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Defective maintenance of pH of stratum corneum is correlated with preferential emergence and exacerbation of atopic-dermatitis-like dermatitis in flaky-tail mice

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ABSTRACT

Background: Neutralization of stratum corneum (SC) pH, which is induced by a variety of stimuli, such as scratching, use of soap and inflammation, can stimulate activity of serine protease (SPase). Activation of SPase induces production of thymic stromal lymphopoietin (TSLP) through protease-activated receptor-2. Both reduced expression of natural moisturizing factors, which are required for maintenance of SC pH, and the preferential development of atopic dermatitis (AD)-like dermatitis are found in flaky-tail mice (FTM) with a loss-of-function mutation in filaggrin.

Objective: We examined possible correlations between disturbance of responses to an exogenous stimulus of SC neutralization and the preferential emergence of AD-like dermatitis in FTM.

Methods: FTM and wild-type mice (C57BL/6) were subjected to an SC-neutralization stimulus *via* application of 1,1,3,3-tetramethylguanidine (TMG). TMG was applied to young mice at a time when FTM had not yet developed significant dermatitis, and we examined their ability to maintain SC acidity and several parameters associated with AD-like dermatitis.

Results: The recovery of SC pH after the application of TMG was delayed in FTM, presumably because of unchanged expression of Na^+/H^+ antiporter 1, which is involved in maintenance of SC acidity. Cutaneous inflammation with elevated SPase activity and serum levels of TSLP, thymus and activation-regulated chemokine and IgE were induced only in TMG-treated FTM.

Conclusion: Our results suggest that defective maintenance of pH of SC is correlated with emergence and exacerbation of AD-like dermatitis in FTM.

1. Introduction

Collaboration of genetic background associated with impaired epidermal barrier and environmental factors is required for the emergence of allergic disorders, such as atopic dermatitis (AD) [1]. In fact, substantial pathological relationships between mutations in filaggrin and allergic conditions, including AD, asthma, and food allergy, have been reported [2]. However, precise mechanisms by which the effects of genetic abnormalities, such as mutations in filaggrin, are influenced by environmental factors, with emergence or augmentation of allergic conditions have not been fully elucidated. We examined possible correlations between disturbance of maintenance of pH of stratum corneum (SC) due to genetic abnormality and the preferential emergence of AD-like dermatitis by an exogenous stimulus of SC neutralization in flaky-tail mice (FTM).

The uppermost epidermal layer, the SC, has an acidic surface pH [3]. The normally acidic pH of SC regulates several key protective functions of the skin, including permeability barrier homeostasis, SC integrity/cohesion [4–6], and antimicrobial defenses [7]. In addition, an acidic SC pH inhibits the activity of serine protease (SPase) [8]. Conversely, neutralization of SC adversely impacts these functions and stimulates activation of SPase [3]. Activation of SPase induces degradation of corneodesmosomes, a decrease in SC integrity/cohesion [8], and production of thymic stromal lymphopoietin (TSLP), which is produced by epithelial cells and triggers dendritic cell-mediated Th2-type inflammation, through protease-activated receptor-2 [9]. A variety of environmental stimuli, such as disturbance of the permeability barrier by scratching, use of soap and allergic cutaneous inflammation including AD [3,10–12],

Abbreviations: AD, atopic dermatitis; FTM, flaky-tail mice; NHE1, sodium/proton pump N^+/H^+ antiporter 1; NMFs, natural moisturizing factors; SC, stratum corneum; SPase, serine protease; sPLA2, secretory phospholipase A2; TARC, thymus and activation-regulated chemokine; TEWL, transepidermal water loss; TMG, 1,1,3,3-tetramethylguanidine; TSLP, thymic stromal lymphopoietin; WT, wild-type mice.

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neutralize the pH of SC. On the other hand, recent studies showed that maintenance of acidic SC prevented the emergence of AD-like dermatitis in a murine model [10], and that acute acidification of SC improved the processing of lipids and inhibit the degradation of corneodesmosomes [13]. Thus, changes in SC pH might be important in the pathogenesis of skin disorders associated with permeability barrier dysfunction and inflammation (in particular, Th2-type inflammation), such as AD, and maintenance of the acidity of SC is very important to skin homeostasis.

Several mechanisms are involved in the maintenance of SC acidity. Free fatty acids of pilosebaceous origin [14], microbial metabolites [15], and eccrine gland-derived products [16,17] appear to decrease SC pH. In addition, a sodium/proton pump N^+/H^+ antiporter 1 (NHE1) [11,18], generation of free fatty acids by secretory phospholipase A2-catalyzd (sPLA2-catalyzed) hydrolysis of phospholipid [19,20], and natural moisturizing factors (NMFs), such as urocanic acid generated from histidines of filaggrin by histidase, also participate in this process [20,21]. In particular, both NHE1 and sPLA2 seem to be essential to SC acidity because, when either the sPLA2- or NHE1-mediated pathways to acidification are compromised, the SC pH rises, indicating that other acidifying mechanisms cannot compensate for their disruption [18–20].

While FTM with a loss-of-function mutation in the gene for filaggrin and the *matted* mutation have a greater tendency than wild-type mice (WT) to exhibit AD-like dermatitis with or without exposure to appropriate stimuli, such as haptens or allergens, the mechanistic link between genetic background and environmental factors is unclear [22,23]. Fluhr et al. had investigated the relationship between SC pH and urocanic acid derived from filaggrin, which can work as NMFs, using FTM. In that study, they hypothesized that if NMFs derived from filaggrin were important to regulate SC pH, SC pH of FTM and histidase-deficient mice should increase, however, those mice could maintain SC acidity *via* enhanced expression of NHE1 and sPLA2 despite decrease of NMFs at steady state [20]. Furthermore, recent study showed that up-regulation of NHE1 and sPLA2 acidification pathway can compensate the filaggrin deficiency, maintaining the skin surface acidity in 3D skin construct [24]. Therefore, the pathway of urocanic acid derived from filaggrin seems to be not essential for SC acidification.

Herein, we hypothesized that, if such compensatory mechanisms were fully exploited at the steady state by genetic abnormality, FTM might be sensitive to an exogenous SC-neutralization stimulus, equivalent to an environmental stimulus such as scratching, soap or cutaneous inflammation, with resultant development of allergic inflammation. We show here that the recovery of SC pH after the exogenous SC-neutralization is delayed and the defective maintenance of pH of SC is correlated with preferential emergence and exacerbation of AD-like dermatitis in FTM.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice, which were used as WT (Japan SLC Inc., Hamamatsu, Japan), and female FTM (Jackson Laboratory, Bar Harbor, ME, USA) were used at indicated ages. All animals were housed under conventional conditions and had free access to a commercial diet and water. All experiments with mice were approved by the Ethics of Animal Experimentation Committee of Oita University.

2.2. Neutralization of SC and physiological assessments

Acute neutralization of SC was achieved with a diluted superbase, 1,1,3,3-tetramethylguanidine (TMG). Topical application of TMG (1:100, v/v) in a mixture of propylene glycol and ethanol (7:3, v/v) on the flank of mice raises the pH of SC without any evidence of toxicity, inflammation or immunological response [5]. In some experiments, we applied TMG which was adjusted with hydrochloric acid into the pH equivalent to that of vehicle (low pH-TMG; pH value is about 5.70). Transepidermal water loss (TEWL), SC hydration and SC surface pH were measured at room temperature (22–26 °C) and 40–55% relative humidity. TEWL was measured with a Tewameter (TM300; Courage & Khazaka, Cologne, Germany). SC hydration was evaluated by analyzing electrical impedance of skin with a Corneometer (CM825; Courage & Khazaka). SC surface pH was evaluated with a skin pH meter (pH 905; Courage & Khazaka). Each value was measured according to each manufacturer's instructions [25,26]. Recovery rate of SC pH was calculated according to the following formula: ([pH value 1 h after TMG treatment] – [pH value at the indicated time])/([pH value 1 h after TMG treatment] – [pH value before TMG treatment]).

2.3. Quantitative assessment of skin morphology

Skin samples from the shaved flank were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4-μm sections were stained with hematoxylin and eosin, toluidine blue and CD3-specific polyclonal antibody (Dako, Tokyo, Japan). The thickness of the epidermis was measured with the scaled ocular lens of a light microscope. The various types of inflammatory cell were counted under high-power magnification.

2.4. Immunohistochemical staining

Skin samples were embedded in Optimal Cutting Temperature compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and frozen in liquid nitrogen. Cryosections of 5-μm thickness (CM3050 cryostat; Leica Microsystems, Wetzlar, Germany) were incubated for 20 min in blocking buffer [PBS plus 5% normal donkey serum (Sigma–Aldrich Co., St. Louis, MO, USA), 2% bovine serum albumin (Sigma–Aldrich Co.) and 0.05% Tween-20]. Then, sections were incubated overnight at 4 °C with primary polyclonal antibodies against NHE1 (Millipore, Temecula, CA, USA), sPLA2 (Santa Cruz Biotechnology Inc., Dallas, TX, USA) or TSLP (R&D Systems, Minneapolis, MN, USA). After washing, specimens were incubated for 1 h at room temperature with Alexa Fluor® 488-conjugated goat anti-rabbit or donkey anti-goat second antibodies (Life Technologies, Carlsbad, CA, USA) and counterstained with propidium iodide. Staining was evaluated under a confocal microscope (LSM710 ZEN; Zeiss, Oberkochen, Germany).

2.5. Western blotting

Epidermal sheets were separated from skin samples by treatment with 1000 IU/ml dispase (Godo Shusei, Tokyo, Japan) for approximately 1 h at 37 °C. Each epidermal sheet was homogenized in protein extraction buffer (40 mM Tris–HCl, pH 7.5, 10 mM EDTA containing protease inhibitors and 0.5% Nonidet P-40). The homogenates were centrifuged; the detergent-insoluble pellet was solubilized in protein extraction buffer containing 6 M urea. These samples were separated by 10% SDS–PAGE before transfer to Immobilon®-P Transfer Membrane (Millipore). We used antibodies against NHE1 (see above) and β-actin (Cell Signaling Technology Inc., Danvers, MA, USA) as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology Inc.) as second antibodies, and visualized with ECL™ Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) [27]. Band intensities were determined with Image Quant LAS