG 503 H) was purchased from Evonik in Essen, Germany. Uranyl Acetate was obtained from Polysciences Europe GmbH, Eppelheim, Germany. Paraformaldehyde was purchased at VWR International in Radnor, PA, USA and glutaraldehyde at Agar Scientific, Stansted, UK. HEPES and Eosin Y solution 0.5% in water were bought at Carl Roth GmbH + Co. KG in Karlsruhe, Germany and RNAse Inhibitor at Fermentas, Waltham, MA, USA. Dulbecco's Modified Eagle Medium (DMEM), the Maxima First Strand cDNA Synthesis Kit and an O'GeneRuler™ 50–1000 bp DNA Ladder were purchased from Thermo Fisher Scientific, Darmstadt, Germany. Buffer RLT and RNeasy Mini Kit used for PCR experiments were bought from Qiagen, Venlo, Netherlands. All pig specific primers were ordered from Applied Biosystems in Darmstadt, Germany: (1) Cldn-1 (CLDN1): Ss033757089, (2) Cldn-3 (CLDN3): Ss03377787, (3) Cldn-4 (CLDN4): Ss03375006, (4) Ocln (OCLN): Ss03377507, (5) ZO-1 (TJP1): Ss03373514, (6) GAPDH (Ss03375435). EZ-Link™-Sulfo-NHS-LC-Biotin (Biotin-SH; 557 Da) was purchased at Pierce, Rockford, IL, USA. Formaldehyde (Formafix® 4%, buffered) was obtained from Formafix Global Technologies, Duesseldorf, Germany. Mayer's haematoxylin solution was provided by Medite GmbH, Burgdorf, Germany. All primary antibodies were purchased from Invitrogen Carlsbad, CA, USA, except Cldn-3 (Abcam, Cambridge, UK). The Alexa-488-coupled secondary antibody F(ab′)2 and Alexa-594-coupled secondary antibody F(ab′)2 were provided by MoBiTec, Goettingen, Germany. Texas-Red®-conjugated Streptavidin was purchased at Merck Biosciences, Darmstadt, Germany. DAPI was bought at Boehringer Mannheim, Mannheim, Germany and Fluoromount-G™ at Southern Biotechnology Associates, Birmingham, AL, USA. Aqueous polyvinyl alcohol, 1% Alcian blue, phosphotungstic acid, dispase, 2-mercaptoethanol, hydrocortisone, fetal calf serum, penicillin/streptomycin and ethylenediaminetetraacetic acid (EDTA) were all obtained from Sigma–Aldrich, St. Louis, MO, USA. RNAlater was purchased from Qiagen, Hilden, Germany.

2.2. Porcine tissue (ears and lung) and mice

Fresh pig ears and pig lungs (Yorkshire/German Large White crossbreed; age: 6 months) were obtained from a slaughterhouse in Zweibruecken, Germany. The ears were excised, brought to the lab immediately and washed thoroughly. Finally they were examined for skin irritations prior to use and only healthy looking skin was selected for experiments. The porcine lung was excised, immediately placed in RNAlater, and delivered to the laboratory. For control experiments on Cldn-3 localization in murine HFs, C57/Bl6 mice (adult) were used.

2.3. Isolation of mRNA for reverse transcription PCR (RT-PCR)

In order to obtain mRNA from the HF, whole HFs of porcine ear skin were pinched from the tissue using a pair of tweezers after incubating 0.3 cm × 0.5 cm sections in a solution consisting of 4.0 mg/ml Dispase, and 1.0 U/μl RNAse Inhibitor in DMEM at 4 °C overnight. They were then washed in phosphate buffered saline (PBS), and stored in 1.0 ml of RLT buffer containing 10 μl 2-Mercaptoethanol. RNA of isolated porcine HFs as well as pig lung was extracted using an RNeasy Mini Kit and tested for purity using the NanoDrop 2000C (Wilmington, DE, USA). After successful isolation of total RNA, cDNA was generated using a Maxima First Strand cDNA Synthesis Kit. RT-PCR was performed as previously described by Brandner et al. (2002). Pig specific primers were used for Cldn-1, -3, -4, Ocln, ZO-1; GAPDH was used as the housekeeping gene.

2.4. Electron microscopy

Visualization of pure magnetite-loaded PLGA NP, which were prepared using a single emulsion-evaporation method (see Supplemental Data (SD) for specifics), was done by negative staining EM following the protocol developed by Laue (2010), and localization of NPs in the HF was analyzed by ultrathin section EM.

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