Acidification of Stratum Corneum Prevents Atopic March in Murine Models

Hae-Jin Lee

The Graduate School

Yonsei University

Department of Medicine

Acidification of Stratum Corneum Prevents Atopic March in Murine Models

Directed by Professor Eung Ho Choi

A Doctoral Dissertation
Submitted to the Department of Medicine
and the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Hae-Jin Lee

December 2015

This certifies that the doctoral dissertation of
Hae-Jin Lee is approved.

Thesis Supervisor: Prof. Eung Ho Choi

Thesis Committee Member #1 : Prof. Soo-Ki Kim

Thesis Committee Member #2 : Prof. Mee Kyung Nam Goong

Thesis Committee Member #3 : Prof. Dong Joon Park

Thesis Committee Member #4 : Prof. Won Yeon Lee

The Graduate School
Yonsei University
December 2015

Acknowledgements

First of all, I would like to thank God, the Almighty, for rendering everything possible by giving me strength and courage.

Until the completion of this Doctor's thesis, Professor Eung Ho Choi, my thesis supervisor, advised and supported me enthusiastically during my Ph.D. course. I am very grateful for his instructions and feedback which kept me focused in my study.

I also appreciate thesis committee members' great support for my research. The precious comments and crucial times given by my commentators, Professor Soo Ki Kim, Mee Kyung Nam Goong, Dong Joon Park and Won Yeon Lee have made the clarification and improvement of this thesis. I am also grateful to professor Sung Ku Ahn and Won-Soo Lee for their commitment in expanding and enriching my training and expertise.

I'd like to thank to Minyoung Jung and Dong Hye Kim in dermatology laboratory for practical helps. I'm also really thankful to Noo Ri Lee, Bo Kyung Kim, Dong In Keum, Hee Chul Jung, Jae Woong Choi and other residents at the department of dermatology, for the great contributions to my experiments.

I really appreciate the endless love and care of my parents, Seung Hun Lee and Soo Kyung Oh, who have always supported me in deep and profound ways and encouraged me to pursue my dreams. I also thank to my brother, Hae Jun Lee for cheering me up anytime and anywhere.

Table of Contents

List of Figures

Abstract

Acidification of stratum corneum prevents atopic march in murine models

Hae-Jin Lee

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Eung Ho Choi)

Maintenance of acidic pH in the stratum corneum(SC) is an important factor for normal skin barrier function. Long standing or repeated skin barrier damage followed by atopic dermatitis(AD) is the initial step of the atopic march that eventually progresses to respiratory allergies. We determined whether various AD murine models, flaky tail, Nc/Nga and multiple oxazolone(Ox) challenged mice, can develop airway inflammation by topical application and nasal inhalation of a house dust mite, Dermatofagoides pteronyssinus(Dp)(defined as a novel 'atopic march animal model'), and whether maintenance of an acidic SC environment by continuous application of acidic cream can interrupt this atopic march. During the course of Dp treatment to the atopic march murine model, an acidic cream with pH 2.8 and neutral cream(pH7.4) adjusted by citric acid and sodium hydroxide mixed with vehicle were applied twice daily. Repeated applications

and inhalations of Dp to AD murine model induced AD skin lesions followed by respiratory allergies. These results indicate that novel atopic march models can be developed by repeated application of house dust mites on flaky tail, Nc/Nga and Ox-induced AD mice, and that the acidification of SC could be a novel intervention method to block the progress of the atopic march.

Key words: atopic march, acid, respiratory allergy, atopic dermatitis, flaky tail mice, Nc/Nga mice, oxazolone, *Dermatofagoides pteronyssinus*

Acidification of stratum corneum prevents atopic march in murine models

Hae-Jin Lee

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Eung Ho Choi)

I. Part 1. Topical acidic cream prevents the development of atopic dermatitis- and asthma-like lesions in murine model

1. Abstract

Long-standing or repeated skin barrier damage followed by atopic dermatitis (AD) is the initial step of the atopic march that eventually progresses to respiratory allergies. Maintenance of an acidic pH in the stratum corneum (SC) is an important factor for normal skin barrier function. To determine whether an oxazolone (Ox)-induced AD murine model can develop airway inflammation by topical application and nasal inhalation of a house dust mite, Dermatofagoides pteronyssinus (Dp), which is a novel

‘atopic march animal model’, and whether an acidic SC environment, made by repeated application of acidic cream, can interrupt this atopic march. During repeated treatment with Ox and Dp to make an atopic march murine model, acidic cream (pH 2.8) and neutral cream (pH 7.4) adjusted by citric acid and sodium hydroxide mixed with vehicle were applied twice daily. Repeated treatment with Ox and Dp to hairless mice induced AD-like skin lesions followed by respiratory allergy, defining it as an atopic march model. Acidic cream inhibited the occurrence of respiratory allergic inflammation as well as AD-like skin lesions. These results indicate that a novel atopic march animal model can be developed by repeated topical and nasal treatments with house dust mite on Ox-induced AD mice, and that the acidification of SC could be a novel intervention method to block the atopic march.

2. Introduction

Atopic dermatitis (AD) is a common, chronic inflammatory skin disease characterized by pruritus, eczematous skin lesions, immunodysregulation, epidermal barrier dysfunction, and IgE-mediated sensitization to food and environmental allergens. The striking increase in the incidence of AD observed in recent decades has been attributed to the resettlement of populations from rural to urban areas, where a lack of early exposure to a variety of microbes purportedly results in reduced immune tolerance.(1, 2) In general, the clinical manifestations of AD predate the development of asthma and allergic rhinitis, suggesting that AD is an 'entry point' for subsequent atopic diseases, in a process called the 'atopic march'.(3, 4)

Many studies suggest that the development of asthma in individuals with AD is secondary to easy allergen sensitization through a primarily defective epidermal barrier, which enables allergens(e.g. house dust mites) to easily penetrate the skin and then make contact with antigen-presenting cells, Langerhans cells (LCs), in the skin(5-7). After LCs capture and process the penetrated allergens, they migrate to draining lymph nodes and interact with naïve T cells and then promote Th2 inflammation leading to systemic allergy(8). In addition, the impaired skin barrier can be further aggravated by chronic colonization by Staphylococcus aureus which secretes superantigens(9). Superantigens in AD skin stimulate keratinocytes to produce thymic stromal lymphopoietin(TSLP) which induces polyclonal activation of T cells, which results in exaggerated Th2 inflammatory responses leading to AD aggravation and promotion of systemic Th2 responses including respiratory allergies.

Thus, prevention of the atopic march is a great challenge for many physicians. Maintenance of acidic pH in the stratum corneum (SC) is an important factor for normal

skin barrier function. Elias et al. hypothesized that a reduced level of filaggrin and, in particular, its acidic derivative, urocanic acid, increases SC pH and alters the activity of multiple serine proteases and two ceramide-generating enzymes that regulate the homeostasis of the SC.(2) The acidic pH of the SC, which is regulated by secretory phospholipase A2 and the sodium/hydrogen antiporter-1, regulates several main protective functions of the skin including permeability barrier homeostasis, SC integrity and cohesion, and antimicrobial defense, as well as primary cytokine activation.(10) Prolonged SC neutralization provokes profound abnormalities in SC function, due to pH-induced high SP activity.(11) In addition, Fluhr et al(12) revealed that the delay in acidification of neonatal SC results in abnormalities in permeability barrier homeostasis and SC integrity and are likely due to pH-induced modulations in enzyme activity in animal model. Simple acidification of the SC substantially prevented AD development by repairing the barrier impairment followed by immune abnormalities in oxazolone (Ox)-treated hairless mice.(13) Based on this information, we assumed that long-standing or repeated barrier damage followed by or occurring in AD is the initial step of the atopic march that eventually progresses to airway allergies. Furthermore, we predicted that maintenance of SC acidity could be used as an interventional modality to block the atopic march. Therefore, our first hypothesis was that an Ox-induced AD murine model could develop airway inflammation after repeated topical and nasal application of house dust mites, and that this model could be used as a novel ‘atopic march animal model.’ Our second hypothesis was that an acidic environment around the SC, created by repeated treatment with an acidic cream, could interrupt this atopic march.

This Ox-induced AD murine model recapitulates virtually all clinical, structural, lipid biochemical, and immunological features of human AD, including: (i) chronic, pruritic,

inflammatory dermatosis, (ii) elevated transepidermal water loss (TEWL) level; (iii) ultrastructural and lipid biochemical abnormalities typical of AD; (iv) tissue eosinophilia, elevated serum IgE level, and Th2-dominant immunophenotype; (v) decreased expression of two key antimicrobial peptides (AMPs), cathelicidin-related antimicrobial peptide (CRAMP) and mBD3; and (vi) decreased expression of epidermal differentiation markers including filaggrin, loricrin, and involucrin.(13-15) Therefore, we established an animal model of the atopic march by modifying this Ox-induced AD mouse model, and assessed the therapeutic and preventative effects of SC acidification in blocking the atopic march.

3. Materials and methods

Animals and Materials

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Wonju Campus. Thirty-six female hairless mice (6 weeks old) were purchased from OrientBio (Seongnam, Korea). Mice were kept under controlled humidity (40%) and temperature (22±2oC) conditions. 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) and acetone were purchased from Sigma-Aldrich (St Louis, MO, USA). Extracted antigen of Dermatofagoides pteronyssinus, a house dust mite, was purchased from the Department of Environmental Medical Biology, Yonsei University (Seoul, Korea). All laboratory measurements were performed under blinded conditions or by blinded researchers. The acidic cream was composed of citric acid purchased from Sigma-Aldrich and vehicle (Cetaphil® cream ; Galderma, Lausanne, Switzerland), adjusted with a pH meter. Neutralization of the acidic cream was performed with NaOH.

Development of an atopic march model by topical application and nasal inhalation of Dp in Ox-induced AD mice

A hapten-induced AD murine model (Ox-AD) with multiple similar features of human AD was developed as done in previous studies.(13-15) One group (Ox-plus-Dp group, n=6) was sensitized by a single topical treatment of 60 μl of 5% Ox and challenged by repeated application of 0.01% Ox, while the other two groups, including a non-treated control group (n=6) and a Dp-only-treated control group (application and inhalation of only Dp, n=6), were not treated with Ox. After the fifth Ox challenge, 40 mg of 0.5% mite (Dp) antigen (Ag) mixed with petrolatum was also applied to the skin of the Dp-only

control group and the Ox-plus-Dp group twice a week for a total of 10 times. During the last 3 days of the experiments, 100 μg of Dp Ag was intra-nasally inhaled daily (Fig. 1a). After a total of 53 days of repeated Ox challenges and Dp applications, the gross appearance, the skin barrier functions such as basal transepidermal water loss (TEWL) and the SC hydration, and the degree of allergic inflammation in skin, blood, and respiratory system were evaluated in all mice.

Intervention of the atopic march model with an acidic cream

For the acidic cream group, 200 mg of acidic cream (pH 2.8, vehicle cream adjusted with citric acid) was applied topically twice a day on the dorsal skin for 46 days after sensitization and of Ox. During the course of Ox challenge, cream was applied after application of Ox. For non-treated control groups, no Ox or interventional cream was applied. For the vehicle group, plain Cetaphil® cream (pH 5.5) was applied as previously performed.(16) For the neutralized cream group, neutralized cream was applied topically twice a day (Fig. 1b).

Basal TEWL was measured with a Tewameter TM210 (Courage and Khazaka, Cologne, Germany), and SC hydration was assessed as capacitance with a Corneometer CM820 (Courage and Khazaka) just before each application of Ox and at 24 h after the last application of Ox and Dp, as described previously.(17) Twenty-four hours after the last treatment, specimens of skin, blood, and lung were collected to evaluate the degree of allergic inflammation, which was measured by mRNA and protein expression.

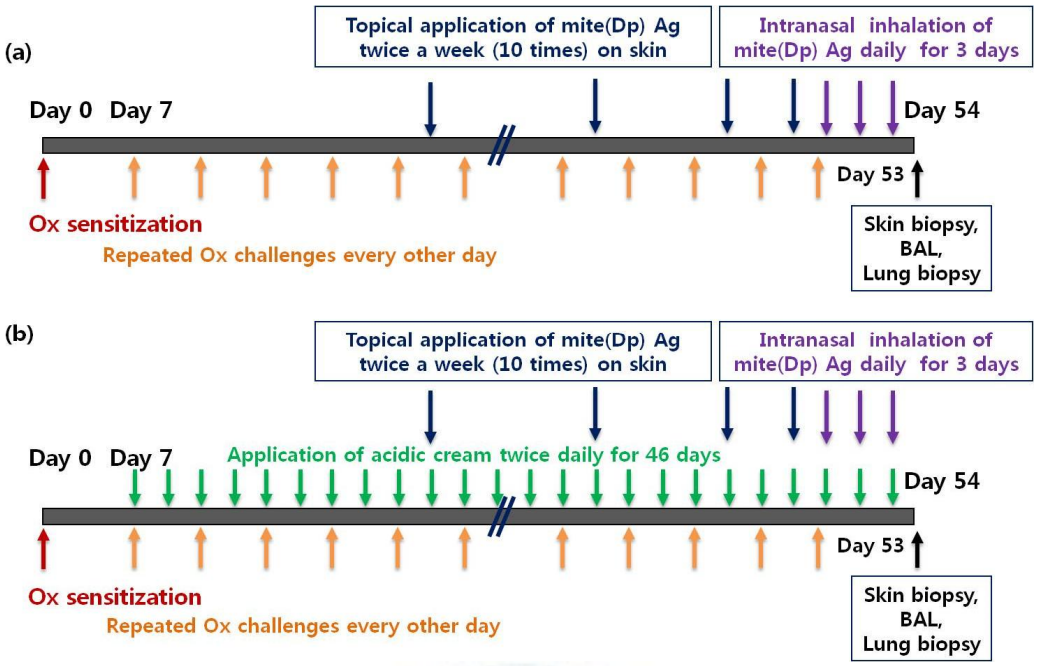

Figure 1. Methods for development of an atopic march model by topical application and nasal inhalation of a house dust mite (Dermatofagoides pteronyssinus; Dp) to oxazolone (Ox)-induced AD mice (a) and interruption of the atopic march model with acidic cream (b).

Tissue preparation, immunohistochemistry, and immunofluorescence

Immunohistochemical stains were performed to assess epidermal differentiation. Briefly, 5-μm-thick paraffin sections were incubated with the primary antibodies of differentiation marker proteins of keratinocytes, including filaggrin, involucrin, loricrin, PAR-2, and TSLP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After three cycles of washes, sections were incubated with the secondary antibody for 30 min. Staining was detected with an ABC-peroxidase kit (Vector Labs, Burlingame, CA, USA), and sections were then counterstained with hematoxylin. To quantify the density of immunohistochemical stain, three pictures from each section were taken at 100x magnification and labeled. An investigator without any information on the specimens ranked the pictures in order of the intensity of immunostaining (highest intensity received first rank). The Mann-Whitney test was used for statistics. Representative pictures from each group were chosen for publication.

ELISA for serum IgE and TSLP

The serum levels of IgE and TSLP were determined using a mouse IgE ELISA kit (Koma Biotech, Seoul, Korea) and a mouse TSLP ELISA kit (R&D Systems, Minneapolis, MN, USA). To detect serum IgE levels, whole blood samples were centrifuged at 5,000 rpm for 5 min at room temperature. The sample serum and standard solution were added to the plates pre-coated with antibodies specific to IgE and TSLP and incubated for 1 hour at room temperature. Then, mouse IgE and TSLP detection antibodies conjugated to horseradish peroxidase were added to each well and incubated for 1 hour at room temperature. Next, a color development with TMB substrate was added to each well for 20 min. Sulfuric acid solution was then added to each well to stop

the reaction. The enzyme-substrate reaction was measured using a microtiter plate reader (BioTek Instruments, Inc., Winooski, VT, USA) to read the plate at 450 nm ± 2 nm wavelength. After triplicate measurements, the sample concentrations of IgE and TSLP were compared to the O.D. of the samples relative to the standard curve.

Bronchoalveolar lavage (BAL)

After each mouse was anesthetized, the trachea was cannulated and the lung was lavaged with 1 mL of Hank's balanced salt solution via the intubation tube.(18) Bronchoalveolar lavage fluid (BALF) was obtained by two rounds of aspiration. Total numbers of cells were counted and cell smear preparations were made. Cells were stained with Wright–Giemsa stain and eosinophils, lymphocytes, macrophages, and neutrophils were counted and expressed as a percentage of 400 total cells. The counts of eosinophils, lymphocytes, macrophages, and neutrophils in the BALF were determined by multiplying their percentages in total cells.

Total RNA preparation and cDNA synthesis with blood and lung tissue

Total RNA preparation and cDNA synthesis were performed using the same methods as our previous report.(14, 16) Total RNA was extracted using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol Reagent; Invitrogen, Carlsbad, CA, USA). RNA concentration was determined by a UV-visible spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA) at 260 nm. Aliquots (1.0 ug) of RNA from each sample were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (MML-V RTase, Promega, Madison, WI, USA). Reverse transcription was performed by adding 1X RT-buffer, 2 mM deoxynucleotide triphosphates (dNTPs,

Promega), 0.2 pM oligo dT primer (16mer) (Bioneer Inc., Daejeon, Korea), and MML-V RTase (2.5 units/ul) in 20 ul reaction volumes. Samples were then incubated at 42℃ for 45 min before storage at -20℃.

Quantitative PCR analysis of gene expression

Expression of specific mRNAs was quantified using a Rotor-Gene™ 3000 (Corbett Life Science, Brisbane, Australia), as in our previous report.(14, 16, 19) Briefly, 10 ul PCR reactions were set up containing: Quantitect SYBR green PCR kit Master mix (Qiagen, Hilden, Germany) in a 2X solution, 8 mM manganese chloride, 200 uM deoxynucleotide triphosphates (dNTPs), 1.25 units Hotstart Taq polymerase, and 0.5 pM/uL each of probes and primers. About 60 ng of cDNA was used per reaction. Primers for PAR-2 and TSLP were designed based on GeneBank sequences using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/, Massachusetts Institute of Technology, Cambridge, MA, USA).

All reactions used GAPDH as a housekeeping gene. Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacturer's guidelines and were used to determine the ΔCt values (Ct of target gene−Ct of housekeeping gene) as raw data for gene expression. Fold changes in gene expression were determined by subtracting ΔCt values for Ox with Dp samples and acidic cream-treated samples from their respective control samples. The resulting ΔΔCt values were then used to calculate fold change in gene expression as 2-ΔΔCt. All reactions were performed in triplicate and the results are expressed as the mean of values from three separate experiments. Samples were amplified under the following conditions: 95℃ for 15 minutes followed by 45 cycles of 95℃ for 15 seconds and 60℃ for 1

minute.

Statistical analysis

All data are expressed as mean ± SE. Statistical analyses were performed using One Way ANOVA or Mann-Whitney test for assaying the density of immunohistochemical staining.

4. Results

Repeated Ox challenge combined with topical application and nasal inhalation of Dp provokes AD-like skin lesions with elevated serum TSLP and IgE levels

In our study, repeated Ox challenge induced AD-like skin lesions, as described in previous reports.(13-15) The Ox-plus-Dp group showed remarkable erythema, edema, lichenification, and scales compared with controls (Fig. 2a). Post-treatment basal TEWL increased and SC hydration decreased significantly in the Ox-plus-Dp group (Figs. 2b and c). Post-treatment serum IgE and TSLP also increased significantly in the Ox-plus-Dp group (Figs. 2d and e). In addition, we evaluated sequential levels of serum TSLP in our model. After the 5th challenge of Ox, serum TSLP significantly increased and was then maintained until the last challenge of the experiments (Fig. 2f). The expression of TSLP and PAR-2, as measured by immunohistochemical stain, increased gradually according to the number of repeats of Ox challenge (Fig. 2g). Eosinophils in the skin also significantly increased in the Ox-plus-Dp group compared with controls (Fig. 3a). The expression levels of epidermal differentiation markers such as filaggrin, involucrin, and loricrin were significantly decreased in the Ox-plus-Dp group after the 23rd challenge of Ox combined topical treatment and nasal inhalation of Dp (Fig. 3b).

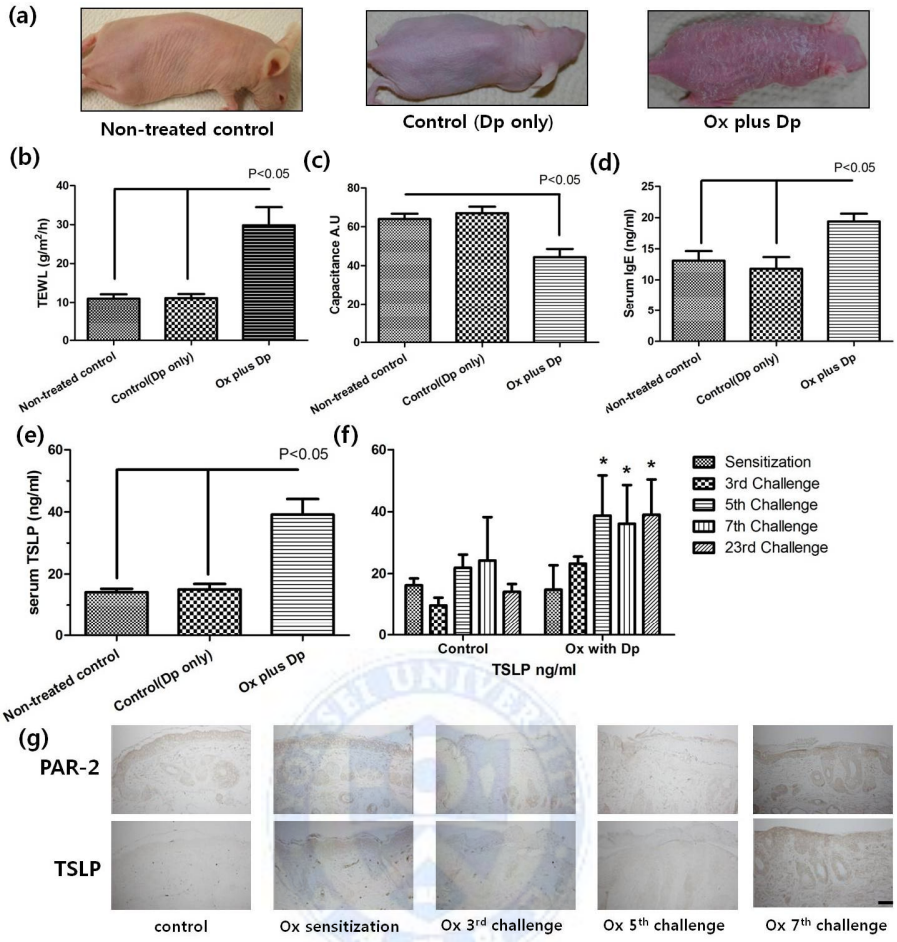

Figure 2. Gross appearance of AD was prominent in the Ox-plus-Dp group (a), which had increased basal TEWL (b), SC hydration (c), serum IgE (d), and TSLP expression (e) compared to controls. According to sequential challenges with Ox, TSLP expression was elevated in the serum and skin (f), while PAR-2 expression was increased in the skin, by immunohistochemical staining (g). *P<0.05, compared to non-treated control, Ox sensitization and Ox 3rd challenge. Control (Dp only), topical application and nasal inhalation of Dp without Ox treatment; Ox-plus-Dp, repeated Ox challenge combined with topical application and nasal inhalation of Dp.

Repeated Ox challenge combined with topical application and nasal inhalation of Dp produces a novel atopic march model by provoking asthma-like lesions, including airway eosinophilia and increased expression of PAR-2 and TSLP in lung tissue

Airway eosinophilia is a hallmark of asthma.(20) BAL was performed after the 23rd challenge of Ox combined topical treatment and nasal inhalation of Dp. Post-treatment BAL showed a significant increase in total cell count in the Ox-plus-Dp group (Fig. 3c), in which eosinophils as well as macrophages and neutrophils were significantly increased compared with the controls (Fig. 3d). The mRNA of PAR-2 and TSLP in the lung tissue significantly increased in the Ox-plus-Dp group compared with the controls (Figs. 3e and f). The expression levels of PAR-2 and TSLP were also increased in the Ox-plus-Dp group compared to the controls, as revealed by immunohistochemical staining (Fig. 3g). These results indicated that this murine model shows 'atopic march' from AD to respiratory allergy.

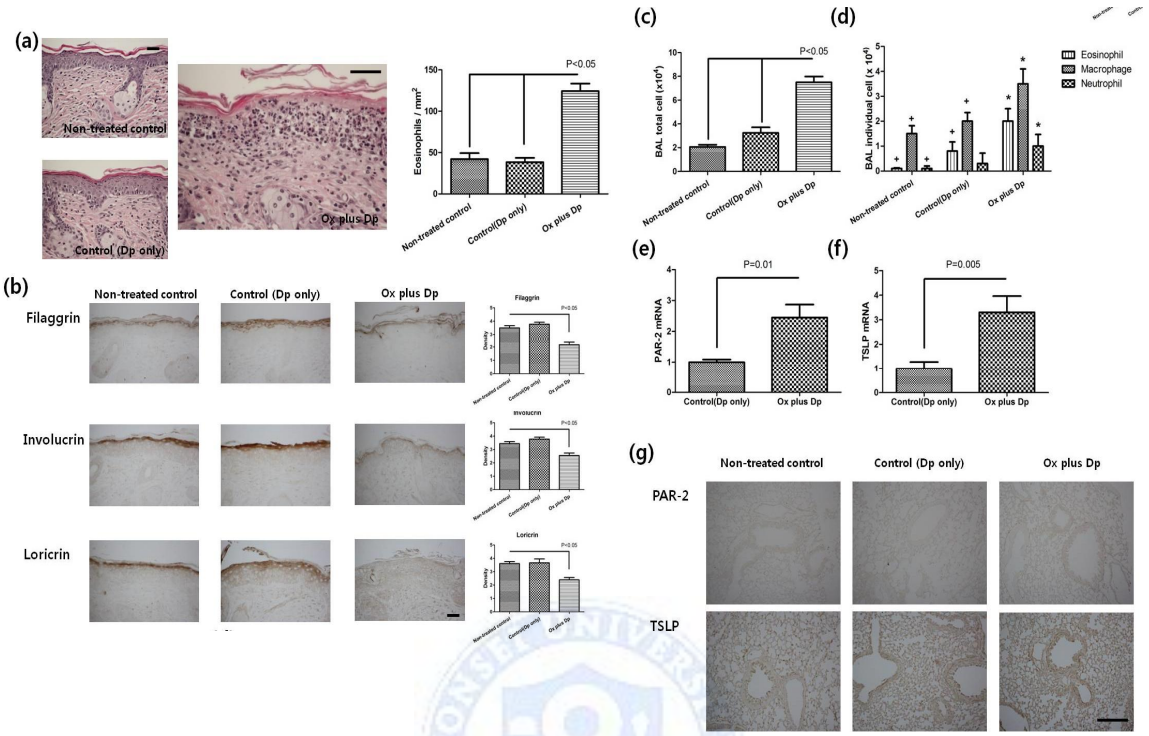

Figure 3. AD-like skin lesions provoked by repeated Ox challenge combined with topical application and nasal inhalation of Dp showed significant increased tissue eosinophils (a) and reduced epidermal differentiation, as presented by filaggrin, involucrin, and loricrin expression levels (b). Total cell count (c) and individual cell count (d) of the BAL were increased in the Ox-plus-Dp group. mRNA (e and f) and tissue expression (g) of PAR-2 and TSLP were also prominent in the Ox-plus-Dp group. Control (Dp only), topical application and nasal inhalation of Dp without Ox treatment; Ox-plus-Dp, repeated Ox challenge combined with topical application and nasal inhalation of Dp. *P<0.05, compared to control; +P<0.05, compared to Ox plus Dp.

Topical applications of acidic cream prevent the occurrence of AD-like skin lesions and the progression to asthma-like lung lesions

We next assessed the effect of the acidic SC environment made by an acidic cream on the process of an initial appearance of AD-like skin lesions followed by asthma-like lung lesions, which was defined as atopic march. Topical application of acidic cream significantly improved gross AD lesions (Fig. 4a), significantly decreased basal TEWL, and significantly increased SC hydration compared to other treatments such as vehicle or neutralized cream (Figs. 4b and c). Skin treated with only acidic cream in the absence of Ox treatment showed no changes in gross appearance or skin barrier functions (data not shown). Post-treatment BAL of the acidic cream group showed a significant decrease in total cell count, and numbers of eosinophils, as well as macrophages and neutrophils, increased significantly compared to the controls (Figs. 4d and e). The mRNA levels of PAR-2 and TSLP in the lung tissue significantly decreased in the Ox-plus-Dp group after acidic cream application compared to other treatments such as vehicle or neutralized cream (Figs. 4f and g).

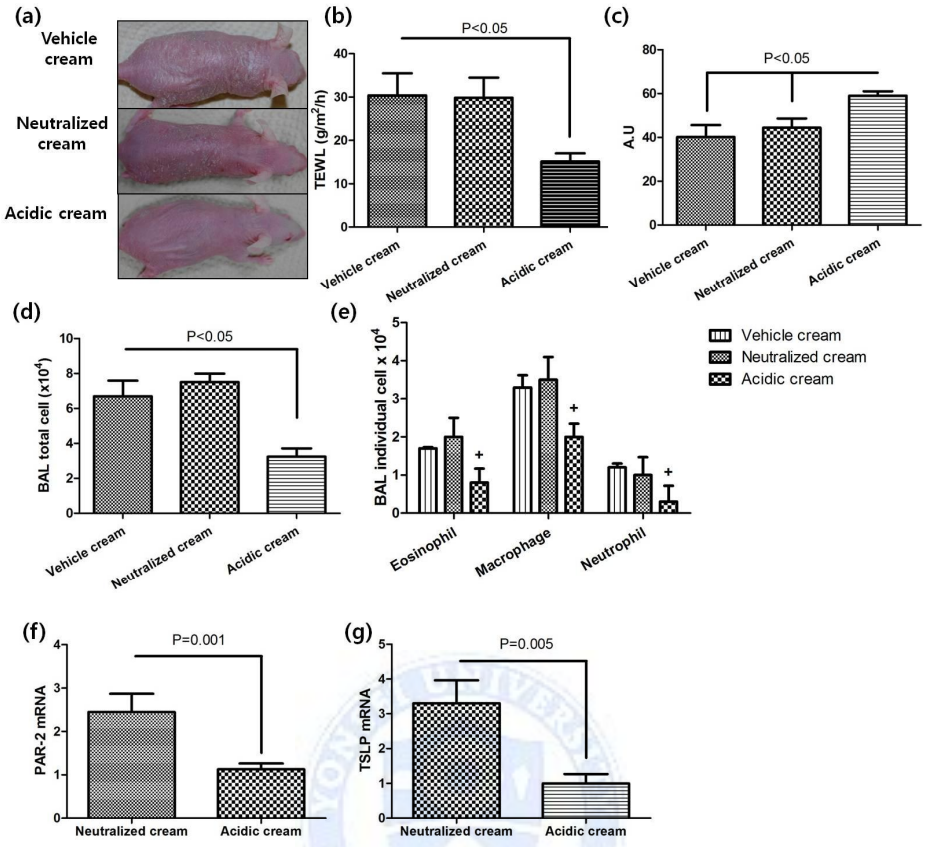

Figure 4. Topical acidic cream prevents the occurrence of AD-like skin lesions and prevents the progression to asthma-like lung lesions provoked by repeated Ox challenge combined with topical application and nasal inhalation of Dp. The acidic cream group showed better gross appearance (a), basal TEWL (b), and SC hydration (c) compared with vehicle or neutralized cream groups. Bronchoalveolar lavage (d and e) performed after the 23rd Ox challenge and topical application and nasal inhalation of Dp. The mRNA levels of PAR-2 (f) and TSLP (g) in the lung tissue were measured after the 23rd Ox challenge combined with topical application and nasal inhalation of Dp. Vehicle cream, vehicle-treated group; neutralized cream, pH 7.4 neutralized citric acid cream-treated group; acidic cream, pH 2.8 citric acid cream-treated group. +P<0.05, compared to vehicle cream and neutralized cream group.

5. Discussion

The concept of the atopic march was hypothesized to describe the progression of atopic diseases from AD in infants to asthma or allergic rhinitis in children.(4, 7) Kubo et al.(1) suggested a theoretical model of barrier disruption followed by percutaneous sensitization, which may apply to the pathogenesis of the atopic march. After abrogation of the SC barrier, protease-active allergens and uncontrolled intrinsic proteases may activate PAR-2 and Toll-like receptors on keratinocytes.(21). Keratinocytes then produce proinflammatory cytokines such as TNF-α, IL-1, and TSLP. During SC perturbation, dendrites of activated LCs penetrate the weakened tight junctions of upper stratum granulosum and then take up exogenous antigens.(22) Such percutaneous sensitization and repeated allergen challenges via different routes such as lung, nasal cavity, or intestinal tract, are speculated to manifest as the atopic march. Many researchers have attempted to establish an animal model of atopic march. There have been several studies done using an occlusive patch of ovalbumin (OVA) to make an atopic march model.(23-26) Although asthma-like lung lesions were developed using these methods, AD-like skin lesions including eczematous lesions and disrupted SC barrier were not fully developed. Although IL-13 transgenic mice with OVA treatment (27), RBP-jCKO mice with OVA (28), and Ox challenge with OVA (29) expressed AD-like skin lesions combined with asthma-like lung lesions, intra-peritoneal injection of OVA was applied for sensitization, not epicutaneous sensitization via damaged skin barrier.

Oxazolone (Ox) are commonly used to provoke allergic contact dermatitis and have been thought to evoke a Th1-dominated response as a hapten.(15) It has been reported, however, that multiple challenges of an extended period with Ox to the skin of hairless mice cause the skin inflammation to shift from Th1-dominated delayed type

hypersensitivity response to a chronic Th2-dominated inflammatory response similar to human AD.(13, 14, 30, 31) Skin barrier abnormality was associated with increased TEWL and impaired lamellar body secretion, decreased SC ceramide contents and hydration, resulting in reduced lamellar membranes, as observed in AD patients. Furthermore, as in human AD, epidermal serine protease activity in SC increased and the expression of two lamellar body-derived antimicrobial peptides, CRAMP and mBD3, declined after multiple Ox challenges, paralleling the decrease of their human homologues in AD skin lesions.(13, 15, 31) Even if the repeated sensitization model of hapten is not a genetically driven model, it may be applicable to extrinsic allergen driven, acquired form of AD.

In order to produce asthma-like lung lesions, we applied Dp antigens, which is one of potent allergens to produce respiratory allergies in human, topically to AD-like skin lesions with disrupted barrier and then caused nasal inhalation of Dp, which revealed a natural mechanism of allergen penetration, systemic circulation, and allergen challenge via different routes such as lung or nasal cavity (Fig. 2).(1) In addition, topical application of Dp antigen combined with Ox challenges created more severe eczematous lesions compared with Ox treatment only (data not shown). Our study indicated that repeated treatment with Ox, a hapten, disrupts the skin barrier, which contributes to the development of AD-like skin lesions by promoting the epicutaneous penetration of allergens such as house dust mites and then leads to the development of asthma-like lung lesions. Indeed, topical application and nasal inhalation of Dp without barrier disruption did not produce any inflammatory lesions in the skin or lung (Figs. 3 and 4). This finding indicates that active allergen uptake by LCs did not occur when the skin barrier was intact and did not lead to an increase in cytokines and TSLP.

TSLP in the pathogenesis of human AD has been well documented to be highly

increased in the lesional skin as well as in the blood of AD patients.(1, 32) Moreover, TSLP was significantly higher in acute AD than in chronic AD (27). Since the Ox-AD murine model was introduced (15), several studies have used this model to elucidate the pathogenesis and treatment of AD-related diseases.(13, 14, 29-31, 33) In the current study, we confirmed that blood TSLP level was increased from the fifth challenge of Ox, which induced early eczema appearance, and was maintained until the last Ox challenge that resulted in severe eczema (Fig. 2f). The elevated TSLP may sufficiently activate LCs. In addition, the expression of PAR-2 and TSLP in murine skin was also increased after Ox challenge (Fig. 2g). Therefore, we showed the additional evidence that Ox-treated hairless mice can be a useful animal model of AD.

Keratinocyte differentiation is also incomplete in AD lesions.(34) In our atopic march model, the expression levels of filaggrin as well as those of involucrin and loricrin decreased after repeated Ox challenges. These decreases compromise the integrity of the skin barrier and then provoke transcutaneous penetration of exogenous allergens, such as Dp (Fig. 3b). This disruption in the skin barrier may also induce asthma-like lung lesions more easily. The expression of the filaggrin gene is down-regulated in AD skin by Th2 cytokines such as IL-4 and IL-13.(35) Therefore, we believe that repeated Ox challenges can alter filaggrin expression by inducing Th2 allergic inflammation.

Expression of PAR2 has been demonstrated in various cells of the skin and the lung. Many studies indicated that the activation of PAR2 by endogenous or exogenous agonists such as allergens contributes to the pathogenesis of AD or asthma which is manifested by airway inflammation and hyper-responsiveness.(36-39) TSLP also takes part in the pathogenesis of atopic diseases including AD and asthma. Kouzaki et al(40) reported that TSLP was induced in airway epithelial cells by exposure to allergen-derived proteases,

and that PAR-2 was involved in this process. Repeated Ox challenge and Dp application increased the expression of PAR-2 and TSLP in the blood and lung tissue used in our study (Fig. 3). From our results, we assume that Dp-induced asthma can be easily produced when the skin barrier is disrupted by repeated Ox challenges, which induced systemic PAR-2 and TSLP as well as eosinophils, although Dp application alone was not sufficient to induce asthma. Increased systemic PAR-2 and TSLP may help to induce asthma-like lung lesions more easily.

The various preventative implications of AD were introduced. Lee et al(41) revealed that in vivo treatment with poly-γ-glutamic acid at early time points can prevent the development of AD in NC mice, which may have therapeutic applications for human AD. Recent study showed that deficiency of n-6 polyunsaturated fatty acids is mainly responsible for AD-like pruritic skin inflammation, which might give us a clue that early administrations of n-6 polyunsaturated fatty acids may prevent pruritic skin inflammation in AD.(42) Kim et al(43) showed consecutive low concentration of formaldehyde exposure in NC/Nga mice might be a key factor to exacerbate house dust mite-mediated AD-like skin inflammation.

The pH of the SC influences at least four key epidermal functions including permeability barrier homeostasis, integrity/cohesion (desquamation), initiation of inflammation, and antimicrobial defense.(44) Therefore, the maintenance of low acidity provides a mechanism that integrates these key functions.(13) Based on this background, Hatano et al(13) suggested that an acidic environment could prevent AD-like skin lesions in an Ox-induced murine model. We also observed similar results of preventive and/or therapeutic effects of topical acidic cream in the Ox-AD murine model (Fig. 4). Moreover, total cell count including eosinophils in the BALF and the expression levels of PAR-2 and

TSLP in the lung tissue decreased significantly in an acidic SC environment made by acidic cream, which revealed the blocking of progression to asthma-like lesions.

Based on our results, we assume that prolonged skin barrier damage induced by repeated Ox challenge provoked the elevation of SC pH. Elevated SC pH increased the expression of PAR-2 and TSLP in the blood and the skin, which contributed to AD-like lesions with elevated IgE and Th2 cytokines. Application of Dp antigen to the AD-like lesions and nasal cavity also induced the respiratory allergy, which was represented by airway eosinophilia and elevated PAR-2 and TSLP levels in the lung tissue. Acidification of SC environment with acidic cream could interrupt this cycle early, thus preventing the atopic march (Fig. 5). In summary, our results show that an atopic march murine model can be developed by Dp application to the Ox-AD mouse model. The acidic SC environment made by application of acidic cream could prevent the progression of atopic march from AD to respiratory allergies, which implies that acidification of the SC could be a novel intervention method to prevent the atopic march.

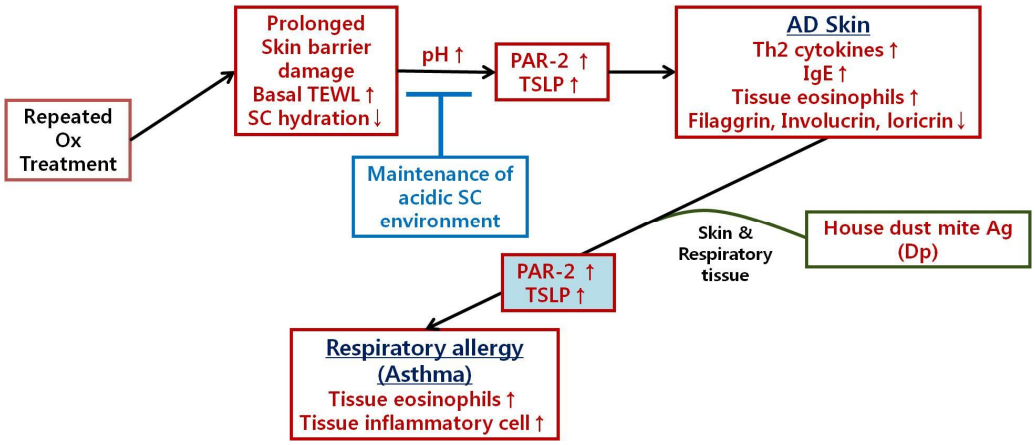

Figure 5. Possible explanation for the development of our atopic march model and intervention

6. References

1. Kubo, A., Nagao, K., and Amagai, M. 2012. Epidermal barrier dysfunction and cutaneous sensitization in atopic diseases. J Clin Invest 122:440-447.

2. Elias, P.M., and Schmuth, M. 2009. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. Curr Opin Allergy Clin Immunol 9:437-446.

3. Gordon, B.R. 2011. The allergic march: can we prevent allergies and asthma? Otolaryngol Clin North Am 44:765-777, xi.

4. Spergel, J.M., and Paller, A.S. 2003. Atopic dermatitis and the atopic march. J Allergy Clin Immunol 112:S118-127.

5. McGrath, J.A., and Uitto, J. 2008. The filaggrin story: novel insights into skin-barrier function and disease. Trends Mol Med 14:20-27.

6. Callard, R.E., and Harper, J.I. 2007. The skin barrier, atopic dermatitis and allergy: a role for Langerhans cells? Trends Immunol 28:294-298.

7. Zheng, T., Yu, J., Oh, M.H., and Zhu, Z. 2011. The atopic march: progression from atopic dermatitis to allergic rhinitis and asthma. Allergy Asthma Immunol Res 3:67-73.

8. Allakhverdi, Z., Comeau, M.R., Jessup, H.K., Yoon, B.R., Brewer, A., Chartier, S., Paquette, N., Ziegler, S.F., Sarfati, M., and Delespesse, G. 2007. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J Exp Med 204:253-258.

9. Bunikowski, R., Mielke, M.E., Skarabis, H., Worm, M., Anagnostopoulos, I., Kolde, G., Wahn, U., and Renz, H. 2000. Evidence for a disease-promoting effect of Staphylococcus aureus-derived exotoxins in atopic dermatitis. J Allergy Clin Immunol 105:814-819.

10. Fluhr, J.W., Behne, M.J., Brown, B.E., Moskowitz, D.G., Selden, C., Mao-Qiang, M., Mauro, T.M., Elias, P.M., and Feingold, K.R. 2004. Stratum corneum acidification in neonatal skin: secretory phospholipase A2 and the sodium/hydrogen antiporter-1 acidify neonatal rat stratum corneum. J Invest Dermatol 122:320-329.

11. Hachem, J.P., Man, M.Q., Crumrine, D., Uchida, Y., Brown, B.E., Rogiers, V., Roseeuw, D., Feingold, K.R., and Elias, P.M. 2005. Sustained serine proteases activity by prolonged increase in pH leads to degradation of lipid processing enzymes and profound alterations of barrier function and stratum corneum integrity. J Invest Dermatol 125:510-520.

12. Fluhr, J.W., Mao-Qiang, M., Brown, B.E., Hachem, J.P., Moskowitz, D.G., Demerjian, M., Haftek, M., Serre, G., Crumrine, D., Mauro, T.M., et al. 2004. Functional consequences of a neutral pH in neonatal rat stratum corneum. J Invest Dermatol 123:140-151.

13. Hatano, Y., Man, M.Q., Uchida, Y., Crumrine, D., Scharschmidt, T.C., Kim, E.G., Mauro, T.M., Feingold, K.R., Elias, P.M., and Holleran, W.M. 2009. Maintenance of an acidic stratum corneum prevents emergence of murine atopic dermatitis. J Invest Dermatol 129:1824-1835.

14. Lee, H.J., Jung, M., Kim, J.H., Yoon, N.Y., and Choi, E.H. 2012. The effect of adipose-derived stem cell-cultured media on oxazolone treated atopic dermatitis-like murine model. Ann Dermatol 24:181-188.

15. Man, M.Q., Hatano, Y., Lee, S.H., Man, M., Chang, S., Feingold, K.R., Leung, D.Y., Holleran, W., Uchida, Y., and Elias, P.M. 2008. Characterization of a hapten-induced, murine model with multiple features of atopic dermatitis: structural, immunologic, and biochemical changes following single versus multiple oxazolone

challenges. J Invest Dermatol 128:79-86.

16. Jung, Y.J., Jung, M., Kim, M., Hong, S.P., and Choi, E.H. 2011. IL-1alpha stimulation restores epidermal permeability and antimicrobial barriers compromised by topical tacrolimus. J Invest Dermatol 131:698-705.

17. Choi, E.H., Brown, B.E., Crumrine, D., Chang, S., Man, M.Q., Elias, P.M., and Feingold, K.R. 2005. Mechanisms by which psychologic stress alters cutaneous permeability barrier homeostasis and stratum corneum integrity. J Invest Dermatol 124:587-595.

18. Shin, Y.S., Sohn, J.H., Kim, J.Y., Lee, J.H., Cho, S.H., Hong, S.J., Lee, J.S., Hong, C.S., and Park, J.W. 2012. Endotoxin is not essential for the development of cockroach induced allergic airway inflammation. Yonsei Med J 53:593-602.

19. Hong, S.P., Oh, Y., Jung, M., Lee, S., Jeon, H., Cho, M.Y., Lee, S.H., and Choi, E.H. 2010. Topical calcitriol restores the impairment of epidermal permeability and antimicrobial barriers induced by corticosteroids. Br J Dermatol 162:1251-1260.

20. Tian, L., Li, W., Wang, J., Zhang, Y., Zheng, Y., Qi, H., Guo, X., Ma, D., Shen, H., and Wang, Y. 2011. The CKLF1-C19 peptide attenuates allergic lung inflammation by inhibiting CCR3- and CCR4-mediated chemotaxis in a mouse model of asthma. Allergy 66:287-297.

21. Briot, A., Deraison, C., Lacroix, M., Bonnart, C., Robin, A., Besson, C., Dubus, P., and Hovnanian, A. 2009. Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. J Exp Med 206:1135-1147.

22. Kubo, A., Nagao, K., Yokouchi, M., Sasaki, H., and Amagai, M. 2009. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction

barriers. J Exp Med 206:2937-2946.

23. Spergel, J.M., Mizoguchi, E., Brewer, J.P., Martin, T.R., Bhan, A.K., and Geha, R.S. 1998. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. J Clin Invest 101:1614-1622.

24. Akei, H.S., Brandt, E.B., Mishra, A., Strait, R.T., Finkelman, F.D., Warrier, M.R., Hershey, G.K., Blanchard, C., and Rothenberg, M.E. 2006. Epicutaneous aeroallergen exposure induces systemic TH2 immunity that predisposes to allergic nasal responses. J Allergy Clin Immunol 118:62-69.

25. He, R., Kim, H.Y., Yoon, J., Oyoshi, M.K., MacGinnitie, A., Goya, S., Freyschmidt, E.J., Bryce, P., McKenzie, A.N., Umetsu, D.T., et al. 2009. Exaggerated IL-17 response to epicutaneous sensitization mediates airway inflammation in the absence of IL-4 and IL-13. J Allergy Clin Immunol 124:761-770 e761.

26. Oyoshi, M.K., Elkhal, A., Scott, J.E., Wurbel, M.A., Hornick, J.L., Campbell, J.J., and Geha, R.S. 2011. Epicutaneous challenge of orally immunized mice redirects antigen-specific gut-homing T cells to the skin. J Clin Invest 121:2210-2220.

27. Zhu, Z., Oh, M.H., Yu, J., Liu, Y.J., and Zheng, T. 2011. The Role of TSLP in IL-13-Induced Atopic March. Sci Rep 1:23.

28. Demehri, S., Morimoto, M., Holtzman, M.J., and Kopan, R. 2009. Skin-derived TSLP triggers progression from epidermal-barrier defects to asthma. PLoS Biol 7:e1000067.

29. Hershko, A.Y., Charles, N., Olivera, A., Alvarez-Errico, D., and Rivera, J. 2012. Cutting edge: persistence of increased mast cell numbers in tissues links dermatitis to enhanced airway disease in a mouse model of atopy. J Immunol 188:531-535.

30. Hatano, Y., Elias, P.M., Crumrine, D., Feingold, K.R., Katagiri, K., and Fujiwara, S. 2011. Efficacy of combined peroxisome proliferator-activated receptor-alpha ligand and glucocorticoid therapy in a murine model of atopic dermatitis. J Invest Dermatol 131:1845-1852.

31. Hatano, Y., Man, M.Q., Uchida, Y., Crumrine, D., Mauro, T.M., Feingold, K.R., Elias, P.M., and Holleran, W.M. 2010. Murine atopic dermatitis responds to peroxisome proliferator-activated receptors alpha and beta/delta (but not gamma) and liver X receptor activators. J Allergy Clin Immunol 125:160-169 e161-165.

32. Liu, Y.J. 2006. Thymic stromal lymphopoietin: master switch for allergic inflammation. J Exp Med 203:269-273.

33. Park, S.Y., Gupta, D., Kim, C.H., and Dziarski, R. 2011. Differential effects of peptidoglycan recognition proteins on experimental atopic and contact dermatitis mediated by Treg and Th17 cells. PLoS One 6:e24961.

34. Elias, P.M. 2010. Therapeutic implications of a barrier-based pathogenesis of atopic dermatitis. Ann Dermatol 22:245-254.

35. Howell, M.D., Kim, B.E., Gao, P., Grant, A.V., Boguniewicz, M., DeBenedetto, A., Schneider, L., Beck, L.A., Barnes, K.C., and Leung, D.Y. 2009. Cytokine modulation of atopic dermatitis filaggrin skin expression. J Allergy Clin Immunol 124:R7-R12.

36. Lee, S.E., Jeong, S.K., and Lee, S.H. 2010. Protease and protease-activated receptor-2 signaling in the pathogenesis of atopic dermatitis. Yonsei Med J 51:808-822.

37. Schmidlin, F., Amadesi, S., Dabbagh, K., Lewis, D.E., Knott, P., Bunnett, N.W., Gater, P.R., Geppetti, P., Bertrand, C., and Stevens, M.E. 2002. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. J Immunol 169:5315-5321.

38. Gu, Q., and Lee, L.Y. 2012. House dust mite potentiates capsaicin-evoked Ca2+ transients in mouse pulmonary sensory neurons via activation of protease-activated receptor-2. Exp Physiol 97:534-543.

39. Ebeling, C., Forsythe, P., Ng, J., Gordon, J.R., Hollenberg, M., and Vliagoftis, H. 2005. Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. J Allergy Clin Immunol 115:623-630.

40. Kouzaki, H., O'Grady, S.M., Lawrence, C.B., and Kita, H. 2009. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. J Immunol 183:1427-1434.

41. Lee, S.W., Park, H.J., Park, S.H., and Hong, S. 2013. Oral administration of poly-gamma-glutamic acid prevents the development of atopic dermatitis in NC/Nga mice. Exp Dermatol 22:561-563.

42. Fujii, M., Nakashima, H., Tomozawa, J., Shimazaki, Y., Ohyanagi, C., Kawaguchi, N., Ohya, S., Kohno, S., and Nabe, T. 2013. Deficiency of n-6 polyunsaturated fatty acids is mainly responsible for atopic dermatitis-like pruritic skin inflammation in special diet-fed hairless mice. Exp Dermatol 22:272-277.

43. Kim, J.Y., Jeong, M.S., Park, K.Y., and Seo, S.J. 2013. Aggravation of atopic dermatitis-like symptoms by consecutive low concentration of formaldehyde exposure in NC/Nga mice. Exp Dermatol 22:219-221.

44. Elias, P.M. 2005. Stratum corneum defensive functions: an integrated view. J Invest Dermatol 125:183-200.

II. Part 2. Atopic march from atopic dermatitis to asthma-like lesions in NC/Nga mice is accelerated or aggravated by neutralization of stratum corneum but partially inhibited by acidification

1. Abstract

Prolonged and/or repeated damage to the skin barrier followed by atopic dermatitis (AD) is an initial step in atopic march that ultimately progresses to respiratory allergy. Maintaining normal SC acidity has been suggested as a therapeutic or preventive strategy for barrier impairment caused by skin inflammation. We determined whether a representative AD murine model, NC/Nga mice, develops airway inflammation after repeated epicutaneous application followed by inhalation of house dust mite (HDM), implying atopic march, and whether prolongation of non-proper SC acidity accelerates respiratory allergy. HDM was applied to the skin of NC/Nga mice, accompanied by the application of neutral cream (pH 7.4) or acidic cream (pH 2.8) for 6 weeks. Intranasal inhalation of HDM was administered daily during the last 3 days. Repeated epicutaneous applications followed by inhalation of HDM in NC/Nga mice induced an atopic march-like progression from AD lesions to respiratory allergy. Concurrent neutral cream treatment aggravated the allergic inflammation in the skin and respiratory system, whereas an acidic cream partially alleviated these symptoms. Collectively, we developed an atopic march in NC/Nga mice by HDM application, and found that prevention of a neutral environment in the SC may be an interventional method to inhibit the march.

2. Introduction

Atopic dermatitis (AD), which is one of the most common chronic inflammatory dermatosis, is characterized by an impaired epidermal barrier, dry skin, pruritus, eczematous lesions, abnormal immune response, and IgE-mediated allergy to various exogenous antigens (Lee and Lee, 2014). The clinical symptoms of AD generally precede the development of asthma and allergic rhinitis. This finding suggests that AD could be a doorway to the successive events of allergic diseases, the so called 'atopic march' (Gordon, 2011; Spergel and Paller, 2003). Researchers have suggested that the occurrence of asthma in AD patients could result from enhanced sensitization of allergens through a defective skin barrier, enabling allergens to penetrate the skin easily, and subsequently to come in contact with to contact with epidermal Langerhans cells (LCs), antigen presenting cells (Callard and Harper, 2007; Zheng et al., 2011). After capturing and processing the penetrated exogenous allergens, LCs move to regional lymph nodes and present the allergens to naïve T cells, which leads to systemic allergy by encouraging Th2 inflammation (Allakhverdi et al., 2007). Besides, thymic stromal lymphopoietin (TSLP) secreted by the keratinocytes of disrupted epidermis activates polyclonal T cells. These T cells, in turn, induce excessive Th2 inflammatory responses, which could aggravate the AD and trigger the systemic Th2 responses including respiratory allergies (Laouini et al., 2003).

The acidity (pH) of the stratum corneum (SC) affects the epidermal key functions including permeability barrier homeostasis, SC integrity and cohesion, initiation of inflammation, processing of the secreted lamellar body (LB) lipids, and antimicrobial defenses (Hachem et al., 2010, Elias et al., 2015). The skin surface of AD lesions exhibits an increased pH, which causes bacterial growth and results in allergic inflammation and

worsening of AD. In a hapten-induced animal model of AD, simple acidification of SC significantly blocks the progression of barrier impairment and the downstream immune abnormalities (Hatano et al., 2009). These studies give us a clue to possible novel modalities that might prevent progression of the atopic march (Lee et al., 2014).

Acidic microdomain was observed within the extracellular matrix of the SC by two-photon fluorescence lifetime imaging (FLIM). The mean pH of the SC increased with depth due to an increase in the ratio of neutral to acidic regions within the SC. FLIM definitely showed that the existence of aqueous acidic pockets within the lipid-rich extracellular matrix leads to the acid mantle of the SC (Hanson et al., 2002). It also revealed that SC acidification occurs through the progressive accumulation of acidic microdomains (Behne et al., 2002). These results give us a clue that surface pH directly reflects changes in the extracellular matrix of the SC as an acidic pH microdomain in the SC. The abnormal acidification demonstrated by FLIM is related to decreased Na+/H+ antiporter (NHE1) expression in the epidermis of moderately aged mice. Diminished NHE1 levels cause the elevation of SC pH, which contributes to suboptimal activation of β-glucocerebrosidase, leading to defective maturation of lipid processing and lamellar membranes in turn. These abnormalities were normalized by exogenous acidification of the SC (Choi et al., 2007).

The NC/Nga mouse has a mutation on chromosome 9, which is related to the increased IgE production and a Th2 dominant inflammation. The skin lesions arise spontaneously or secondarily to exposure of various aeroallergens, which are similar to AD in humans. The mice also present abnormalities of skin barrier functions, such as increased basal transepidermal water loss (TEWL) and abnormal SC hydration under conventional conditions, and diminished ceramide metabolism (Aioi et al., 2001). In addition, exposure

to a standard environment alone is not enough to reveal AD-like symptoms in these mice; however, epicutaneous stimulation with certain environmental allergens (e.g., house dust mite allergens) provokes AD-like symptoms (Kim et al., 2014).

Based on previous reports, we first hypothesized that NC/Nga mice, as a murine model of inherited AD, would establish respiratory inflammation after epicutaneous treatment followed by subsequent inhalation of house dust mite (HDM), similar to the model of the atopic march. Second, we hypothesized that the atopic march could be interrupted by maintaining SC acidity or accelerated by neutralization of the SC.

3. Materials and methods

Animals and Materials

All animal procedures were approved by the Yonsei University Wonju Campus Institutional Animal Care and Use Committee (IACUC). Twenty-four female NC/Nga mice (5 weeks old) were purchased from SLC Japan (Shizuoka, Japan). Mice were kept under conditions of controlled humidity (40%) and temperature (22 ± 2°C). Extracted antigen of Dermatofagoides pteronyssinus (Dp), a HDM, was purchased from the Department of Environmental Medical Biology, Yonsei University (Seoul, Korea). All laboratory measurements were performed under blinded conditions. An acidic cream (pH 2.8) was made by the addition of citric acid (Sigma-Aldrich; St Louis, MO, USA) to vehicle cream. A neutral cream (pH 7.4) was made by neutralization of the acidic cream with sodium hydroxide (NaOH). The vehicle was plain Cetaphil® cream with a pH of 5.5 (Galderma, Lausanne, Switzerland), as previously described (Jung et al., 2011).

Development of an atopic march model in NC/Nga mice by epicutaneous application and subsequent nasal inhalation of Dp, application of acidic or neutral cream, and measurement of skin barrier function including surface pH

Five-week-old NC/Nga mice and BALB/c mice (as a control) were used in this study. Once a week the mice were anesthetized and the hair on their dorsal skin was shaved. Dp antigen (Ag) was mixed with petrolatum to make a 0.5% ointment. Four percent sodium lauryl sulfate (SLS) and 0.5% Dp Ag were applied to the dorsal skin of the NC/Nga mice (NC/Nga + Dp) and the control mice (BALB/c + Dp) twice a week for 6 weeks (a total of 12 applications). During the last 3 days of the experiment, 100 μg of Dp Ag was given daily by intranasal inhalation (Lee et al., 2014). While Dp Ag was being applied

epicutaneously, 200 mg of acidic cream (pH 2.8) was applied twice a day on the same skin sites for 45 days (acidic cream group). For the non-treated control groups (BALB/c), no cream was applied. For the neutral cream group, neutralized cream (pH 7.4) was applied twice a day (Fig. 6). After a total of 46 days of epicutaneous and intranasal application with Dp Ag, the gross appearance and barrier functions of the skin, including basal TEWL and SC hydration, skin surface pH, and the degree of allergic inflammation of the skin, blood, and respiratory system were evaluated in all mice. Surface pH was measured with pH meter (WTW, Weilheim, Germany). The Basal TEWL was measured with the Tewameter TM210 (Courage and Khazaka, Cologne, Germany), and SC hydration was assessed as capacitance with the Corneometer CM820 (Courage and Khazaka) 24 h after the final application of Dp, that is 12 h after the final application of cream, as described previously (Choi et al., 2005; Lee et al., 2014). Twenty-four hours after the last treatment of Dp, specimens of skin, blood, and lung were collected to evaluate the degree of allergic inflammation as measured by mRNA and protein expression. In addition, in order to evaluate the maintenance time of lowered or elevated pH by application of acidic or neutral cream, skin surface pH was measured at pre-treatment and 0 h, 1 h, 3 h, 6 h, 9 h and 12 h right after the application of acidic (pH 2.8) or neutral (pH 7.4) creams as well as vehicle (pH 5.5) cream. (each group, n=4)

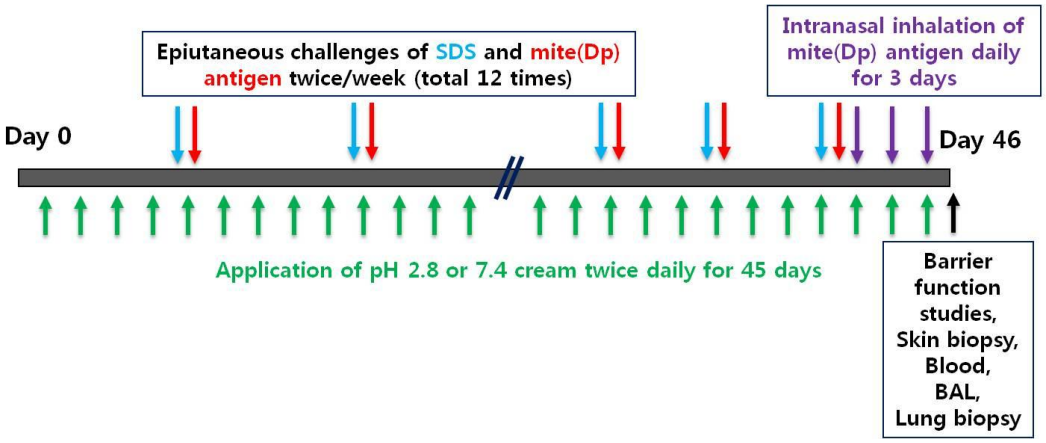

Figure 6. Establishment of a model of atopic march in NC/Nga mice by epicutaneous application and subsequent nasal inhalation of HDM, and the intervention by topical acidic or neutral cream.

Evaluation of macroscopic AD-like skin lesions

Scoring of dermatitis was assessed macroscopically in a blinded fashion using the following scoring procedure. The development of (i) erythema, (ii) edema, (iii) excoriation, (iv) scale, and (v) lichenification was each scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The total symptom severity score was defined as the sum of the individual scores.

Tissue preparation, immunohistochemistry, and immunofluorescence

Immunohistochemical staining was performed to assess epidermal differentiation and inflammation. Briefly, 5-μm thick paraffin sections were incubated with primary antibodies against epidermal differentiation marker proteins such as filaggrin, involucrin, and loricrin, protease-activated receptor-2 (PAR-2), and thymic stromal lymphopoietin (TSLP) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After three cycles of washing, the sections were incubated with the appropriate secondary antibody for 30 min. Staining was detected with the ABC-peroxidase kit (Vector Lab, Burlingame, CA, USA) and counterstaining with hematoxylin was performed. To quantify the density of immunohistochemical staining, three pictures from each section were taken at 100x magnification and labeled. An investigator without any information on the specimen ranked the pictures in order of the intensity of immunostaining (the highest intensity received the first rank). Representative images of each group are provided.

Enzyme-linked immunoassay (ELISA) for serum IgE and TSLP

Serum levels of IgE and TSLP were determined using ELISA kits for mouse IgE (Koma

Biotech, Seoul, Korea) and mouse TSLP (R&D systems, Minneapolis, MN, USA). To detect serum IgE levels, whole blood samples were centrifuged at 5,000 rpm for 5 min at room temperature (RT). The sample serum and standard solutions were added to plates that were pre-coated with specific antibodies for IgE and TSLP, and incubated for 1 h at RT. Horseradish peroxidase-conjugated mouse secondary detection antibodies were added to each well and incubated for 1 hour at RT. TMB substrate was added to each well and incubated for 20 min for color development. A sulfuric acid solution was added to each well to stop the reaction and the enzyme-substrate reaction was measured using a microtiter plate reader (BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 450 ± 2 nm. After triplicate measurements, the concentrations of IgE and TSLP in the samples were determined by comparison with a standard curve.

Bronchoalveolar lavage (BAL)

BAL was performed as previously described.(Lee et al., 2014) After each mouse was anesthetized, the trachea was cannulated and the lung was lavaged with 1 mL of Hank's balanced salt solution via the intubation tube. Bronchoalveolar lavage fluid (BALF) was obtained by two rounds of aspiration. Total numbers of cells were counted and cell smear preparations were made. Cells were stained with Wright–Giemsa stain and eosinophils, lymphocytes, macrophages, and neutrophils were counted and expressed as a percentage of 400 total cells. The counts of eosinophils, lymphocytes, macrophages, and neutrophils in the BALF were determined by multiplying their percentages in total cells.

Total RNA preparation and cDNA synthesis with blood and lung tissue, and Quantitative PCR analysis of gene expression

Total RNA preparation and cDNA synthesis were performed as previously described (Jung et al., 2011; Lee et al., 2012). Expression of specific mRNAs was quantified using a Rotor-Gene™ 3000 (Corbett Life Science, Brisbane, Australia), as in our previous reports (Hong et al., 2010; Jung et al., 2011; Lee et al., 2012). Briefly, 10-μl PCR reactions were set up containing Quantitect SYBR green PCR kit Master mix (Qiagen, Hilden, Germany) in a 2X solution, 8 mM manganese chloride, 200 μM deoxynucleotide triphosphates (dNTPs), 1.25 units Hotstart Taq polymerase, and 0.5 pM/μl each of probes and primers. Approximately 60 ng of cDNA was used per reaction. Primers for PAR-2 and TSLP were designed based on GeneBank sequences using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/, Massachusetts Institute of Technology, Cambridge, MA). All reactions included GAPDH as a housekeeping gene. Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacturer's guidelines and were used to determine ΔCt values (Ct of target gene−Ct of housekeeping gene) as raw data for gene expression. Changes in gene expression were determined by subtracting ΔCt values for samples and acidic cream-treated samples from their respective control samples. The resulting ΔΔCt values were used to calculate fold change in gene expression as 2-ΔΔCt. All reactions were performed in triplicate, and the results are expressed as the mean of values from three separate experiments. Samples were amplified under the following conditions: 95°C for 15 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Statistical analysis

All data were expressed as mean ± SEM. ANOVA or Kruskal-Wallis test were

performed when three or more groups were compared or to compare the density of immunohistochemical staining. For individual comparison, Student's unpaired t-test was used in case of normal distribution, and Mann-Whitney test was used in case of non-normal distribution or ranking variables. *$p<0.05$ was regarded as statistically significant. Statistical analysis was conducted using SPSS software 10.1 (SPSS, Chicago, IL).

4. Results

Repeated epicutaneous application of HDM extract provokes remarkable AD-like skin lesions and barrier dysfunction in NC/Nga mice, a representative murine model of inherited AD, which are aggravated by a neutral cream.

Repeated epicutaneous application of a HDM, Dermatofagoides pteronyssinus (Dp) antigen provoked remarkable AD-like skin lesions with a high eczema score in the NC/Nga mice but not in the control mice (BALB/c mice). BALB/c mice did not exhibit eczema lesions despite epicutaneous treatment of SLS and Dp antigen. Treatment with SLS alone to NC/Nga mice also induced eczema lesions. Moreover, application of a neutral cream with Dp extract (NC/Nga SLS + Dp + pH 7.4 cream) resulted in more prominent eczema lesions compared to other NC/Nga treatment groups. Time course of skin surface pH after the application of creams on the skin of NC/Nga mice revealed that skin surface pH is maintained by acidic or neutral cream for approximately 6 hours after application of the creams (Fig. 7a). Application of an acidic cream (NC/Nga SLS + Dp + pH 2.8) alleviated eczema lesions in Dp-treated NC/Nga mice, but this effect was not significant (Fig. 7b). Post-treatment basal TEWL was increased and SC hydration was significantly decreased in the group treated with neutral cream (NC/Nga SLS + Dp + pH 7.4 group; Fig. 7c and d). However, there was no significant difference in skin surface pH among all groups, measured 24 h after the final application of Dp, that is 12 h after the final application of cream (Fig. 7e).

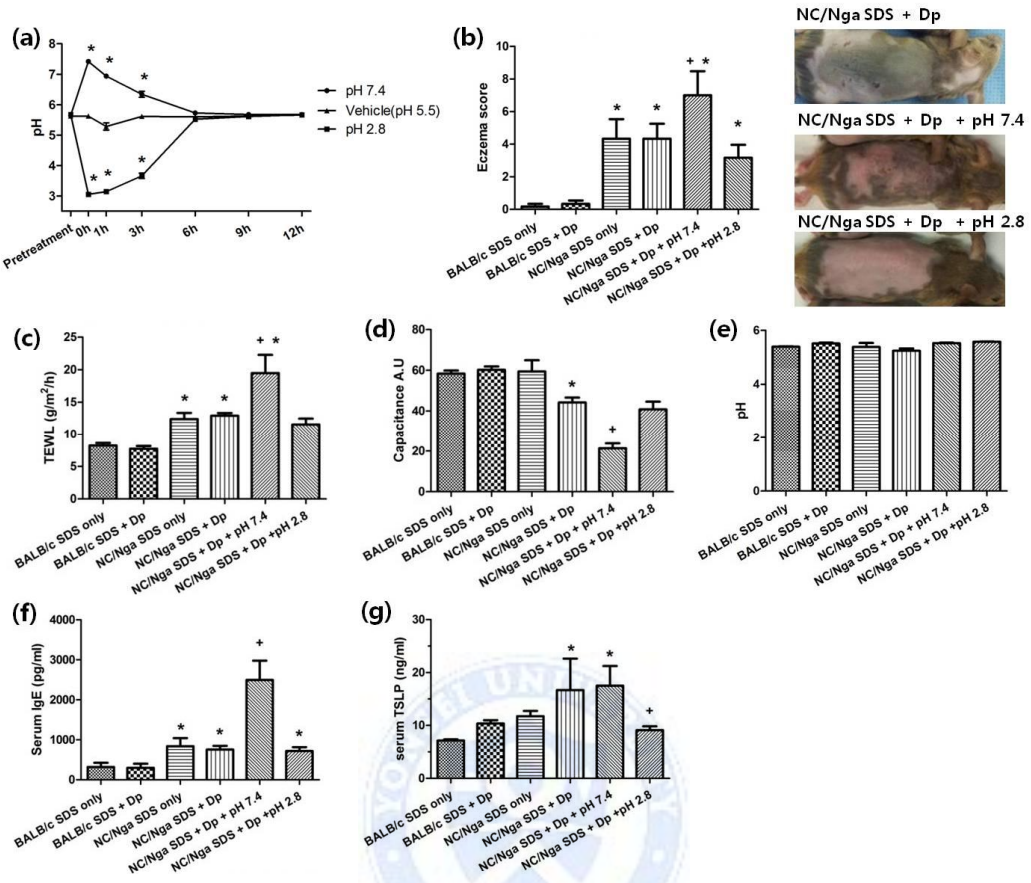

Figure 7. Repeated epicutaneous application of HDM (Dp) extract followed by nasal inhalation provokes remarkable AD-like lesions and barrier disruption in NC/Nga mice. Symptoms were aggravated by neutral cream, but neither aggravated nor improved by acidic cream. Systemic allergic inflammation was aggravated by neutral cream but partially improved by acidic cream. (a) Time course of skin surface pH after application of acidic or neutral cream (n=4, each group). (b) Eczema scores and gross appearance, (c) basal TEWL, (d) SC hydration, (e) skin surface pH, (f) serum IgE, and (g) TSLP after Dp treatment were compared among treatment groups. ANOVA showed $p<0.05$ in all measurements except (e) surface pH. Results are mean ± SEM. *$p < 0.05$, compared to control (BALB/c SLS+Dp); +$p<0.05$, compared to NC/Nga SLS+Dp.

Systemic and cutaneous allergic inflammation and abnormal epidermal differentiation induced in NC/Nga mice are aggravated by neutral cream but partially improved by acidic cream.

After treatment, serum total IgE levels significantly increased in all of the NC/Nga mice compared to control BALB/c mice. Among NC/Nga mice, mice that were treated with neutral cream showed the most prominent increase in serum IgE. In contrast, acidic cream did not decrease serum IgE in NC/Nga mice treated with SLS and Dp antigen (Fig. 7f). Post-treatment serum TSLP was significantly increased in the NC/Nga SLS+Dp group. Mice treated with neutral cream (NC/Nga SLS + Dp + pH 7.4) showed an increase in serum TSLP similar to that of the NC/Nga SLS + Dp group. Application of acidic cream significantly decreased serum TSLP in the NC/Nga SLS + Dp group (Fig. 7g).

Also, the number of tissue eosinophils was noticeably increased in the skin of NC/Nga SLS + Dp + pH 7.4 group (Fig. 8a). Semiquantitative analysis of the expression of epidermal differentiation markers (filaggrin, involucrin, and loricrin) by immunohistochemical staining showed that their expression was significantly decreased in the NC/Nga SLS + Dp group compared to the control BALB/c SLS + Dp group (Fig. 8b). Application of neutral cream to the NC/Nga SLS + Dp group significantly decreased expression of these markers, whereas acidic cream significantly increased their expression. Semiquantitative analysis demonstrated that epidermal expression of protease activated receptor - 2 (PAR-2) and TSLP significantly increased in the NC/Nga SLS + Dp group compared to the control BALB/c SLS + Dp group (Fig. 8b). Application of neutral cream to the NC/Nga SLS + Dp group significantly increased expression of PAR-2 and TSLP, whereas acidic cream significantly decreased their expression.

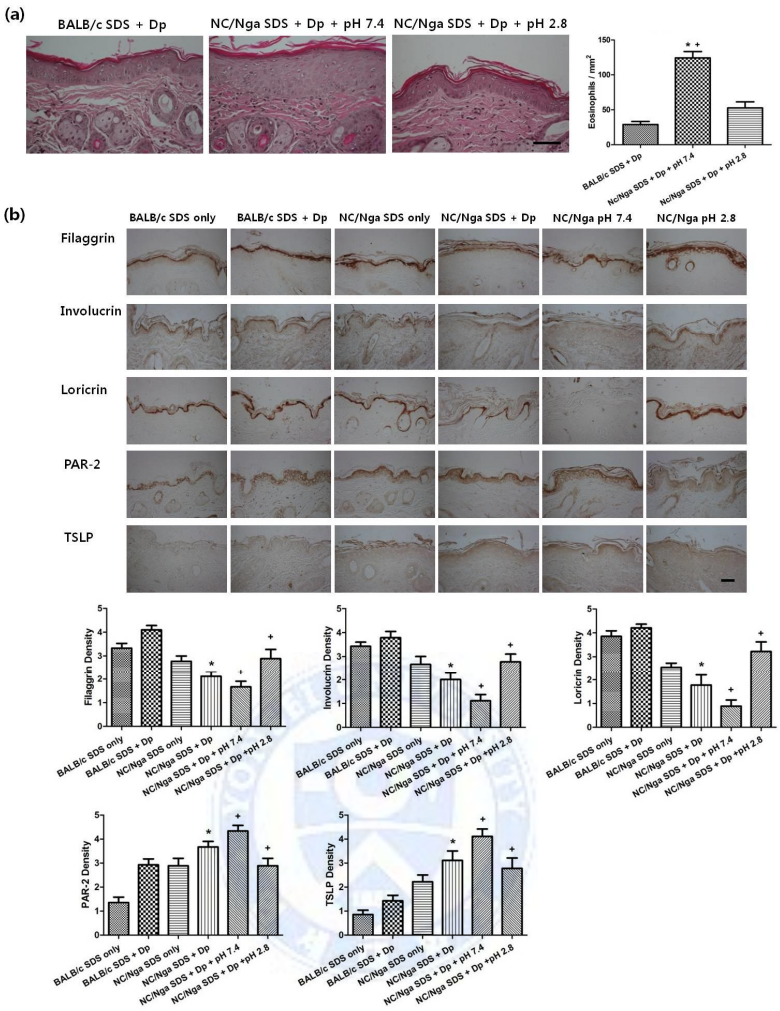

Figure 8. Cutaneous allergic inflammation and abnormal epidermal differentiation induced in NC/Nga mice are aggravated by neutral cream but partially improved by acidic cream. (a) Tissue eosinophils were counted with H&E staining, and (b) expression of epidermal differentiation markers (filaggrin, involucrin, loricrin), PAR-2, and TSLP were measured by immunohistochemical staining in skin specimens taken after repeated epicutaneous treatment and nasal inhalation of Dp. (a) ANOVA and (b) Kruskal-Wallis test showed $p<0.05$ in all measurements. Results are mean ± SEM. *$p<0.05$, compared to control (BALB/c SLS + Dp); +$p<0.05$, compared to NC/Nga SLS + Dp. (n=6, each group)

Repeated epicutaneous application followed by nasal inhalation of HDM extract produces asthma-like airway inflammation and AD-like skin lesions in NC/Nga mice, implying atopic march; these symptoms are aggravated by neutral cream and partially improved by acidic cream.

Because airway eosinophilia is a characteristic manifestation of asthma (Tian et al., 2011), we performed bronchoalveolar lavage (BAL) after 3 consecutive days of nasal inhalation of Dp. After treatment, the total cell count of the BAL was significantly increased in the NC/Nga SLS + Dp group; this increase was aggravated by treatment with neutral cream (NC/Nga SLS + Dp + pH 7.4 group) and decreased by application of the acidic cream with statistical significance (Fig. 9a). The number of eosinophils, macrophages, and neutrophils were significantly increased in the NC/Nga SLS + Dp group, similar to the results of total cell count (Fig. 9b). Quantitation of mRNA and immunohistochemical staining revealed increased expression of PAR-2 and TSLP in the lung tissue of the NC/Nga SLS + Dp group, which was further aggravated by application of neutral cream application and decreased by acidic cream with statistical significance (Fig. 9c, d and e).

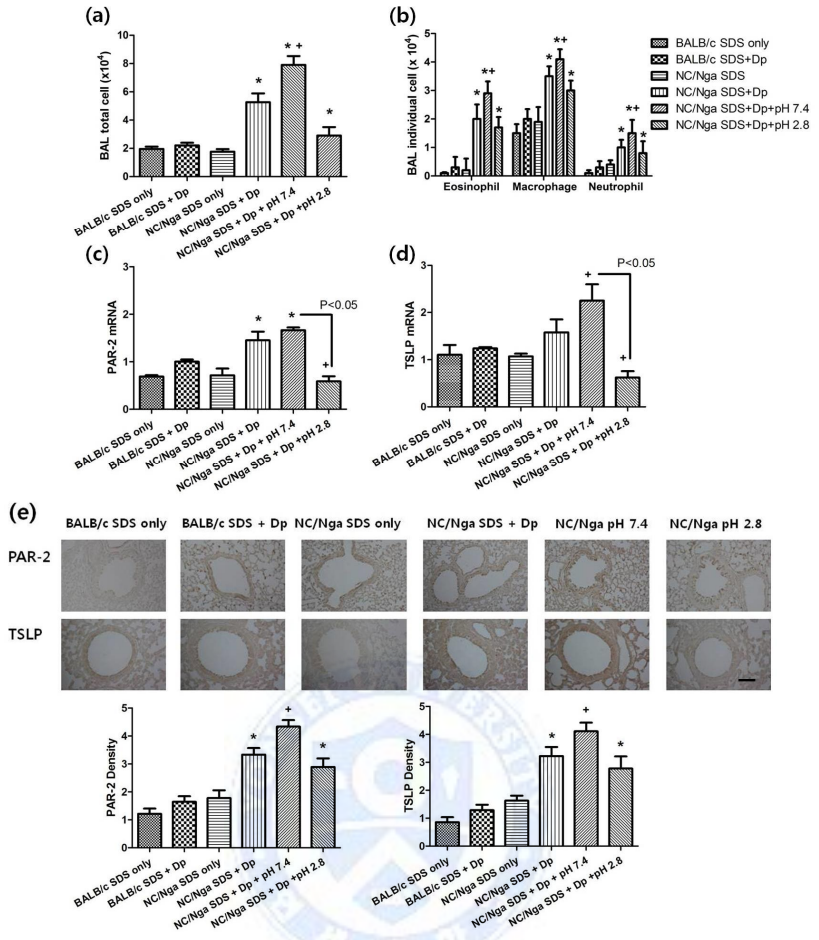

Figure 9. Repeated epicutaneous application of HDM (Dp) extract followed by nasal inhalation produces asthma-like inflammatory response as well as AD lesions in NC/Nga mice, implying atopic march. Symptoms are aggravated by neutral cream, but partially improved by acidic cream. BAL, RQ-PCR for mRNA expression, and immunohistochemical staining of PAR-2 and TSLP were performed in the lung tissue after Dp treatment. (a) Total cells and (b) individual cell count in the BAL, and (c, d) mRNA and (e) expression of PAR-2 and TSLP in the lung tissue were measured. (a-d) ANOVA and (e) Kruskal-Wallis test showed $p<0.05$ in all measurements. Results are mean ± SEM. *$p<0.05$, compared to control (BALB/c SLS + Dp); +$p<0.05$, compared to NC/Nga SLS + Dp and NC/Nga SLS + Dp + pH 2.8. (n=6, each group)

5. Discussion

Since Strid et al. suggested the idea of a skin barrier defect initiating allergic sensitization and showed its mechanism in mice (Strid et al., 2006), the theoretical model described by Kubo et al. (Kubo et al., 2012) has been considered applicable to the atopic march pathogenesis. Break down of the epidermal barrier activates the intrinsic proteases and permits the penetration of protease-active allergens, which could activate Toll-like receptors and PAR-2 on keratinocytes (Briot et al., 2009). The keratinocytes subsequently produce proinflammatory cytokines including IL-1, TNF-α, and TSLP. Along with the disruption of SC, the activated LCs extend their dendrites through the weakened tight junctions and attach to the penetrating allergens (Kubo et al., 2009). Such percutaneous sensitization of exogenous allergens, followed by repeated challenges from various routes, including lung, nasal cavities, and gastrointestinal tract, is thought to develop the atopic march. In our study, only epicutaneous treatment and nasal inhalation of HDM with no disruption of permeability barrier in the control group of Balb/c mice did not demonstrate such remarkable inflammatory lesions in the skin, as well as the lung (Figs. 7-9). This result suggests that the uptake of active allergens and the activation of LCs did not take place when the epidermal barrier was not disrupted, therefore there was no increase in the levels of cytokines and TSLP. It is well known that the expression of TSLP is highly increased in lesional skin and the blood of patients with AD, and that this elevation in TSLP expression is sufficient to activate LCs during AD development (Kubo et al., 2012; Liu, 2006).

There have been many attempts to develop animal models of atopic march. Although all of the results present asthma-like lung lesions as well as AD-like skin lesions, intraperitoneal injections of ovalbumin were used for sensitization rather than

epicutaneous application via a disrupted barrier (Li, 2014). To develop asthma-like lung lesions, HDM antigen was treated to AD lesions presenting a broken barrier before the nasal inhalation in NC/Nga mice, a murine model of inherited AD. This provided a more natural and physiologic mechanism for allergen sensitization compared to previous sensitization methods. Recently, we reported that a murine model of acquired AD induced by repeated hapten (oxazolone) application progresses to asthma-like lesions under more natural and physiologic mechanisms (Lee et al., 2014).

The NC/Nga mouse shows an abnormal skin permeability barrier, which presents increased TEWL and abnormal skin hydration. These are combined with Th2 inflammatory responses and impaired ceramide metabolism even in normal condition (Matsuda et al., 1997). Our results show that the eczema score was increased in the group of NC/Nga mice treated with SLS only, which confirms that NC/Nga mice have inherited abnormalities in skin barrier functions. Their AD-like skin changes are suggested to be independent of IgE and Th2 reaction, as the skin lesions developed in STAT6-deficient NC/Nga mice with undetectable IgE serum levels (Yagi et al., 2002). Although the Th2 response may not be necessary for AD development in NC/Nga mice, additional applications of SLS and HDM may cause earlier barrier disruption and allergen exposure, which could contribute to the Th2 immune response. Therefore, in our study a greater increase in eczema scores was associated with poorer barrier functions and a greater increase in serum TSLP and IgE.

In AD lesions, filaggrin expression is downregulated by Th2 cytokines such as IL-4, IL-13, IL-22, and IL-25 regardless of FLG gene mutations and the decrease in NMF is dependent on the severity of lesional inflammation, rather than the FLG mutation (Jung et al., 2014; Novak, 2012). In addition, epidermal differentiation in AD lesions is

incomplete (Elias, 2010). In this atopic march-like model using NC/Nga mice, the expression of filaggrin, involucrin and loricrin decreased after multiple epicutaneous applications of HDM antigens. Repeated application of HDM antigen to the NC/Nga mouse model aggravated AD skin lesions and then severely decreased filaggrin expression, which could disrupt the skin barrier and eventually permit skin permeation of exogenous antigens including HDM (Fig. 8b). This process may also result in the asthma-like lesions.

PAR-2 and TSLP expression has been demonstrated in various tissues in addition to skin and lung, which are also involved in the occurrence of AD and respiratory allergy (Ebeling et al., 2005; Gu and Lee, 2012; Lee et al., 2014). TSLP expression is induced in airway epithelial cells following exposure to allergen-derived proteases, and PAR-2 plays a role in this process (Kouzaki et al., 2009). In our study, repeated HDM application to damaged skin increase the levels of TSLP, IgE, or PAR-2 in the systemic circulation or lung tissue (Fig. 9). Based on our results, we presume that HDM-induced asthma could occur more easily when the skin barrier is more severely impaired and AD lesions are more severe. These conditions provoke an increase in systemic TSLP and IgE as well as eosinophils, whereas HDM application alone does not easily induce asthma. Systemic TSLP levels may affect the PAR-2 and TSLP in distant epithelial organs such as the lung, which might be readily affected by the HDM allergen.

There are controversies as to whether filaggrin deficiency per se increases the SC pH or not (Ohman et al., 1998; Fluhr et al., 2010; Vávrová et al., 2014). However, decrease in filaggrin degradation products in AD lesions might contribute to an increase in the SC pH high enough to induce serine proteases (SP) activation in the SC which optimally acts under neutral to alkaline pH (Brattsand et al., 2005). An elevated SC pH also increases

the activity of SP such as kallikrein 5 (KLK5) and KLK7 (stratum corneum chymotryptic enzyme), resulting in overdegradation of corneodesmosomes (CD) and decrease of SC integrity and cohesion (Lee et al., 2010). On the other hand, the elevation of SC pH decreases the activity of lipid-processing enzymes, which are indispensable for the production of free fatty acids and ceramide in the SC, and thus impairs lipid processing and eventually disrupts the permeability barrier of epidermis (Hachem et al., 2010; Hatano et al., 2009).

Several reports present some indirect evidence that acidification of SC prevents the penetration of the SC by external allergens including HDM. This evidence was demonstrated with ultrastructural changes of SC and SG shown by fine EM pictures (Hachem et al., 2003, 2005, 2010; Hatano et al., 2009). Therefore, we believe that allergens from Dp extract with high molecular weight over 18,000 dalton (Lind, 1985) only limitedly penetrate the skin barrier which is partially improved by SC acidification in NC/Nga mice. Hatano et al. reported that an acidic environment can inhibit the formation of AD-like skin lesions in the hapten-induced AD model (Hatano et al., 2009). We previously reported that acidification of SC inhibits the development of both asthma-like lesions and AD-like lesions by HDM in the murine model (Lee et al., 2014). In the present study using NC/Nga mice, we observed an aggravating or harmful effect of neutral cream and a partial preventive effect of acidic cream on the atopic march. A previous study already confirmed that acidic treatment (pH 2.0 with 2M HCl) to HDM (Dp) preserved the allergenicity and antigenicity of Dp. No significant differences were observed in the antigenic profile of native and acid treated extracts (Fernández-Caldas et al., 2008). The difference in the degree of response might reflect differences in skin structure between these mouse models. Compared to hairless mice, NC/Nga mice with

abundant terminal hairs have a thinner SC and weaker skin barrier function. Also, the transient receptor potential vanilloid type 1(TRPV1) channel, which is activated by diverse noxious stimuli including capsaicin, low pH or heat, is deeply associated with permeability barrier function. Blockade of its activation accelerates barrier recovery and alleviates AD-like symptoms in NC/Nga mice and Ox-AD mice (Yun et al., 2011). Therefore, we assume that the acidic cream might more easily activate TRPV1 in the epidermis of NC/Nga mice with relatively thinner SC compared to hairless mice. Another considerable factor is that AD-like skin changes in NC/Nga mice are IgE/Th2-independent, which is different with AD lesions of hairless mice and humans (Yagi et al., 2002). This immunologic difference might be the cause of the partial or minimal pH-dependent preventative effects in the NC/Nga mice, and might also explain the lack of statistical difference in surface skin pH among the groups. In addition, skin surface pH was maintained approximately for 6 hours after application of the creams, but the creams were applied every 12 hours (Fig. 7a). Further study is needed to evaluate whether the acidic cream is more effective if applied every 6 hours.

In summary, we demonstrate that a murine model of atopic march can be developed by application of HDM in NC/Nga mice. Maintenance of a neutral skin environment aggravates barrier function and AD lesions which in turn, eventually, accelerates the atopic march. Therefore, prevention of a neutral environment on the SC may be an intervention to inhibit the progression of AD to respiratory allergies, an atopic march.

6. References

Aioi A, Tonogaito H, Suto H, et al. (2001) Impairment of skin barrier function in NC/Nga Tnd mice as a possible model for atopic dermatitis. Br J Dermatol 144:12-8.

Allakhverdi Z, Comeau MR, Jessup HK, et al. (2007) Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J Exp Med 204:253-8.

Behne MJ, Barry NP, Hanson KM, et al. (2002) NHE1 regulates the stratum corneum permeability barrier homeostasis. Microenvironment acidification assessed with fluorescence lifetime imaging. J Biol Chem 277:47399-406.

Brattsand M, Stefansson K, Lundh C, et al. (2005) A proteolytic cascade of kallikreins in the stratum corneum. J Invest Dermatol 124:198-203.

Briot A, Deraison C, Lacroix M, et al. (2009) Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. J Exp Med 206:1135-47.

Callard RE, Harper JI (2007) The skin barrier, atopic dermatitis and allergy: a role for Langerhans cells? Trends Immunol 28:294-8.

Choi EH, Brown BE, Crumrine D, et al. (2005) Mechanisms by which psychologic stress alters cutaneous permeability barrier homeostasis and stratum corneum integrity. J Invest Dermatol 124:587-95.

Choi EH, Man MQ, Xu P, et al. (2007) Stratum corneum acidification is impaired in moderately aged human and murine skin. J Invest Dermatol 127:2847-56.

Ebeling C, Forsythe P, Ng J, et al. (2005) Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. J Allergy Clin Immunol 115:623-30.

Elias PM (2010) Therapeutic implications of a barrier-based pathogenesis of atopic dermatitis. Ann Dermatol 22:245-54.

Elias PM (2015) Stratum corneum acidification: how and why? Exp Dermatol 24:179-80.

Fernández-Caldas E, Gallego M, Carnés J, et al (2008) Enzymatic activity of Dermatophagoides pteronyssinus extracts after acidic treatment. Int Arch Allergy Immunol 145:298-304

Fluhr JW, Elias PM, Man MQ, et al. (2010) Is the filaggrin-histidine-urocanic acid pathway essential for stratum corneum acidification? J Invest Dermatol 130: 2141-4.

Gordon BR (2011) The allergic march: can we prevent allergies and asthma? Otolaryngol Clin North Am 44:765-77, xi.

Gu Q, Lee LY (2012) House dust mite potentiates capsaicin-evoked Ca2+ transients in mouse pulmonary sensory neurons via activation of protease-activated receptor-2. Exp Physiol 97:534-43.

Hachem JP, Crumrine D, Fluhr J, et al (2003) pH directly regulates epidermal permeability barrier homeostasis, and stratum corneum integrity/cohesion. J Invest Dermatol 121:345-53.

Hachem JP, Man MQ, Crumrine D, et al (2005) Sustained serine proteases activity by prolonged increase in pH leads to degradation of lipid processing enzymes and profound alterations of barrier function and stratum corneum integrity. J Invest Dermatol 125:510-20.

Hachem JP, Roelandt T, Schurer N, et al. (2010) Acute acidification of stratum corneum membrane domains using polyhydroxyl acids improves lipid processing and inhibits degradation of corneodesmosomes. J Invest Dermatol 130:500-10.

Hanson KM, Behne MJ, Barry NP et al. (2002) Two-photon fluorescence lifetime imaging of the skin stratum corneum pH gradient. Biophys J 83:1682-90.

Hatano Y, Man MQ, Uchida Y, et al. (2009) Maintenance of an acidic stratum corneum prevents emergence of murine atopic dermatitis. J Invest Dermatol 129:1824-35.

Hong SP, Oh Y, Jung M, et al. (2010) Topical calcitriol restores the impairment of epidermal permeability and antimicrobial barriers induced by corticosteroids. Br J Dermatol 162:1251-60.

Jung M, Choi J, Lee SA, et al. (2010) Pyrrolidone carboxylic acid levels or caspase-14 expression in the corneocytes of lesional skin correlates with clinical severity, skin barrier function and lesional inflammation in atopic dermatitis. J Dermatol Sci 76:231-9.

Jung YJ, Jung M, Kim M, et al. (2011) IL-1alpha stimulation restores epidermal permeability and antimicrobial barriers compromised by topical tacrolimus. J Invest Dermatol 131:698-705.

Kim JY, Jeong MS, Park MK, et al. (2014) Time-dependent progression from the acute to chronic phases in atopic dermatitis induced by epicutaneous allergen stimulation in NC/Nga mice. Exp Dermatol 23:53-7.

Kouzaki H, O'Grady SM, Lawrence CB, et al. (2009) Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. J Immunol 183:1427-34.

Kubo A, Nagao K, Amagai M (2012) Epidermal barrier dysfunction and cutaneous sensitization in atopic diseases. J Clin Invest 122:440-7.

Kubo A, Nagao K, Yokouchi M, et al. (2009) External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. J Exp Med 206:2937-46.

Laouini D, Kawamoto S, Yalcindag A, et al. (2003) Epicutaneous sensitization with

superantigen induces allergic skin inflammation. J Allergy Clin Immunol 112:981-7.

Lee HJ, Jung M, Kim JH, et al. (2012) The effect of adipose-derived stem cell-cultured media on oxazolone treated atopic dermatitis-like murine model. Ann Dermatol 24:181-8.

Lee HJ, Lee SH (2014) Epidermal permeability barrier defects and barrier repair therapy in atopic dermatitis. Allergy Asthma Immunol Res 6:276-87.

Lee HJ, Yoon NY, Lee NR, et al. (2014) Topical acidic cream prevents the development of atopic dermatitis- and asthma-like lesions in murine model. Exp Dermatol 23:736-41.

Lee SE, Jeong SK, Lee SH (2010) Protease and protease-activated receptor-2 signaling in the pathogenesis of atopic dermatitis. Yonsei Med J 51:808-22.

Li M (2014) Current evidence of epidermal barrier dysfunction and thymic stromal lymphopoietin in the atopic march. European respiratory review : an official journal of the European Respiratory Society 23:292-8.

Lind P (1985) Purification and partial characterization of two major allergens from the house dust mite Dermatophagoides pteronyssinus. J Allergy Clin Immunol 76:753-61.

Liu YJ (2006) Thymic stromal lymphopoietin: master switch for allergic inflammation. J Exp Med 203:269-73.

Matsuda H, Watanabe N, Geba GP, et al. (1997) Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. International immunology 9:461-6.

Novak N (2012) An update on the role of human dendritic cells in patients with atopic dermatitis. J Allergy Clin Immunol 129:879-86.

Ohman H, Vahlquist A (1998) The pH gradient over the stratum corneum differs in X-linked recessive and autosomal dominant ichthyosis: a clue to the molecular origin of the "acid skin mantle"? J Invest Dermatol 111: 674-7.

Shin YS, Sohn JH, Kim JY, et al. (2012) Endotoxin is not essential for the development of cockroach induced allergic airway inflammation. Yonsei Med J 53:593-602.

Spergel JM, Paller AS (2003) Atopic dermatitis and the atopic march. J Allergy Clin Immunol 112:S118-27.

Strid J, Callard R, Strobel S (2006) Epicutaneous immunization converts subsequent and established antigen-specific T helper type 1 (Th1) to Th2-type responses. Immunology 119:27-35

Tian L, Li W, Wang J, et al. (2011) The CKLF1-C19 peptide attenuates allergic lung inflammation by inhibiting CCR3- and CCR4-mediated chemotaxis in a mouse model of asthma. Allergy 66:287-97.

Vávrová K, Henkes D, Strüver K, et al. (2014) Filaggrin deficiency leads to impaired lipid profile and altered acidification pathways in a 3D skin construct. J Invest Dermatol 134: 746-53.

Yagi R, Nagai H, Iigo Y, et al. (2002) Development of atopic dermatitis-like skin lesions in STAT6-deficient NC/Nga mice. J Immunol 168:2020-7.

Yun JW, Seo JA, Jeong YS, et al. (2011) TRPV1 antagonist can suppress the atopic dermatitis-like symptoms by accelerating skin barrier recovery. J Dermatol Sci 62:8-15.

Zheng T, Yu J, Oh MH, et al. (2011) The atopic march: progression from atopic dermatitis to allergic rhinitis and asthma. Allergy Asthma Immunol Res 3:67-73.

III. Part 3. Maintenance of Stratum Corneum Acidity Interrupts Atopic March in Flaky Tail Mice with Congenital Skin Barrier Defect due to Inherited Filaggrin Deficiency

1. Abstract

Congenitally impaired skin barrier followed by atopic dermatitis (AD) is an initial step in atopic march that eventually progresses to respiratory allergy. Maintenance of an acidic pH in the stratum corneum (SC) is an important factor for normal epidermal barrier function. We determined whether an AD murine model, flaky tail(Flg^{ft}) mice with congenital barrier defect due to filaggrin deficiency, can develop airway inflammation by repeated topical application followed by nasal inhalation of a house dust mite(HDM), (defined as a novel 'atopic march-like animal model'), and whether maintenance of an acidic SC environment by continuous application of acidic cream can interrupt this atopic march. During the course of HDM treatment to the Flg^{ft} mice, acidic cream (pH 2.8) or neutral cream(pH7.4) adjusted by citric acid or sodium hydroxide mixed with vehicle were applied twice daily. Repeated applications and inhalations of HDM to AD murine model induced AD skin lesions followed by respiratory allergies, defining it as an atopic march model. Maintenance of SC acidity inhibited the occurrence of respiratory allergic inflammation as well as AD-like skin lesions. These results indicate that a novel atopic march models can be developed by repeated application of HDM on Flg^{ft} mice, and that the acidification of SC could be a novel intervention method to block the progress of the atopic march.

2. Introduction

Atopic dermatitis(AD) is one of the most frequent chronic inflammatory skin diseases characterized by epidermal barrier dysfunction, xerosis, eczematous lesions, pruritus, immunodysregulation, and IgE-mediated sensitization to exogenous allergens.(1) The obvious increase in the incidence of AD observed in recent decades has been due to the resettlement of populations from rural to urban areas, where a lack of early exposure to a variety of microbes results in reduced immune tolerance.(2, 3) The clinical manifestations of AD predate the development of asthma and allergic rhinitis in a process called the 'atopic march', suggesting that AD is an 'entry point' for subsequent allergic disease.(4, 5) The concept of the atopic march was hypothesized to describe the progression of atopic disorders from AD in infants to asthma and allergic rhinitis in children.

It has been reported that the development of asthma in the individuals with AD is secondary to easy allergen sensitization through a primarily defective epidermal barrier, which enables allergens(e.g. house dust mites (HDM)) to easily penetrate the skin and then make contact with antigen-presenting cells, Langerhans cells(LCs), in the skin(6-9). LCs, then, capture and process the penetrated allergens, migrate to draining lymph nodes and interact with naïve T cells, which promote Th2 inflammation leading to systemic allergy(10). Meanwhile, thymic stromal lymphopoietin (TSLP) secreted by the keratinocytes of disrupted epidermis activates polyclonal T cells. These T cells, in turn, induce excessive Th2 inflammatory responses, which could aggravate the AD and trigger the systemic Th2 responses including respiratory allergies.(11)

Many physicians, therefore, have attemted to prevent of the atopic march. The acidic pH of SC is a key factor for many protective functions including permeability barrier homeostasis, SC integrity and cohesion, antimicrobial defense, because it is important for

the functional activity of enzymes involved in processing of the SC lipids, and modulating the activity of the serine protease required for coordinated epidermal differentiation and cornified cell envelope formation.(12, 13) In AD, there is an increase of pH in lesional skin resulting in bacterial growth, allergic inflammation and aggravation of AD.(14) In a hapten-induced and immune-dysregulated(NC/Nga) animal model of AD, simple acidification of SC significantly blocks the progression of barrier impairment and the downstream immune abnormalities(9, 15, 16). This study gives us the clue and possibility of novel AD treatment in that maintenance of a normal or hyperacidic pH could reverse or prevent AD in human. However, haten-induced atopic march model only represents acquired AD without hereditary abnormalities and NC/Nga mice have IgE/Th2 independent pathogenesis which may not fully represent immune status of atopic march.(9, 16)

Filaggrin(FLG) mutation is a significant predisposition to an increased risk of developing atopic diseases including AD, atopic asthma, allergic rhinitis, and nickel and food allergies, although filaggrin is not found in bronchial epithelium.(17) In general, AD associated with FLG mutations is earlier onset, more 'atopic' and likely to be associated with asthma as well as more severe and persistent manifestations.(18) If FLG mutation is occurred, the FLG–associated SC barrier is disrupted; decreased formation and secretion of lamellar body(LB), cornified envelope, and corneodesmosome, elevated skin surface pH and decreased tight junction proteins, which leads to increased episodes of percutaneous allergen exposure.(2, 19) Flaky tail(Flg^{ft}) mouse, which showing the deficiency of filaggrin and natural moisturizing factors(NMF), has been used to investigate the role of filaggrin in AD. The spontaneous flaky tail(ft) mouse arose on the background of an existing recessive hair phenotype, matted(ma). It also presents

spontaneous dermatitis with increased IgE levels, endogenous protease (KLKs 5, 7, and 14) activity, TSLP expression, and basophil in the skin, which shows many similarities of AD phenotype.(20, 21)

For that reasons, we believed that the flaky tail mouse could be the most proper animal model similar to human AD patient with congenital barrier defect due to inherited deficiency of filaggrin. Therefore, we developed an atopic march murine model using flaky tail mouse. Secondly, we elucidated that this atopic march was interrupted by maintenance of the SC acidity and accelerated by neutralization of the SC.

3. Materials and methods

Mice and materials

All animal procedures were approved by the Yonsei University Wonju Campus Institutional Animal Care and Use Committee (IACUC). C57BL/6J (B6) female mice were purchased from OrientBio (Sungnam, Korea). Flg^{ft} (STOCK a/a ma ft/ma ft/J) mice, which carry double-homozygous flaky tail (ft) and matted (ma) mutations, were outcrossed onto B6 at Kyoto university (Kyoto, Japan).(48) Mice were kept under controlled humidity (40%) and temperature (22±2oC) conditions. Extracted antigen of *Dermatofagoides pteronyssinus*, a house dust mite, was purchased from the Department of Environmental Medical Biology, Yonsei University (Seoul, Korea). All laboratory measurements were performed under blinded conditions or by blinded researchers. The acidic cream was composed of citric acid purchased from Sigma-Aldrich (St Louis, MO, USA) and vehicle (Cetaphil®; Lausanne, Switzerland), adjusted with a pH meter. Neutralization of the acidic cream was performed with NaOH.

Development of an atopic march model by topical application and nasal inhalation of *Dermatofagoides pteronyssinus*(Dp) in Flg^{ft} mice and intervention of the atopic march model with an acidic cream

Five-week-old Flg^{ft} mice and B6 mice were used in this study. Once a week the mice were anesthetized and the hair on their dorsal skin was shaved. Dp antigen (Ag) was mixed with petrolatum to make a 0.5% ointment. Forty mg of 0.5% mite (Dp) Ag mixed with petrolatum was applied on the skin of the control group(B6 + Dp, n=6) and the Flg^{ft} with Dp groups(n=6, each) twice a week for a total of 20 times. During the last 3 days of the experiment, 100 μg of Dp Ag was given daily by intranasal inhalation.(9) While

appling Dp on the Flg^{ft} mice, 200 mg of acidic cream (pH 2.8, vehicle cream adjusted with citric acid) was applied topically twice a day on the dorsal skin for 45 days (acidic cream group). For non-treated control groups(B6, n=6), no interventional cream was applied. For the neutralized cream group(pH 7.4, vehicle cream adjusted with sodium hydroxide, n=6), neutralized citric acid cream was topically applied twice a day (Fig. 10). The vehicle was plain Cetaphil® cream with pH 5.5 (Galderma, Lausanne, Switzerland) as previously performed.(9, 16, 37) After a total of 70 days of Dp application, gross appearance, skin barrier functions such as basal transepidermal water loss (TEWL), SC hydration and skin surface pH, and the degree of allergic inflammation in the skin, blood, and respiratory system were evaluated in all mice. Surface pH was measured with pH meter (WTW, Weilheim, Germany). Basal TEWL was measured with the Tewameter TM210 (Courage and Khazaka, Cologne, Germany), and SC hydration was assessed as capacitance with the Corneometer CM820 (Courage and Khazaka) at 24 h after the final application of Dp, as described previously.(49) Twenty-four hours after the last treatment, specimens of skin, blood, and lung were collected to evaluate the changes in allergic inflammation as measured by mRNA and protein expression.

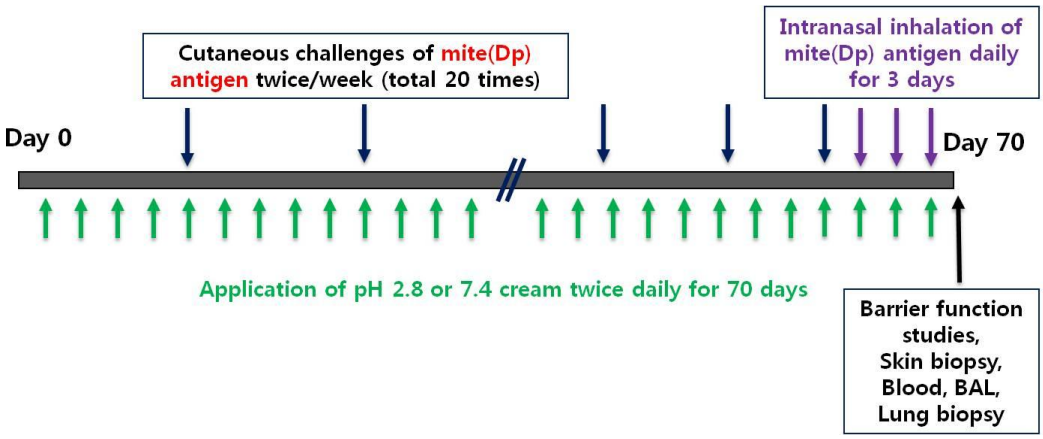

Figure 10. Development of an atopic march model in Flg^{ft} mice by epicutaneous application and subsequent nasal inhalation of house dust mite (Dp) and the application of acidic or neutral cream.

Evaluation of macroscopic AD-like skin lesions

Scoring of dermatitis was assessed macroscopically in a blinded fashion using the following scoring procedure described by Saunders et al.(29) and adapted from assessment of skin inflammation in the NC/Nga mouse model.(50) The development of (i) erythema, (ii) edema, (iii) excoriation, (iv) scale, and (v) lichenification was each scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The total score for each mouse was calculated from the sum of individual scores for each parameter.

Tissue preparation and immunohistochemistry

Immunohistochemical staining was performed to assess epidermal differentiation and inflammation. Briefly, 5-μm thick paraffin sections were incubated with primary antibodies against epidermal differentiation marker proteins such as filaggrin, involucrin, and loricrin, protease-activated receptor-2 (PAR-2), and thymic stromal lymphopoietin (TSLP) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After three cycles of washing, the sections were incubated with the appropriate secondary antibody for 30 min. Staining was detected with the ABC-peroxidase kit (Vector Lab, Burlingame, CA, USA) and counterstaining with hematoxylin was performed. To quantify the density of immunohistochemical staining, three pictures from each section were taken at 100x magnification and labeled. An investigator without any information on the specimen ranked the pictures in order of the intensity of immunostaining (the highest intensity received the first rank). Representative images of each group are provided.

ELISA for serum IgE and TSLP

Serum levels of IgE and TSLP were determined using ELISA kits for mouse IgE (Koma Biotech, Seoul, Korea) and mouse TSLP (R&D systems, Minneapolis, MN, USA) as previously reported.(9, 16) To detect serum IgE and TSLP levels, whole blood samples were centrifuged at 5,000 rpm for 5 min at room temperature (RT). The sample serum and standard solutions were added to plates that were pre-coated with specific antibodies for IgE and TSLP, and incubated for 1 h at RT. Horseradish peroxidase-conjugated mouse secondary detection antibodies were added to each well and incubated for 1 hour at RT. TMB substrate was added to each well and incubated for 20 min for color development. A sulfuric acid solution was added to each well to stop the reaction and the enzyme-substrate reaction was measured using a microtiter plate reader (BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 450 ± 2 nm. After triplicate measurements, the concentrations of IgE and TSLP in the samples were determined by comparison with a standard curve.

Bronchoalveolar lavage (BAL)

BAL was performed as previously described.(9, 16) After each mouse was anesthetized, the trachea was cannulated and the lung was lavaged with 1 mL of Hank's balanced salt solution via the intubation tube. Bronchoalveolar lavage fluid (BALF) was obtained by two rounds of aspiration. Total numbers of cells were counted and cell smear preparations were made. Cells were stained with Wright–Giemsa stain and eosinophils, lymphocytes, macrophages, and neutrophils were counted and expressed as a percentage of 400 total cells. The counts of eosinophils, lymphocytes, macrophages, and neutrophils in the BALF were determined by multiplying their percentages in total cells.

Total RNA preparation and cDNA synthesis with blood and lung tissue and Quantitative PCR analysis of gene expression

Total RNA preparation and cDNA synthesis were performed as previously described.(37, 51) Expression of specific mRNAs was quantified using a Rotor-Gene™ 3000 (Corbett Life Science, Brisbane, Australia), as in our previous reports (37, 51, 52). Briefly, 10-ul PCR reactions were set up containing Quantitect SYBR green PCR kit Master mix (Qiagen, Hilden, Germany) in a 2X solution, 8 mM manganese chloride, 200 μM deoxynucleotide triphosphates (dNTPs), 1.25 units Hotstart Taq polymerase, and 0.5 pM/μl each of probes and primers. Approximately 60 ng of cDNA was used per reaction. Primers for PAR-2 and TSLP were designed based on GeneBank sequences using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/, Massachusetts Institute of Technology, Cambridge, MA). All reactions included GAPDH as a housekeeping gene. Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacturer's guidelines and were used to determine ΔCt values (Ct of target gene−Ct of housekeeping gene) as raw data for gene expression. Changes in gene expression were determined by subtracting ΔCt values for samples and acidic cream-treated samples from their respective control samples. The resulting ΔΔCt values were used to calculate fold change in gene expression as 2-ΔΔCt. All reactions were performed in triplicate, and the results are expressed as the mean of values from three separate experiments. Samples were amplified under the following conditions: 95°C for 15 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Statistical analysis

All data were expressed as mean ± SEM. ANOVA or Kruskal-Wallis test were performed when three or more groups were compared or to compare the density of immunohistochemical staining. For individual comparison, Student's unpaired t-test was used in case of normal distribution, and Mann-Whitney test was used in case of non-normal distribution or ranking variables. *$p<0.05$ was regarded as statistically significant. Statistical analysis was conducted using SPSS software 10.1 (SPSS, Chicago, IL).

4. Results

Repeated epicutaneous application of HDM extract provokes remarkable AD-like skin lesions and barrier dysfunction in Flg^{ft} mice, a representative murine model of inherited AD, which are prevented by acidic cream and aggravated by a neutral cream.

Repeated epicutaneous application of a HDM, Dermatofagoides pteronyssinus (Dp) antigen provoked remarkable AD-like skin lesions with a high eczema score in Flg^{ft} mice. However the control mice (B6 mice) did not exhibit eczema lesions despite same epicutaneous treatment with Dp antigen. Moreover, application of a neutral cream with Dp extract (Flg^{ft} + Dp + pH 7.4) resulted in more prominent eczema lesions compared to other treatment groups. On the contrary, application of an acidic cream (Flg^{ft} + Dp + pH 2.8) alleviated eczema lesions in Dp-treated Flg^{ft} mice significantly (Fig. 11a). Post-treatment basal TEWL was increased and SC hydration was significantly decreased in the group treated with neutral cream(Flg^{ft} + Dp + pH 7.4 group) but significantly improved in the group with acidic cream(Flg^{ft} + Dp + pH 2.8 group)(Fig. 11b and c). Moreover, there was significant difference in skin surface pH among cream treated groups, which was measured 24 h after the final application of Dp, that is, 12 h after the final application of cream. Post-treatment SC pH decreased significantly in the group treated with acidic cream(Flg^{ft} + Dp + pH 2.8 group) and increased in the group with neutral cream(Flg^{ft} + Dp + pH 7.4 group) (Fig. 11d).

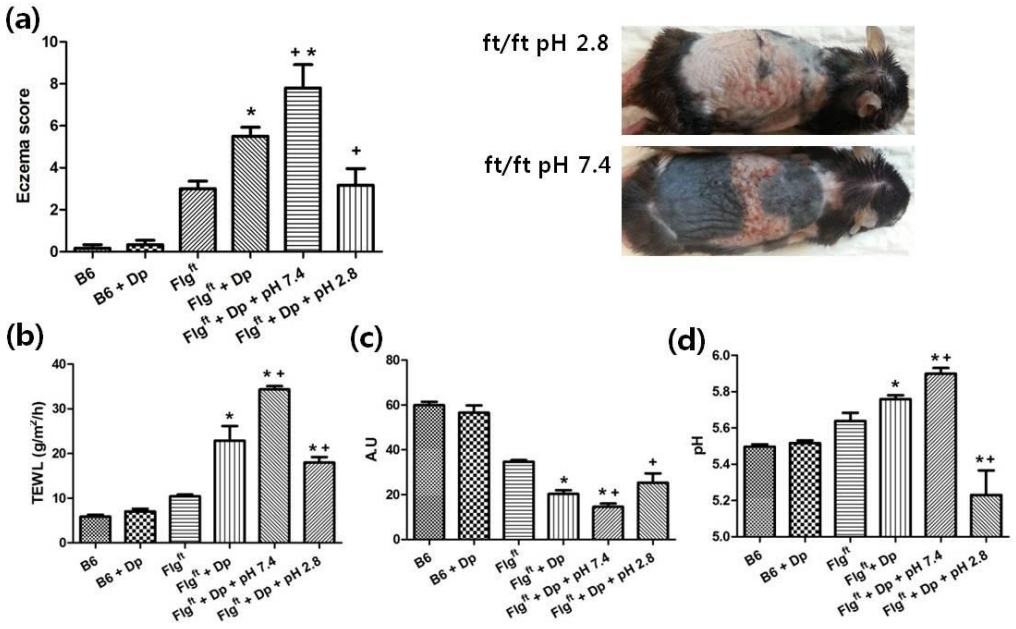

Figure 11. Repeated epicutaneous application of house dust mite (Dp) extract followed by nasal inhalation provokes remarkable AD-like skin lesions and barrier disruption in Flg^{ft} mice, a representative murine model of inherited AD. Those symptoms were improved by acidic cream but aggravated by neutral cream. Systemic allergic inflammation induced in Flg^{ft} mice was improved by acidic cream but aggravated by neutral cream. (a) Eczema scores and gross appearance, (b) basal TEWL, (c) SC hydration, (d) skin surface pH, serum IgE (e) and TSLP (f) after repeated epicutaneous application of house dust mite extract (Dp) followed by nasal inhalation were compared among treatment groups. ANOVA showed $p<0.05$ in all measurements. Results are mean ± SEM. *$p<0.05$, compared to control (B6 + Dp and Flg^{ft}); +$p < 0.05$, compared to Flg^{ft} + Dp. (n=6, each group)

Cutaneous and systemic allergic inflammation in Flg^{ft} mice were aggravated due to HDM and affected by SC acidity

The number of tissue eosinophils in the skin was also significantly increased in the Flg^{ft} + Dp + pH 7.4 group and decreased in the Flg^{ft} + Dp + pH 2.8 goup compared to the Flg^{ft} + Dp group(Fig. 12a). The expression of epidermal differentiation markers such as involucrin, and loricrin by immunohistochemical staining and semiquantitative analysis showed that their expression was significantly decreased in the Flg^{ft} + Dp group compared to the control B6 + Dp group(Fig. 12a). Application of neutral cream to the Flg^{ft} + Dp group significantly decreased expression of these markers, whereas acidic cream significantly increased their expression except filaggrin. There was minimal expression of filaggrin among Flg^{ft} groups. Semiquantitative analysis of epidermal expression of PAR-2 and TSLP was significantly increased in the Flg^{ft} mice compared to control. Dp treatment increased their expression more. Application of neutral cream to the Flg^{ft} + Dp group significantly increased expression of PAR-2 and TSLP, whereas acidic cream significantly decreased their expression(Fig. 12b).

Serum levels of total IgE and TSLP significantly increased in all of the Flg^{ft} mice with or without Dp treatment compared to control mice. Among Flg^{ft} mice, the mice that were treated with neutral cream showed the most prominent increase in serum IgE and TSLP. On the contrary, acidic cream significantly decreased serum IgE and TSLP in Flg^{ft} mice treated with Dp antigen (Fig. 12c and d).

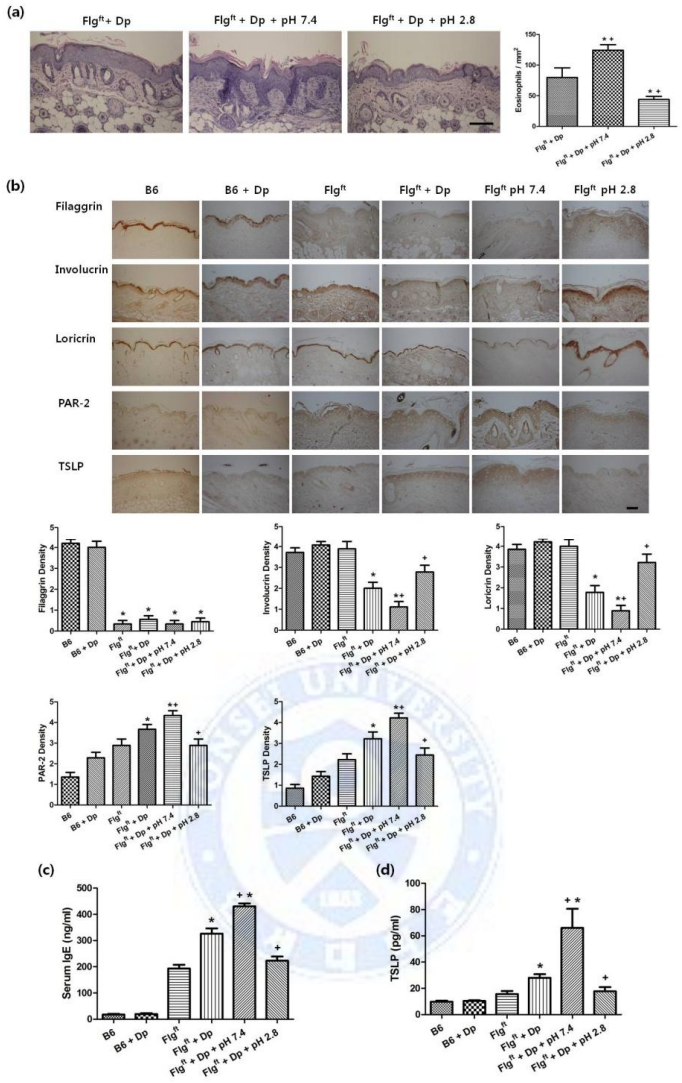

Figure 12. Cutaneous allergic inflammation and abnormal epidermal differentiation induced in Flg^{ft} mice is aggravated by neutral cream but improved by acidic cream. Tissue eosinophils were counted with H&E staining (a) and expression of epidermal differentiation markers such as filaggrin, involucrin, and loricrin, PAR-2, and TSLP was measured by immunohistochemical staining (b) in skin specimens taken after repeated epicutaneous application and nasal inhalation of Dp. ANOVA (a) and Kruskal-Wallis test (b) showed $p<0.05$ in all measurements. Results are mean ± SEM. *$p<0.05$, compared to control (B6 + Dp); +$p<0.05$, compared to Flg^{ft} + Dp.

Repeated epicutaneous application followed by nasal inhalation of HDM extract produces asthma-like airway inflammation as well as AD-like skin lesions in Flg^{ft} mice, implying atopic march; it was interrupted by SC acidification, whereas aggravated by SC neutralization.

Quantitation of mRNA and immunohistochemical staining revealed increased expression of PAR-2 and TSLP in the lung tissue of the Flg^{ft} + Dp group, which was further aggravated by application of neutral cream application and improved by acidic cream with statistical significance (Fig. 13a, b and c). Since airway eosinophilia is a hallmark of asthma (Tian et al., 2011), we performed BAL after 3 consecutive days of nasal inhalation of Dp. Total cell count of the BAL was significantly increased in the Flg^{ft} mice with Dp application. This increase was significantly aggravated by treatment with neutral cream (Flg^{ft} + Dp + pH 7.4 group) and improved by application of the acidic cream(Fig. 13d). Each number of eosinophils, macrophages, and neutrophils was also significantly increased in the Flg^{ft} + Dp group, similar to the results of total cell count (Fig. 13e).

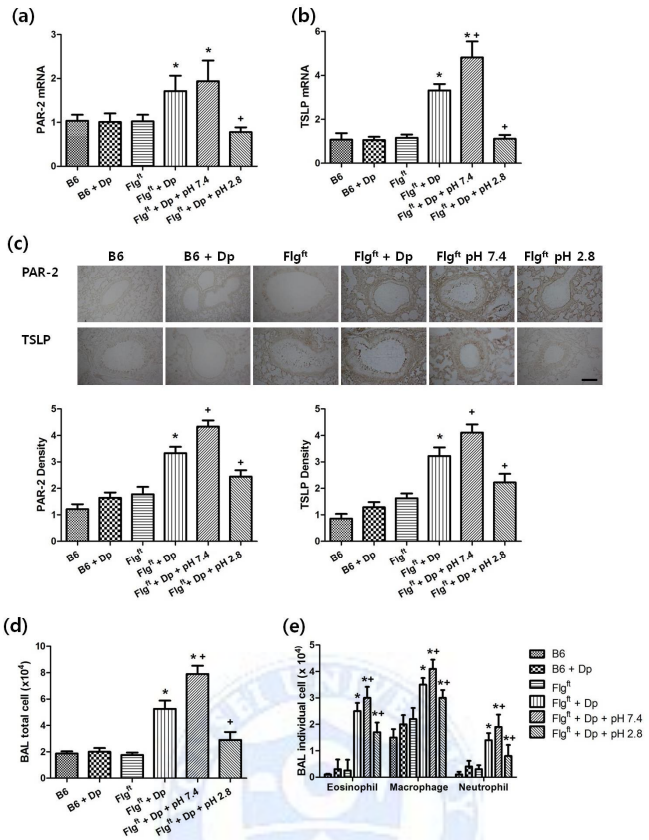

Figure 13. Repeated epicutaneous application followed by nasal inhalation of a house dust mite extract (Dp) produces asthma-like airway inflammation as well as AD-like skin lesions in Flg^{ft} mice, implying atopic march. Those symptoms were improved by acidic cream but aggravated by neutral cream. Bronchoalveolar lavage (BAL), real-time RT-PCR for mRNA expression, and immunohistochemical staining for PAR-2 and TSLP in the lung tissue were performed after epicutaneous application and nasal inhalation of Dp. Total cell count (a) and individual cell count (b) of the BAL, and mRNA (c, d) and tissue expression (e) of PAR-2 and TSLP were measured after treatment. ANOVA (a-d) and Kruskal-Wallis test (e) showed $p<0.05$ in all measurements. Results are mean ± SEM. *$p < 0.05$, compared to control (B6 + Dp); +$p < 0.05$, compared to Flg^{ft} + Dp. (n=6, each group)

5. Discussion

The concept of the atopic march was hypothesized to describe the progression of atopic disorders from AD in infants to asthma and allergic rhinitis in children(5, 8). Since Strid et al.(22) suggested the idea of epidermal barrier defect initiating allergic sensitization and showed its pathogenesis in murine model, the theoretical model described by Kubo et al. (2) has been considered applicable to the pathogenesis of atopic march. Abrogation of SC barrier promotes the penetration of protease-active allergens and uncontrolled intrinsic proteases, which may activate PAR-2 and Toll-like receptors on keratinocytes.(23). Keratinocytes then produce proinflammatory cytokines such as TNF-α, IL-1, and TSLP. Meanwhile, dendrites of activated LCs penetrate the weakened tight junctions of the upper stratum granulosum and take up exogenous antigens(24). Such percutaneous sensitization and chronic repeated allergen challenges via different routes, such as lung, nasal cavities, and intestinal tract, are speculated to manifest as the allergic march. In this study, along with our previous studies(9, 16), epicutaneous treatment and nasal inhalation of HDM with no disruption of SC barrier in the control mice without barrier defect did not provoke remarkable inflammatory lesions in the skin as well as the lung (Figs. 11-13). This result also suggests that the uptake of active allergens and the activation of LCs did not take place when the epidermal barrier was not disrupted, therefore there was no increase in the levels of TSLP. It is well known that the expression of TSLP is highly increased in lesional skin and the blood of patients with AD, and that this elevation in TSLP expression is sufficient to activate LCs during AD development(2, 25).

Since PAR-2 and TSLP expression has been reported in various tissues including skin and lung, they also take part in the occurrence of AD and respiratory allergy(9, 26, 27). TSLP

expression is induced when airway epithelial cells are exposed to allergen-derived proteases, and PAR-2 involves in this process(28). In our study, repeated HDM application to defected SC barrier increases the levels of TSLP, PAR-2, and IgE in the systemic circulation or lung tissue (Fig. 13). In addition, we could assume that HDM-induced asthma could occur more easily when the skin barrier is more severely impaired and AD lesions are more severe. These conditions provoke an elevation of systemic TSLP and IgE as well as eosinophils, whereas HDM application alone on the impaired skin barrier rarely induce asthma. Systemic TSLP levels may affect the PAR-2 and TSLP in distant epithelial organs such as the lung, which might be readily affected by the HDM allergen.

Flg^{ft} mice, homozygous mutation of 5303delA in the FLG gene and Tmem79/Matt gene, have been used to investigate the role of filaggrin in AD(29). Topical application of HDM in Flg^{ft} mice induces skin lesions which are more clinically and histologically similar to those seen in AD with outside-to-inside skin barrier dysfunction(20). The relative contribution of Flg and ma to the compound phenotype has yet to be fully defined. Recently, Matted mouse gene is a predisposing gene for atopic dermatitis and a common SNP in MATT has an association with AD in human subjects(29). In addition, Sasaki et al(30) showed the Tmem79(ma/ma) mutation is responsible for the spontaneous dermatitis phenotype in matted mice, probably as a result of impaired LB secretory system and altered SC barrier function, which can also be found in Caucasians who have FLG mutations in dose dependant alterations(31). Thus far, Flg^{ft} mice might be one of the most represented animal model of AD corresponding with filaggrin mutation in human AD.

There are many studies which attempted to establish an animal model of the allergic

march. There have been several studies done using an occlusive patch of ovalbumin (OVA) to make an atopic march model. Although majority of these studies expressed atopic dermatitis-like skin lesions combined with asthma-like lung lesions, they used intraperitoneal injections for sensitization, not epicutaneous sensitization via damaged skin barrier. In addition, allergic reaction of OVA is disappeared in adulthood, whereas one of HDM still remains(32-35). Recently, we reported the murine models of acquired and inherited AD progresses to asthma-like lesions under more natural and physiologic mechanisms with favorable results(9, 16). In order to produce asthma-like lung lesions, we applied Dp antigens topically to skin lesions on the Flg^{ft} mice, a murine model of inherited AD, with disrupted barrier before nasal inhalation of Dp, which revealed more physiologic pathway of allergen penetration, systemic circulation, and allergen challenge via different routes, such as lung and nasal cavities(Fig. 10)(2). Recent study using filaggrin-deficient mice showed that innate immunity initiates dermatitis and the adaptive immunity required for subsequent development of compromised lung function.(36) Along with previous report, our results also showed similar results that there is no significant change on the lung without inhalation of HDM on Flg^{ft} mice(Fig. 13).

In AD skin, decreased SC hydration leads to a steeper water gradient across the SC as a result of filaggrin deficiency, thereby 'driving' increased TEWL(37). Filaggrin (FLG) mutations are a significant predisposition to an increased risk of developing atopic diseases including AD, atopic asthma, allergic rhinitis, and nickel and food allergies, although filaggrin is not found in bronchial epithelium(1, 8, 17). Besides, to promote the compaction of corneocytes, filaggrin monomers are degraded into natural moisturizing factors (NMFs) to maintain the hydration of the upper SC and to reduce the pH of the skin surfaces(3, 38, 39). If these proteins are decreased or absent due to FLG mutations,

the FLG–associated SC barrier is disrupted, which leads to increased episodes of percutaneous allergen exposure(2). Keratinocyte differentiation is also incomplete in AD lesions(38). In our allergic march model, the expressions of involucrin and loricrin were decreased after treatment of HDM, with minimal expression of filaggrin, which may imply disruption of the SC barrier and provocation of transcutaneous infiltration of allergens such as Dp (Fig. 12b). This may also induce asthma-like lesions easily.

Decrease in filaggrin degradation products in AD lesions might contribute to an increase in the SC pH high enough to induce activation of serine proteases(SP) in the SC which optimally acts under neutral to alkaline pH(40), although there are controversies whether filaggrin deficiency per se increases the SC pH or not (41-43). An elevated SC pH also increases the activity of SP such as kallikrein 5 (KLK5) and KLK7 (stratum corneum chymotryptic enzyme), resulting in overdegradation of corneodesmosomes (CD) and weakening of SC integrity and cohesion(44). On the other hand, the elevation of SC pH decreases the activity of lipid-processing enzymes, which are crucial for the production of free fatty acids and ceramide in the SC, and thus impairs lipid processing and eventually disrupts the permeability barrier of epidermis(12, 15). Based on our results, we assume that prolonged skin barrier damage induced by repeated HDM in Flg^{ft} mice provoked the elevation of SC pH. Elevated surface pH increased PAR-2 and TSLP in the blood as well as in the skin, which contributes to AD-like lesions with elevated IgE and Th2 cytokines. Application of Dp antigen on the AD-like lesions and respiratory system also induced respiratory allergies, represented by airway eosinophilia and elevated PAR-2 and TSLP in the lung tissue. Maintenance of an acidic skin environment would block this cycle early; hence, the allergic march could be prevented (Fig. 5).

Several indirect evidence of reports show that acidification of SC prevents the

penetration of the SC by external allergens including HDM(9, 12, 15, 16, 45, 46). Hatano et al. reported that an acidic environment can inhibit the formation of AD-like skin lesions in the hapten-induced AD model(15). We previously reported that acidification of SC inhibits the development of both asthma-like lesions and AD-like lesions by HDM in the murine models(9, 16). In the present study using Flg^{ft} mice, we observed a preventive effect of acidic cream and an aggravating or harmful effect of neutral cream on the atopic march. A previous study already confirmed that acidic treatment to HDM (Dp) preserved the allergenicity and antigenicity of Dp with no significant differences in the antigenic profile of native and acid treated extracts(47).

In summary, this animal study demonstrates that epicutaneous allergen sensitization through an inherited defective skin barrier can induce respiratory allergic inflammation. We demonstrate that a murine model of atopic march can be developed by application of Dp to Flg^{ft} mice. Our previous studies and present results indicate that application of Dp antigen to the AD-like lesions, regardless of acquired(hapten-induced) and inherited(defect of immune or SC barrier) etiology, and respiratory system readily induced respiratory allergies, as represented by airway eosinophilia and elevated PAR-2 and TSLP expression in the lung tissue. Maintenance of a neutral skin environment aggravated and an acidic skin interrupted this cycle(Fig. 14). Therefore, maintenance of an acidic environment on the SC may be a novel method of intervention to block the progression of AD to respiratory allergies (the atopic march).

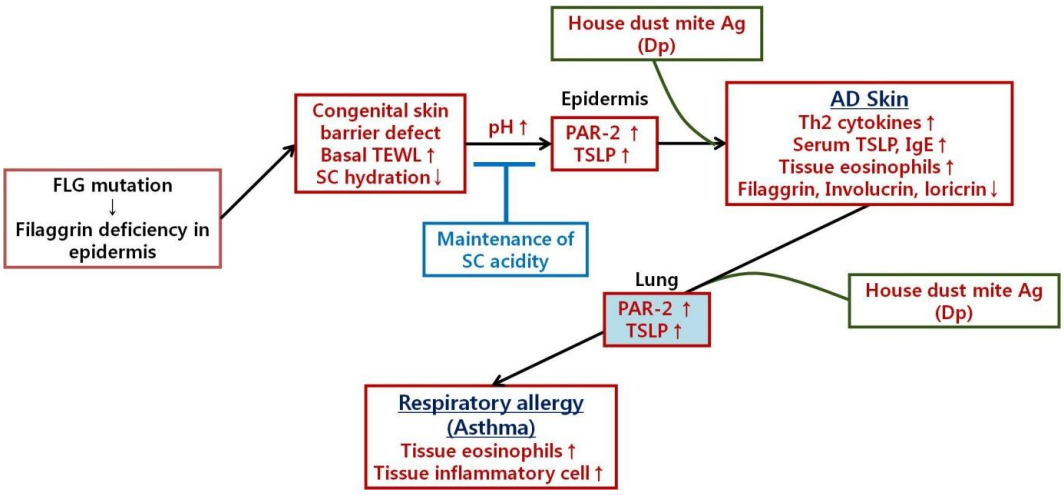

Figure 14. Possible explanations for the development of atopic march models using flaky tail mouse and intervention.

6. References

1. Lee, H.J., and Lee, S.H. 2014. Epidermal permeability barrier defects and barrier repair therapy in atopic dermatitis. Allergy Asthma Immunol Res 6:276-287.

2. Kubo, A., Nagao, K., and Amagai, M. 2012. Epidermal barrier dysfunction and cutaneous sensitization in atopic diseases. J Clin Invest 122:440-447.

3. Elias, P.M., and Schmuth, M. 2009. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. Curr Opin Allergy Clin Immunol 9:437-446.

4. Gordon, B.R. 2011. The allergic march: can we prevent allergies and asthma? Otolaryngol Clin North Am 44:765-777, xi.

5. Spergel, J.M., and Paller, A.S. 2003. Atopic dermatitis and the atopic march. J Allergy Clin Immunol 112:S118-127.

6. McGrath, J.A., and Uitto, J. 2008. The filaggrin story: novel insights into skin-barrier function and disease. Trends Mol Med 14:20-27.

7. Callard, R.E., and Harper, J.I. 2007. The skin barrier, atopic dermatitis and allergy: a role for Langerhans cells? Trends Immunol 28:294-298.

8. Zheng, T., Yu, J., Oh, M.H., and Zhu, Z. 2011. The atopic march: progression from atopic dermatitis to allergic rhinitis and asthma. Allergy Asthma Immunol Res 3:67-73.

9. Lee, H.J., Yoon, N.Y., Lee, N.R., Jung, M., Kim, D.H., and Choi, E.H. 2014. Topical acidic cream prevents the development of atopic dermatitis- and asthma-like lesions in murine model. Exp Dermatol 23:736-741.

10. Allakhverdi, Z., Comeau, M.R., Jessup, H.K., Yoon, B.R., Brewer, A., Chartier, S., Paquette, N., Ziegler, S.F., Sarfati, M., and Delespesse, G. 2007. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or

inflammation and potently activates mast cells. J Exp Med 204:253-258.

11. Laouini, D., Kawamoto, S., Yalcindag, A., Bryce, P., Mizoguchi, E., Oettgen, H., and Geha, R.S. 2003. Epicutaneous sensitization with superantigen induces allergic skin inflammation. J Allergy Clin Immunol 112:981-987.

12. Hachem, J.P., Roelandt, T., Schurer, N., Pu, X., Fluhr, J., Giddelo, C., Man, M.Q., Crumrine, D., Roseeuw, D., Feingold, K.R., et al. 2010. Acute acidification of stratum corneum membrane domains using polyhydroxyl acids improves lipid processing and inhibits degradation of corneodesmosomes. J Invest Dermatol 130:500-510.

13. Elias, P.M. 2015. Stratum corneum acidification: how and why? Exp Dermatol 24:179-180.

14. McAleer, M.A., and Irvine, A.D. 2013. The multifunctional role of filaggrin in allergic skin disease. J Allergy Clin Immunol 131:280-291.

15. Hatano, Y., Man, M.Q., Uchida, Y., Crumrine, D., Scharschmidt, T.C., Kim, E.G., Mauro, T.M., Feingold, K.R., Elias, P.M., and Holleran, W.M. 2009. Maintenance of an acidic stratum corneum prevents emergence of murine atopic dermatitis. J Invest Dermatol 129:1824-1835.

16. Lee, H.J., Lee, N.R., Jung, M., Kim, D.H., and Choi, E.H. 2015. Atopic March from Atopic Dermatitis to Asthma-Like Lesions in NC/Nga Mice Is Accelerated or Aggravated by Neutralization of Stratum Corneum but Partially Inhibited by Acidification. J Invest Dermatol.

17. Ring, J., Mohrenschlager, M., and Weidinger, S. 2012. Molecular genetics of atopic eczema. Chem Immunol Allergy 96:24-29.

18. McLean, W.H., and Irvine, A.D. 2012. Heritable filaggrin disorders: the paradigm of atopic dermatitis. J Invest Dermatol 132:E20-21.

19. Gruber, R., Bornchen, C., Rose, K., Daubmann, A., Volksdorf, T., Wladykowski, E., Vidal, Y.S.S., Peters, E.M., Danso, M., Bouwstra, J.A., et al. 2015. Diverse Regulation of Claudin-1 and Claudin-4 in Atopic Dermatitis. Am J Pathol 185:2777-2789.

20. Moniaga, C.S., Jeong, S.K., Egawa, G., Nakajima, S., Hara-Chikuma, M., Jeon, J.E., Lee, S.H., Hibino, T., Miyachi, Y., and Kabashima, K. 2013. Protease activity enhances production of thymic stromal lymphopoietin and basophil accumulation in flaky tail mice. Am J Pathol 182:841-851.

21. Moniaga, C.S., Egawa, G., Kawasaki, H., Hara-Chikuma, M., Honda, T., Tanizaki, H., Nakajima, S., Otsuka, A., Matsuoka, H., Kubo, A., et al. 2010. Flaky tail mouse denotes human atopic dermatitis in the steady state and by topical application with Dermatophagoides pteronyssinus extract. Am J Pathol 176:2385-2393.

22. Strid, J., Callard, R., and Strobel, S. 2006. Epicutaneous immunization converts subsequent and established antigen-specific T helper type 1 (Th1) to Th2-type responses. Immunology 119:27-35.

23. Briot, A., Deraison, C., Lacroix, M., Bonnart, C., Robin, A., Besson, C., Dubus, P., and Hovnanian, A. 2009. Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. J Exp Med 206:1135-1147.

24. Kubo, A., Nagao, K., Yokouchi, M., Sasaki, H., and Amagai, M. 2009. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. J Exp Med 206:2937-2946.

25. Liu, Y.J. 2006. Thymic stromal lymphopoietin: master switch for allergic inflammation. J Exp Med 203:269-273.

26. Ebeling, C., Forsythe, P., Ng, J., Gordon, J.R., Hollenberg, M., and Vliagoftis, H.

2005. Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. J Allergy Clin Immunol 115:623-630.

27. Gu, Q., and Lee, L.Y. 2012. House dust mite potentiates capsaicin-evoked Ca2+ transients in mouse pulmonary sensory neurons via activation of protease-activated receptor-2. Exp Physiol 97:534-543.

28. Kouzaki, H., O'Grady, S.M., Lawrence, C.B., and Kita, H. 2009. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. J Immunol 183:1427-1434.

29. Saunders, S.P., Goh, C.S., Brown, S.J., Palmer, C.N., Porter, R.M., Cole, C., Campbell, L.E., Gierlinski, M., Barton, G.J., Schneider, G., et al. 2013. Tmem79/Matt is the matted mouse gene and is a predisposing gene for atopic dermatitis in human subjects. J Allergy Clin Immunol 132:1121-1129.

30. Sasaki, T., Shiohama, A., Kubo, A., Kawasaki, H., Ishida-Yamamoto, A., Yamada, T., Hachiya, T., Shimizu, A., Okano, H., Kudoh, J., et al. 2013. A homozygous nonsense mutation in the gene for Tmem79, a component for the lamellar granule secretory system, produces spontaneous eczema in an experimental model of atopic dermatitis. J Allergy Clin Immunol 132:1111-1120 e1114.

31. Gruber, R., Elias, P.M., Crumrine, D., Lin, T.K., Brandner, J.M., Hachem, J.P., Presland, R.B., Fleckman, P., Janecke, A.R., Sandilands, A., et al. 2011. Filaggrin genotype in ichthyosis vulgaris predicts abnormalities in epidermal structure and function. Am J Pathol 178:2252-2263.

32. Spergel, J.M., Mizoguchi, E., Brewer, J.P., Martin, T.R., Bhan, A.K., and Geha, R.S. 1998. Epicutaneous sensitization with protein antigen induces localized allergic

dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. J Clin Invest 101:1614-1622.

33. Akei, H.S., Brandt, E.B., Mishra, A., Strait, R.T., Finkelman, F.D., Warrier, M.R., Hershey, G.K., Blanchard, C., and Rothenberg, M.E. 2006. Epicutaneous aeroallergen exposure induces systemic TH2 immunity that predisposes to allergic nasal responses. J Allergy Clin Immunol 118:62-69.

34. He, R., Kim, H.Y., Yoon, J., Oyoshi, M.K., MacGinnitie, A., Goya, S., Freyschmidt, E.J., Bryce, P., McKenzie, A.N., Umetsu, D.T., et al. 2009. Exaggerated IL-17 response to epicutaneous sensitization mediates airway inflammation in the absence of IL-4 and IL-13. J Allergy Clin Immunol 124:761-770 e761.

35. Oyoshi, M.K., Elkhal, A., Scott, J.E., Wurbel, M.A., Hornick, J.L., Campbell, J.J., and Geha, R.S. 2011. Epicutaneous challenge of orally immunized mice redirects antigen-specific gut-homing T cells to the skin. J Clin Invest 121:2210-2220.

36. Saunders, S.P., Moran, T., Floudas, A., Wurlod, F., Kaszlikowska, A., Salimi, M., Quinn, E.M., Oliphant, C.J., Nunez, G., McManus, R., et al. 2015. Spontaneous atopic dermatitis is mediated by innate immunity, with the secondary lung inflammation of the atopic march requiring adaptive immunity. J Allergy Clin Immunol.

37. Jung, Y.J., Jung, M., Kim, M., Hong, S.P., and Choi, E.H. 2011. IL-1alpha stimulation restores epidermal permeability and antimicrobial barriers compromised by topical tacrolimus. J Invest Dermatol 131:698-705.

38. Elias, P.M. 2010. Therapeutic implications of a barrier-based pathogenesis of atopic dermatitis. Ann Dermatol 22:245-254.

39. Sandilands, A., Sutherland, C., Irvine, A.D., and McLean, W.H. 2009. Filaggrin in the frontline: role in skin barrier function and disease. J Cell Sci 122:1285-1294.

40. Brattsand, M., Stefansson, K., Lundh, C., Haasum, Y., and Egelrud, T. 2005. A proteolytic cascade of kallikreins in the stratum corneum. J Invest Dermatol 124:198-203.

41. Fluhr, J.W., Elias, P.M., Man, M.Q., Hupe, M., Selden, C., Sundberg, J.P., Tschachler, E., Eckhart, L., Mauro, T.M., and Feingold, K.R. 2010. Is the filaggrin-histidine-urocanic acid pathway essential for stratum corneum acidification? J Invest Dermatol 130:2141-2144.

42. Ohman, H., and Vahlquist, A. 1998. The pH gradient over the stratum corneum differs in X-linked recessive and autosomal dominant ichthyosis: a clue to the molecular origin of the "acid skin mantle"? J Invest Dermatol 111:674-677.

43. Vavrova, K., Henkes, D., Struver, K., Sochorova, M., Skolova, B., Witting, M.Y., Friess, W., Schreml, S., Meier, R.J., Schafer-Korting, M., et al. 2014. Filaggrin deficiency leads to impaired lipid profile and altered acidification pathways in a 3D skin construct. J Invest Dermatol 134:746-753.

44. Lee, S.E., Jeong, S.K., and Lee, S.H. 2010. Protease and protease-activated receptor-2 signaling in the pathogenesis of atopic dermatitis. Yonsei Med J 51:808-822.

45. Hachem, J.P., Crumrine, D., Fluhr, J., Brown, B.E., Feingold, K.R., and Elias, P.M. 2003. pH directly regulates epidermal permeability barrier homeostasis, and stratum corneum integrity/cohesion. J Invest Dermatol 121:345-353.

46. Hachem, J.P., Man, M.Q., Crumrine, D., Uchida, Y., Brown, B.E., Rogiers, V., Roseeuw, D., Feingold, K.R., and Elias, P.M. 2005. Sustained serine proteases activity by prolonged increase in pH leads to degradation of lipid processing enzymes and profound alterations of barrier function and stratum corneum integrity. J Invest Dermatol 125:510-520.

47. Fernandez-Caldas, E., Gallego, M., Carnes, J., and Iraola, V. 2008. Enzymatic

activity of Dermatophagoides pteronyssinus extracts after acidic treatment. Int Arch Allergy Immunol 145:298-304.

48. Presland, R.B., Boggess, D., Lewis, S.P., Hull, C., Fleckman, P., and Sundberg, J.P. 2000. Loss of normal profilaggrin and filaggrin in flaky tail (ft/ft) mice: an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris. J Invest Dermatol 115:1072-1081.

49. Choi, E.H., Brown, B.E., Crumrine, D., Chang, S., Man, M.Q., Elias, P.M., and Feingold, K.R. 2005. Mechanisms by which psychologic stress alters cutaneous permeability barrier homeostasis and stratum corneum integrity. J Invest Dermatol 124:587-595.

50. Matsuda, H., Watanabe, N., Geba, G.P., Sperl, J., Tsudzuki, M., Hiroi, J., Matsumoto, M., Ushio, H., Saito, S., Askenase, P.W., et al. 1997. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. Int Immunol 9:461-466.

51. Lee, H.J., Jung, M., Kim, J.H., Yoon, N.Y., and Choi, E.H. 2012. The effect of adipose-derived stem cell-cultured media on oxazolone treated atopic dermatitis-like murine model. Ann Dermatol 24:181-188.

52. Hong, S.P., Oh, Y., Jung, M., Lee, S., Jeon, H., Cho, M.Y., Lee, S.H., and Choi, E.H. 2010. Topical calcitriol restores the impairment of epidermal permeability and antimicrobial barriers induced by corticosteroids. Br J Dermatol 162:1251-1260.

IV. Conclusion

In conclusion, these results indicate that novel atopic march models can be developed by repeated application of house dust mites on flaky tail, Nc/Nga and Ox-induced AD mice, and that the acidification of SC could be a novel intervention method to block the progress of the atopic march.

Abstract in Korean (국문 요약)

아토피행진 동물모델에서 각질층의 산도유지에 의한 아토피행진의 진행예방

< 지도교수 최 응 호 >

연세대학교 대학원 의학과

이 해 진

각질층의 적당한 산도유지는 정상적인 피부장벽기능을 유지하는데 중요한 역할을 한다. 아토피피부염에서 지속적 또는 반복적인 피부장벽기능의 손상은 아토피행진을 통하여 호흡기 알레르기로 이행되는 첫 단계이다. 저자는 Oxazolone(Ox)이 반복 도포된 무모마우스, NC/Nga 마우스, Flaky tail 마우스와 같은 세 종류의 아토피피부염 동물모델에 집먼지진드기의 한가지인 *Dermatofagoides pteronyssinus* (Dp)를 피부에 도포하고 흡입시켜서 아토피행진 동물모델을 만들 수 있는지, 이 동물모델의 각질층에 산성크림을 지속적으로 도포하여 적절한 산성환경을 유지하였을 경우에, 아토피행진을

효과적으로 예방할 수 있는지에 대한 연구를 진행하였다. 각질층의 산도를 유지하기 위하여 구연산과 수산화나트륨을 섞어서 만든 산성(pH2.8) 및 중성(pH7.4) 크림을 아토피행진 동물모델에 하루 두번씩 도포하였다. Dp를 반복적으로 도포시킨 후 흡입시킨 아토피피부염 동물모델에서 호흡기알레르기가 발생하였다. 산성크림을 도포하여 각질층의 산도를 유지하자 아토피행진을 예방하는 효과를 얻었고, 중성크림을 도포하였을 때는 이를 더욱 악화되는 결과를 보였다. 결론적으로 Oxazolone을 반복 도포한 무모마우스, NC/Nga 마우스, Flaky tail 마우스와 같은 아토피피부염 동물모델에 지속적으로 Dp를 국소도포하고 흡입시켜서 아토피행진 동물모델을 만들 수 있으며, 각질층의 산도를 적절하게 유지하는 방법으로 아토피행진을 효과적으로 억제할 수 있었다.

핵심 되는 말: 아토피행진, 호흡기 알레르기, 아토피피부염, Flaky tail 마우스, NC/Nga 마우스, Oxazolone, 집먼지진드기

Publication list

Lee HJ, Yoon NY, Lee NR, Jung M, Kim DH, Choi EH. Topical acidic cream prevents the development of atopic dermatitis and asthma-like lesions in murine model. *Exp Dermatol.* 2014;23:736–741

Lee HJ, Lee NR, Jung M, Kim DH, Choi EH. Atopic March from Atopic Dermatitis to Asthma-Like Lesions in NC/Nga Mice is Accelerated or Aggravated by Neutralization of Stratum Corneum but Partially Inhibited by Acidification. *J Invest Dermatol.* 2015;135(12):3025-33