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Cutaneous permeability barrier function in signal transducer and activator of transcription 6-deficient mice is superior to that in wild-type mice

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ABSTRACT

Background: Th2 cytokines exhibit a variety of inhibitory effects on permeability barrier function via signal transducer and activator of transcription 6 (STAT6). However, the role of STAT6 signaling on the construction and/or homeostasis of permeability barrier function in the physiological state has not been fully assessed.
Objective: We compared permeability barrier function between Stat6-deficient and wild-type C57BL/6 mice at steady state.
Methods and results: Measurement of transepidermal water loss and quantitative penetration assay revealed that permeability barrier function was superior in Stat6-deficient mice. Accordingly, expressions of loricrin, acidic sphingomyelinase (aSMase) and β-glucocerebrosidase (β-GlcCer'ase) in epidermis and ceramide levels in stratum corneum were elevated in STAT6-deficient mice. On the other hands, up-regulations of loricrin, aSMase and β-GlcCer'ase were not observed in 3-dimensionally cultured human keratinocytes transfected with siRNA for STAT6. Meanwhile, number of mast cells in the dermis was decreased in Stat6-deficient mice.
Conclusions: These results suggest that STAT6 signaling negatively affects permeability barrier function in vivo, even in the physiological state. However, the superior permeability barrier function in Stat6-deficient mice may be a secondary effect exerted via cells other than keratinocytes, such as mast cells, since mast cells are known to influence permeability barrier function in vivo. Blockade of STAT6 signaling might be a strategy to augment the permeability barrier function.
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1. Introduction

Numerous negative effects of Th2 cytokines, IL-4 and IL-13 on cutaneous permeability barrier functions have been reported. Production of ceramide, an integral part of the extracellular lipid bilayer of the stratum corneum (SC) that forms the permeability barrier in the skin [1], and expressions of epidermal differentiation-related molecules such as filaggrin (FLG), loricrin (LOR) and involucrin (IVL), are negatively regulated by Th2 cytokines [2–6]. Those deleterious effects of Th2 cytokines on the permeability barrier are thought to be involved in the vicious cycle of allergic inflammation and cutaneous permeability barrier dysfunction in the pathogenesis of atopic dermatitis [7,8]. However, the role of Th2 cytokines in the homeostasis and/or construction of the cutaneous permeability barrier in the steady state has not been fully assessed, although Sehra et al. demonstrated that SC integrity was increased with elevated expressions of IVL and FLG but not LOR in IL-4-deficient mice [9].

Functions of IL-4 and IL-13 are well known to be mediated by a signal transduction molecule, signal transducer and activator of transcription 6 (STAT6) [10]. The possibility of alternative pathways of STAT6 activation have also been observed [10]. As for the regulation of expressions of epidermal differentiation-related molecules, loricrin, involucrin, and filaggrin expression have been demonstrated to be regulated by Th2 cytokines through STAT-6 [6,11].

Abbreviations: aCEase, acid-ceramidase; β-GlcCer'ase, β-glucocerebrosidase; EDTA, ethylenediaminetetraacetic acid; FLG, filaggrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; IVL, involucrin; LOR, loricrin; mRNA, messenger RNA; NHKs, normal human keratinocytes; PCR, polymerase chain reaction; SC, stratum corneum; siRNA, small interfering RNA; SMase, sphingomyelinase; STAT, signal transducer and activator of transcription; 3D, 3-dimensionally; TEWL, transepidermal water loss; Th, T helper.

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The present study examined the cutaneous permeability barrier function of Stat6-deficient mice to assess the role of STAT6 in cutaneous permeability barrier homeostasis in the steady state.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (Japan SLC, Hamamatsu, Japan) were purchased for use as wild-type mice. Female Stat6-knockout mice were donated by Prof. Yokozeki (Tokyo Medical and Dental University, Tokyo, Japan). Background strain of Stat6-knockout used here was C57BL/6 mice. The mice were used at 9–10 weeks old. All animals were housed under conventional conditions and had ad libitum access to commercial diet and water. All experiments with mice were approved by the Ethics of Animal Experimentation Committee at Oita University.

2.2. Physiological assessments

TEWL, SC hydration and SC surface pH on flanks were measured using a Tewameter (TM300; Courage & Khazaka, Cologne, Germany), Corneometer (CM825; Courage & Khazaka) and skin pH meter (PH 905; Courage & Khazaka), as described previously [12], 2 days after hair shaving.

2.3. Skin sample preparation

Skin samples were collected 3 days after hair shaving. Whole skin samples for penetration assay, hematoxylin and eosin stain, Giemsa stain, and electromicroscopic analysis were collected from flanks. Epidermal sheet for real-time PCR and Western blotting was collected from each flank by 1-h incubation at 37 °C with 1000 IU/ml of dispase (Godo Shusei, Tokyo, Japan). SC for lipid analysis was collected from 2 cm^2 of each abdomen by incubation for 2.5 h with 0.5% trypsin (Difco Laboratories, Detroit, MI) at 37 °C.

2.4. Penetration assay

Quantitative evaluation of outside-to-inside penetration of the skin was assessed with Evans blue dye, as described previously [13]. Each sample was floated on MCDB 153 medium (Sigma-Aldrich, St. Louis, MO) containing 1.8 mM of $CaCl_2$ with the outer epidermal surface of each sample exposed to the air. Next, 50 μl of 2% Evans blue in phosphate-buffered saline was pipetted onto the outer epidermal surface of each skin explant. The dye was allowed to penetrate the skin for 2 h at room temperature, then the surface of the skin was washed with phosphate-buffered saline and gently wiped with a Kimwipe (Nippon Paper Crecia, Tokyo, Japan). After washing procedures had been repeated three times, the center of each explant was biopsied with a 4-mm punch and each 4-mm disk was placed into 100 μl of 1 N KOH. After overnight incubation at 37 °C, each sample was neutralized by the addition of 900 μl of a mixture of 0.6 N H_3PO_4 and acetone (5:13, v/v). After vigorous vortexing for a few seconds, the mixture was centrifuged at 3000 rpm for 15 min in an RA-150AM centrifuge (Kubota, Tokyo, Japan), and absorbance of supernatants was measured at 360 nm.

2.5. Quantitative morphology under electron microscopy

Skin biopsies from mice were fixed in Karnovsky's fixative, and post-fixed with either 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide. Ultrathin sections were examined using an electron microscope (JEM-1200EX II; JEOL, Tokyo, Japan) operated at 80 kV. Measurement of the number of SC layers was performed using electron microscopic images taken by an investigator blinded to the source of the specimen.

2.6. Counts of mast cells in murine dermis

Mast cells in Giemsa-stained murine dermis were counted under high-power magnification. Counts were performed in 9–16 areas in each skin sample. The average number in each sample was analyzed.

2.7. Culture of keratinocytes transfected with siRNA at the air-liquid interface

Culture of normal human keratinocytes (NHKs) transfected with siRNA was performed as previously reported [14]. Third- or fourth-passage NHKs (Cell Applications, San Diego, CA) were cultured in Keratinocyte Basal Medium 2 with Supplement Pack Keratinocyte Growth Medium 2 (PromoCell, Heidelberg, Germany) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Stealth siRNA transfection was performed once cells reached 70–90% confluence. The Stealth siRNA against human Stat6 (HSS110291, Thermo Fisher Scientific, Lafayette, CO) and Stealth RNAi Negative Control (Thermo Fisher Scientific) were used. Cells were transfected with 100 nM siRNAs in Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and OPTI-MEM (Invitrogen), according to the instructions from the manufacturer. Transfected cells were harvested with ethylenediaminetetraacetic acid (EDTA)-trypsin (DS Pharma Biomedical, Osaka, Japan), then seeded into Cell Culture Inserts (pore size, 0.4 μm; BD Biosciences, Durham, NC) with Keratinocyte Basal Medium 2, which were placed into a Companion Plate (BD Biosciences) containing the same medium 24 h after the transfection. The medium in Cell Culture Inserts was aspirated 24 h later, and the medium in the Companion Plate was changed to assay medium (EPI-MODEL; Japan Tissue Engineering, Aichi, Japan). Keratinocytes transfected with siRNA were grown at the air-liquid interface. The medium in the plate was changed every three day, and keratinocytes were cultured for 7 days. Then, stratified keratinocytes and conditioned medium were harvested for real-time PCR or Western blotting.

2.8. Real-time PCR

Total RNA was isolated from epidermal sheets and cultured NHKs using an RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany), and reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the instructions from the manufacturer. Complementary DNA products were amplified in a LightCycler 480 System (Roche Diagnostics GmbH), as described previously [14]. The primers used for real-time PCR are shown in Supplementary Table S1. Product specificity was evaluated by melting curve analysis, and relative gene expression was calculated from a standard curve included in each run. Relative mRNA expression levels were normalized with the housekeeping gene, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or mouse β-actin.

2.9. Western blotting

Western blotting was performed as previously described [12,14]. Each epidermal sheet and cultured NHKs were homogenized in Pierce RIPA buffer (Thermo Scientific, Rockford, IL) with Halt Protease Inhibitor Cocktail (Thermo Scientific). These samples were separated by 10% SDS-PAGE before transfer to Immobilon-P Transfer Membrane (Millipore, Billerica, MA) or Hybond™-P (GE Healthcare, Buckinghamshire, UK). We used antibodies against FLG

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