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Tight junction protein claudin-4 is modulated via ΔNp63 in human keratinocytes

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ABSTRACT

In the epidermis, tight junction (TJ) structure is specifically located in the stratum granulosum, where the expression of ΔNp63, a p53 family transcription factor, is attenuated. Since the relationship between ΔNp63 and barrier function has not been fully uncovered, we assessed expression profiles of TJ proteins in skin tissues and cultured keratinocytes. The results showed that expression of ΔNp63 and that of claudin-4 were inversely correlated in healthy human epidermis. *In vitro* studies using HaCaT keratinocytes revealed functional relevance of ΔNp63 and claudin-4. Curiously, Toll-like receptor (TLR)-3 ligand, which is known to be liberated from damaged cells, suppressed ΔNp63 expression and concomitantly upregulated claudin-4 expression in primary keratinocytes. More interestingly, a broad expression pattern of claudin-4 was found in the epidermis of atopic dermatitis (AD), a barrier defect disorder, which contains ΔNp63-lacking keratinocytes as we reported previously. Therefore, upregulation of claudin-4 expression regulated by ΔNp63 might be associated with complementary or repair responses of damaged keratinocytes with AD.

1. Introduction

The skin is the first site of exposure to the external environment and plays a crucial role in prevention of chemical, physical or biological invasion as well as maintenance of moisture and temperature of the body. Dysfunction of this barrier in cases such as an extensive burn is often lethal. Keratinocytes, the main components of the epidermis, are stratified epithelia composed of several distinct characteristic layers by their differentiation stages: stratum corneum, granulosum, spinosum, and basale. The stratum corneum is composed of fully keratinized cell layers and works as a forefront of the physical barrier. In the stratum granulosum, just beneath the stratum corneum, tight junctions work as the secondary barrier preventing the entry of small molecules and water loss [1,2].

Tight junctions are the apical-most constituents of the intercellular junctional complex in mammalian epithelia and act as a semipermeable barrier to the paracellular transport of restricted small molecules. Claudins are essential components of tight junctions and consist of 27 or more members of a gene family. In addition, distinct sets of claudins are generally expressed in a cell- and tissue-specific manner. For instance, several leaktight claudins such as claudin-1 and -4 are expressed in the skin for protection against invasion of outer pathogens and prevention of water loss, whereas leaky, paracellular channel-forming claudins such as claudin-2 and -15 are expressed in the intestine for nutritional absorption [3]. Therefore, in the stratum granulosum, claudin expression and distribution should be strictly regulated to maintain the skin barrier, but the mechanisms of regulation remain largely unknown.

A p53 family transcription factor p63 is expressed in stratified epithelia and is known to rule epithelial cellular fate and function. In epidermal keratinocytes, ΔNp63, a p63 variant lacking the N-terminal transactivation domain, is dominantly expressed and plays a crucial role in regulating the differentiation of keratinocytes [4]. Experiments using inducible ΔNp63 knockout mice experiments revealed impaired keratinocyte differentiation and wound healing, suggesting ΔNp63 plays a fundamental role in

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epidermal integrity [5]. While we previously reported that p53 family transcription factors regulate some claudin proteins in thymic epithelial cells and a knockout mouse study has shown that ΔNp63 regulates claudin-1 expression, the relationships between human ΔNp63 and other tight junction proteins in keratinocytes have not been fully investigated [6,7].

In this study, we found that the expression of ΔNp63 was inversely correlated with claudin-4 in the human epidermis. *In vitro* study using cultured keratinocytes and the RNA interfering method revealed that ΔNp63 negatively regulated claudin-4, consistent with their distribution pattern in the epidermis. Treatment with TLR3 ligand, potentially released from damaged epithelial cells [8], suppressed ΔNp63 expression and concomitantly increased claudin-4 expression. Interestingly, claudin-4 protein was expressed more widely in the epidermis of the affected region of atopic dermatitis (AD), which is considered to be a barrier defect disorder, than in healthy skin. Since claudins are considered to have non-barrier forming activities, increased claudin-4 in AD would contribute to an unknown repair response of damaged keratinocytes as well as complementary responses of the impaired barrier in AD [9].

2. Materials and methods

2.1. Tissues

Skin tissues were obtained from patients with AD and patients presenting without pathological findings who underwent skin biopsies at Sapporo Medical University Hospital. The diagnosis of AD was established by both dermatologists and pathologists. Human tissue specimens were obtained with written informed consent and the study was approved by the institutional review board of Sapporo Medical University Hospital.

2.2. Cell cultures

Commercially available human primary keratinocytes were obtained from DS Pharma Biomedical (Tokyo, Japan) and cultured in a serum-free medium for human keratinocytes (DS Pharma Biomedical). Human HaCaT epidermal cells (RIKEN, Tsukuba, Japan) were maintained in DMEM (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin and 5% fetal bovine serum. For stimulation of cells, culture media were supplemented with the following reagents: 10 μg/ml zymozan (InvivoGen, San Diego, CA), 25 μg/ml polyinosine-polycytidylic acid (poly I:C, InvivoGen), 100 ng/ml lipopolysaccharide (LPS, InvivoGen), 5 μM Type B CpG oligonucleotide (ODN2006, InvivoGen) and 10 ng/ml TNFα (R&D Systems, Minneapolis, ME). All cells were cultured at 37 °C in a humidified atmosphere in 5% CO_2.

2.3. RT-PCR and real-time PCR analyses

Total RNA was extracted and purified using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). For RT-PCR, 2 μg of total RNA was reverse-transcribed into cDNA using a reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time PCR was performed as described in the manufacturer's protocol for Assays-on-Demand Gene Expression products (Applied Biosystems). The amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in each sample was used to standardize the quantities of p63 mRNA (Hs00978344), claudin-1 mRNA (Hs00221623) and claudin-4 mRNA (Hs00976831). To calculate the relative mRNA expression of triplicate specimens, the ΔΔCT method was employed according to the manufacturer's instructions.

2.4. siRNA preparation and transfection

A cocktail of three siRNAs for human p63 (siTrio full set) was purchased from B-Bridge International (Sunnyvale, CA). The sense sequences of the siRNAs for human p63 were as follows: 5′-CAGAAGAUGGUGCGACAAATT-3′, 5′-GUGAAUUCAACGAGGGACATT-3′, and 5′-GCAAAAAAGAGUUGGGUGUTT-3′. Negative control siRNA was obtained from Invitrogen (Carlsbad, CA). Cells were cultured at a density of 2×10^5 cells/well in a 6-well plate in 2.0 ml culture medium. After 24 h, the culture medium was replaced by a medium containing a complex of the siRNAs specific for p63 and Lipofectamine RNAi MAX (Invitrogen), giving a final concentration of siRNA of 40 nM following the manufacturer's instructions. After 72 h, cells were harvested to be subjected to quantitative PCR, Immunoblot or immunofluorescence staining analysis.

2.5. Antibodies

The primary antibodies used were a rabbit polyclonal antibody (pAb) anti ΔNp63 (p40; Merck KGaA, Darmstadt, Germany), a rabbit anti-claudin-1 pAb (JAY.8; Invitrogen), mouse anti-claudin-4 monoclonal antibody (Clone 3E2C1; Invitrogen) and a rabbit anti-cytokeratin pAb (Biogenesis, Poole, England). Alexa 488 (green)- and Alexa 594 (red)-conjugated anti-mouse and anti-rabbit IgGs were purchased from Invitrogen. Peroxidase-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from KPL (Gaithersburg, MD).

2.6. Immunostaining analysis

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. The cultured cells were fixed with cold acetone–ethanol (1:1 volume mixture). After rinsing these specimens with PBS, they were incubated with optimally diluted primary antibodies at room temperature for 1 h and secondary antibodies under the same conditions for another 1 h. Then in the immunofluorescence, 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich) was employed for counterstaining of the cell nuclei. Specimens were examined under a fluorescence microscope (IX81, Olympus, Tokyo, Japan).

2.7. Immunoblot analysis

Cell lysis was performed with 0.5% NP-40, 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and protease inhibitors (Roche, Basel, Switzerland) for 30 min at 4 °C. Aliquots of the supernatants were applied to SDS-10% polyacrylamide gels under reducing conditions and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were incubated with blocking buffer and then incubated with optimally diluted antibodies for 1 h at room temperature. After washing with wash buffer (0.1% Tween 20 in PBS), the membranes were reacted with a peroxidase-labeled secondary antibody for 1 h. After washing again with wash buffer three times, signals were visualized using an enhanced chemiluminescence detection system (Amersham Life Science, Arlington Height, IL). The levels of intensity of signals detected in immunoblots were quantified using NIH Image-J software. The intensity levels were normalized to the corresponding levels of β-actin, and their relative levels were shown in histograms.

2.8. Statistical analysis

Statistical significance was examined using the unpaired *t* test or ANOVA with Tukey's post hoc tests. Graph bars in the figures present means ± SD.