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Functional tight junction barrier localizes in the second layer of the stratum granulosum of human epidermis☆

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ABSTRACT

Background: Mammalian epidermis has two diffusion barriers, the stratum corneum (SC) and tight junctions (TJs). We reported previously that a single living cell layer exists between the SC and TJ-forming keratinocytes in mice; however, the exact location of the TJ barrier in human epidermis has not been defined.
Objective: To investigate the precise distribution of epidermal TJs in relation to various cell–cell junction proteins and the SC and to clarify the barrier function of TJs against macromolecules in human skin.
Methods: The localization of various junctional proteins was investigated in human skin sections and in the roofs of bullae formed by *ex vivo* exfoliative toxin (ET) treatment in three dimensions. ET and single-chain variable fragments (scFv) against desmoglein 1 were used as large diffusion probes.
Results: Human stratum granulosum (SG) cells have a distinct distribution of TJ, adherens junction, and desmosome proteins in the uppermost three layers (SG1–SG3 from the surface inward). *Ex vivo* injection of ET or scFv demonstrated that only SG2–SG2 junctions function as a TJ barrier, limiting the inside-out diffusion of these proteins. The roofs of bullae formed by *ex vivo* ET treatment consisted of SC, SG1 cells, and TJ-forming SG2 cells, probably mimicking bulla formation in bullous impetigo.
Conclusion: Human epidermis has three SG cell layers with distinct properties just beneath the SC, of which only SG2 cells have functional TJs. Our results suggest that human epidermal TJs between SG2 cells form a paracellular diffusion barrier against soluble proteins, including immunoglobulins and bacterial toxins.

1. Introduction

Skin prohibits the entry of microbial pathogens and allergens, as well as the leakage of water, solutes, and nutrients. These outside-in and inside-out barrier functions are dependent on the epidermis, a stratified epithelial cellular sheet. In mammals, cornified cellular sheets called the stratum corneum (SC) constitute the outermost epidermal barrier [1]. Beneath the SC, apical paracellular spaces between the outer layer of living cells are sealed by tight junctions (TJs) in amphibians [2], reptiles [3], mice [4,5], and humans [6–8], suggesting that TJs constitute another fundamental skin barrier system.

Early morphological studies demonstrated vertical alignment of corneocytes in rodent ear epidermis [9–12]. Underneath the corneocytes, stratum granulosum (SG) cells are also aligned vertically [5,9,10]. The uppermost three SG layers are named (from the surface inward) SG1, SG2, and SG3 in mice [4,5]. These cells have distinct claudin-1 expression patterns: SG1 cells do not express claudin-1, SG2 cells have TJs at their apical cell–cell contacts and express claudin-1 on their basolateral membranes but not on their apical membranes, and SG3 cells express claudin-1 over their entire surface but do not form TJs in between [4,5]. As

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keratinocytes undergo continuous turnover, these observations suggest that the distinct distribution patterns of claudin-1 in SG cells are dependent on the differentiation of SG cells from SG3 to SG1. In contrast to claudin-1, occludin or zonula occludens-1 (ZO-1) proteins were shown to exclusively localize to TJs of SG2 cells [4,5]. The *en face* observation of the epidermal cellular sheets of the mouse ear demonstrated that a single layer of ZO-1 honeycombs between SG2 cells demarcates the entire surface of the epidermis [5].

In contrast to rodent ear epidermis, human corneocytes and SG cells have been shown to be more disordered [13,14]. When observed *en face*, occludin-positive junctions appeared to be multiplied [15]. This complexity of the human epidermis make it difficult to determine whether human epidermis has a counterpart to SG1–3 of mouse epidermis, particularly the SG1 cells that form a single viable cell layer between the SC and TJ-forming keratinocytes (SG2 cells). As we have demonstrated in mice that the SG1 layer is important for Langerhans cells to take up foreign antigens outside the TJ barrier [5,16], we have focused much attention on whether the SG1 layer also exists outside the TJ barrier in human epidermis. Previous immunofluorescence observations of human skin sections showed that the uppermost living cells have TJs beneath the SC; no counterparts of the mouse SG1 layer existing outside the TJ barrier were described [6,7,15]. In electron microscopic analysis, viable nucleated cells have been occasionally observed on the apical side of cells that form a permeability barrier against lanthanum [15,17], suggesting the existence of a counterpart to the SG1 layer. As both methods visualize a single section of the epidermis at a single time point, it is difficult to determine whether the single viable cell layer is constantly present outside the TJ barrier in human epidermis.

Here, we demonstrated that the SG1–3 cells identified in mice are well conserved in human skin, and that the TJ barrier is single-layered and exists between SG2 cells. *En face* three-dimensional imaging showed that a single layer of nucleated SG1 cells, partly cornified, is constantly present between the SC and TJ-forming SG2 cells in human epidermis, as in mice.

2. Materials and methods

2.1. Study participants

Healthy skin samples from seven men and nine women patients with a mean age of 52 years (24–79 years) were obtained from leftover skin collected during excisional surgery. Sample collection was approved by the institutional review board of Keio University School of Medicine (ethics committee reference number, 20090052), and written informed consent was obtained from patients prior to surgery. This study was conducted according to the principles of the Declaration of Helsinki. The diagnosis of bullous impetigo in one patient (a 24-year-old man) was based on clinical, histological, and skin culture findings.

2.2. TJ permeability assays and preparation of cellular sheets

TJ permeability assays used the surface biotinylation technique [18]. Skin specimens were trimmed by removing the fat tissue and then cut into 5 mm × 5 mm pieces. Following intradermal injection of 50 μL of 10 mg/mL Sulfo-NHS-LC-Biotin (biotin-SH; #21335; Thermo Fisher Scientific, Rockford, IL, USA) in phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$, the skin specimens were placed on the inserts of Transwell plates (Corning Inc., Corning, NY, USA). Defined keratinocyte serum-free medium (Invitrogen, Carlsbad, CA, USA) containing 1 mM $CaCl_2$ was placed in the outer compartment, and the Transwell system was incubated for 30 or 60 min. For the single-chain variable fragment (scFv) permeability assay, an scFv against desmoglein 1 (Dsg1; defined by scFv 3–30/3h [19]) was obtained as follows. cDNA coding the scFv 3–30/3h tagged with a C-terminal 6× histidine and a HA [19] was cloned into a pET-16b expression vector (Novagen, UK). The BL21(DE3)pLysS strain of *Escherichia coli* (Promega, Madison, WI) was transformed with this expression vector. The expression of the scFv was induced in *E. coli* by introducing 0.5 mM isopropylthio-β-galactoside and lysed using Fastbreak (Promega). The insoluble fraction was obtained by centrifugation, washed with 1% Triton X-100 in PBS twice and with distilled water to purify the scFv. The scFv was solubilized by 4 M guanidine–HCl/10 mM 2-mercaptoethanol in 50 mM Tris–HCl (pH 8.0) and refolded *via* dialysis in PBS. Solubilized scFv (50 μL, approx. 0.75 mg/mL in concentration) was injected intradermally, and the skin specimens were incubated as described above. For the preparation of cellular sheets, 50 μL of 1.3 mg/mL recombinant exfoliative toxin (ET)-A [20] in PBS containing 1 mM $CaCl_2$ was injected intradermally, and the skin specimens were incubated for 30 min at 37 °C. The bulla roof was stripped away and fixed through incubation in 95% ethanol on ice for 30 min. The crystal structure of ET-A has been described previously [21,22].

2.3. Fluorescence microscopic observation

The human skin samples were embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan), frozen in liquid nitrogen and sectioned by cryostat. The frozen sections were immediately processed for immunostaining when using the scFv or fixed through incubation in 95% ethanol at 4 °C for 30 min, and then incubated in 100% acetone at room temperature for 1 min before staining. Skin sections or epidermal cellular sheets were blocked with PBS containing 10% fetal bovine serum and 5% goat serum (Dako, Tokyo, Japan) for 30 min at room temperature, incubated with primary antibodies in the blocking solution at 4 °C overnight, washed three times with PBS, and incubated with secondary antibodies in the blocking solution at room temperature for 1 h. Samples were washed with PBS, mounted in Mowiol (Merck, Darmstadt, Germany), and observed using a Leica TCS sp5 laser scanning confocal microscope equipped with a 63× objective. To obtain en face images of epidermal cellular sheets, multiple 0.4–0.5-μm optical slices were obtained and reconstructed in 3D using the Leica sp5 software. Images and movies were processed using Adobe Photoshop CS4 and Apple QuickTime Pro.

2.4. Antibodies

The following primary antibodies were used: polyclonal antibodies (pAbs) against claudin-1 (51-9000; Invitrogen), claudin-4 (ab15104; Abcam, Cambridge, UK), occludin (40-4700; Invitrogen), ʟ-afadin (ab11337; Abcam), gp135 (kindly provided from Dr. Kai Simons, Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany) [23] and HA (H6908; Sigma–Aldrich, St. Louis, MO, USA) at 1:200 dilution; and monoclonal antibodies (mAbs) against ZO-1 (T8-754, kindly provided from Dr. Mikio Furuse, Kobe University, Kobe, Japan) [24] at a 1:10 dilution, desmoplakin I/II (DP-2.15 + DP-2.17 + DP-2.20 antibody cocktail; #65146; Progen, Heidelberg, Germany) at a 1:2 dilution, α-catenin (alpha18, kindly provided from Dr. Akira Nagafuchi, Nara Medical University, Nara, Japan) [25] at a 1:1000 dilution, E-cadherin (ECCD2; Takara, Tokyo, Japan) at a 1:200 dilution, and occludin (MOC37, kindly provided from Dr. Furuse) [26] at a 1:20 dilution. Species-specific secondary antibodies and