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# Changes of Antimicrobial Peptides and Transepidermal Water Loss After Topical Application of Tacrolimus and Ceramide-dominant Emollient in Patients with Atopic Dermatitis

Increased transepidermal water loss (TEWL) and downregulated antimicrobial peptides (AMPs) are observed in patients with atopic dermatitis (AD). Tacrolimus and ceramide-dominant emollients are effective in the treatment of AD by preventing the production of inflammatory cytokines and by correcting skin barrier dysfunctions, respectively. Present study was designed to investigate the relationship between antimicrobial and barrier factors by measuring the changes of AMPs and TEWL after topical application of tacrolimus and ceramide-dominant emollient in the patients with AD. A total of three patients with AD were treated with tacrolimus in one lesion and ceramide-dominant emollient in another lesion for 4 weeks. RT-PCR and western blotting revealed that the mRNA and protein expression levels of hBD-2 and LL-37 were increased on the both study sites. Immunohistochemical analysis showed significant increase of AMPs and IL-1$\alpha$, while, IL-4 was decreased on the both study sites. The mean changes of TEWL and AMPs showed no statistical difference between both sites. Tacrolimus and ceramide-dominant emollient influence on both TEWL and AMPs expression in patients with AD, namely they have similar effects on both of the two. This study shows that restoration of permeability barrier function is accompanied by the concomitant improvement of antimicrobial defense in patients with AD.

Key Words : *Antimicrobial peptide; Dermatitis, Atopic; Ceramides; Permeability Barrier; Tacrolimus*



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## INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with impaired cell-mediated immune function and compromised skin barrier function. The incidence of AD is generally considered to be increasing worldwide (1, 2).

The presence of a defect in cutaneous permeability barrier function is generally viewed as a downstream consequence of inflammation ("inside-outside" hypothesis) (1). Yet, alternatively, it has been hypothesized that the xerosis and permeability barrier abnormality, or both could drive disease activity in AD and other inflammatory dermatoses ("outside-inside" hypothesis) (3). The primary cytokines, such as, IL-1$\alpha$ and IL-1$\beta$ initiate the cytokine cascade in AD (4). IL-4 inhibits ceramide synthesis, and exogenous applications of the IL-4 impede permeability barrier recovery after acute perturbations (5, 6). Two members of human AMPs, hBD2 and hCAP18 (LL-37) are down-regulated in a Th2-dependent fashion in AD. They are co-localized with lipids within epidermal lamellar bodies before their secretion, hence some hypothesized that these two functions could be linked, and explored the relationship between the cutaneous permeability and antimicrobial barriers (7-9).

Recently, several studies have shown that permeability barrier function and antimicrobial defense are not discrete but rather regulated in parallel (7, 10, 11). However, there are no reports confirming that the mechanism is working in patients with AD. Here, we investigated whether restoration of permeability barrier is accompanied by the concomitant improvement of antimicrobial defense in patients with AD.

## MATERIALS AND METHODS

### Subjects

The diagnostic criteria for AD were established according to Hanifin and Rajka (12). The study protocol was approved

Table 1. Characteristics of subjects at study entry

| Type | Sex/Age (yr) | Duration (yr) | SCORAD | Biopsy site |
|---|---|---|---|---|
| 1 | F/24 | 11 | 20.1 | Popliteal |
| 2 | M/22 | 15 | 29.5 | Popliteal |
| 3 | F/14 | 7 | 23.1 | Buttock |

SCORAD, Severity Scoring of Atopic Dermatitis.

by the Ethical Committee of Chung-Ang University Hospital Institute Review Board (I2007014). Informed consents were obtained from all subjects after they were fully informed about the details and the potential risk of the study. The characteristics of subjects are summarized in Table 1. Two, clinically similar and symmetric lesions of the patients were selected, after measuring transepidermal water loss (TEWL), punch biopsies were performed on the involved skin of 3 patients with AD. Thin layers of tacrolimus ointment 0.1% (Protopic) and ceramide-dominant emollient (TriCeram) were applied twice daily for 4 weeks, respectively.

### TEWL assessment

To assess epidermal permeability barrier function, we performed measurements of TEWL, which have been utilized to provide information about status of permeability barrier under either normal, experimentally perturbed, or diseased conditions.

The published guidelines for the measurement of TEWL were followed (13). In detail, all subjects' conditions were first stabilized for 15 to 20 min in a climate- and humidity-controlled room. Ambient temperature ranged between 21℃ and 24℃, with a mean relative humidity of 45%. TEWL was measured with a Tewameter TM 210 (Courage & Khazaka, Cologne, Germany) and estimated over 2 representative involved sites, tacrolimus-applied skin and emollient-applied skin, respectively.

### Reverse transcription-polymerase chain reaction (RT-PCR)

The tissues from punch biopsy were cut by scissor. Total RNA was isolated from skin using TRIZol reagent (Invitrogen, Carlsbad, CA, USA) after adding 1 mL of TRIZol reagent. And the tissue was homogenized by homogenizer. After 5 min at room temperature, 0.2 mL of chloroform per 1mL of TRIZol reagent was added. Tubes were shaken vigorously. The mixtures were centrifuged with 12,000 rpm at 4℃ for 20 min, the upper aqueous phase were transferred to a fresh tube, and the same amount of 2-propanol was added. After the mixture was incubated at 4℃ for 15 min, it was centrifuged with 12,000 rpm for 15 min. The supernatant was removed, then the RNA pellet was washed with 70% ethanol and centrifuged with 12,000 rpm at 4℃ for 15 min, and briefly dried. The purified RNA was dissolved in DEPC-DW. Total cellular RNA was reverse transcribed at 42℃ for 30 min in containing reverse trancriptase (TaKaRa, Shiga, Japan), 10×buffer, 10 mM dNTP (dNTP mix), oligo dT primer, RNase inhibitor, and 25 mM $MgCl_2$.

RT-PCR analysis of LL-37 and hBD-2 mRNA using specific primers were performed.

We synthesized the PCR primer on the basis of GenBank data. Primers were chemically synthesized by DNA synthesizer (Pharmacia, Biogatan, Uppsala, Sweden). Their sequences were as follows:

hBD-2 (128 bp),
5′-ATC TCC TCT TCT CGT TCC TC-3′ (sense)
5′-ACC TTCTAG GGC AAA AGA CT-3′ (anti-sense);

LL-37 (208 bp),
5′-CTG ATG CCT CTT CCA GGT GT-3′ (sense)
5′-GAG GGA GCC CTT TCT GAA TC-3′ (anti-sense);

GAPDH (593 bp),
5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ (sense),
5′-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3′ (anti-sense).

Two μL of each cDNA sample from the RT-PCR was amplified by PCR in containing *Taq* polymerase (TaKaRa), 10×buffer, 25 mM $MgCl_2$ and 10 pM primer.

### Electrophoresis

The products were run in 1.5% agarose gel contain 1 μg ethidium bromide per millimeter. Twenty μL of reaction mixture was mixed with loading buffer, separated by electrophoresis for 15 min at 100 volts and visualized by UV transillumination. PCR products of hBD-2 and LL-37 were normalized with GAPDH by using densitometer (volume of hBD-2/volume of GAPDH×100, volume of LL-37/volume of GAPDH×100).

### Western blotting

The tissues were cut by scissor. Skin were lysed in a buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/mL phenylmethanesulfonyl fluoride, 1 μg/mL aprotinin, 1% Triton×100, the tissue was homogenized by homogenizer. After centrifuging with 12,000 rpm at 4℃ for 30 min, the supernatant was transferred into new tube, 30 μg of soluble protein were loaded in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with sample buffer containing 1 M Tris and 50% glycerol. Samples were heated at 95℃ for 5 min prior to gel loading. For hBD-2 detection, separated protein on gel electrophoresis was transferred to nitrocellulose membrane (Osmonics, Minnesota, MN, USA) at 0.16 A for 1 hr. The membrane was washed 3 times with Tris-buffered saline Tween 20 (TBST), and blocked with 5% skim milk for 1 hr at room temperature. Following this, the membrane was incubated overnight

at 4℃ with goat anti-hBD-2 polyclonal antibody (1:1,500 in 5% bovine serum albumin, SantaCruz, Delaware, CA, USA) and goat anti-LL-37 polyclonal antibody (1:1,500 in 5% bovine serum albumin, SantaCruz, Delaware, CA) and then washed 3 times with TBST. The secondary mouse anti-goat peroxidase conjugated antibody (1:2,000 in blocking solution, SantaCruz) was incubated for 1 hr at room temperature. After washing with ECL solution (SantaCruz) for 3 min, the membrane was then exposed to radiography film (Roche, Indianapolis, IN, USA).

Table 2. Comparison of mean TEWL levels between tacrolimus-applied and emollient-applied lesion in AD

| Subjects No. | Application | TEWL (g/hm²) | | ΔTEWL |
|---|---|---|---|---|
| | | Before | After | |
| 1 | Tacrolimus | 60 | 46 | 14 |
| | Ceramide | 60 | 44 | 16 |
| 2 | Tacrolimus | 55 | 31 | 24 |
| | Ceramide | 46 | 30 | 16 |
| 3 | Tacrolimus | 36 | 21 | 15 |
| | Ceramide | 34 | 21 | 13 |

ΔTEWL, mean changes of TEWL before and after application.

## Immunohistochemistry (IHC)

IHC was carried out using tissue section of involved sites of atopic patients. In brief, 4 μm thick sections were deparaffinized in xylene 2 times for 15 min each, and epitopes were retrieved by autoclaving (60℃) for 1 hr in citrate-buffered saline (pH 6.0). After 40 min of cooling at room temperature, the activity of endogenous peroxidase was quenched by treatment with 3% $H_2O_2$ in TBST for 10 min. The sections were blocked with normal goat serum for 1 hr, and incubated with mouse anti-human LL-37, hBD-2, IL-1α and IL-4 polyclonal antibodies, respectively. After 5 washes with PBS, the sections were incubated with peroxidase conjugated goat anti-mouse secondary antibody, FITC anti-mouse secondary antibody and color was developed with diaminobenzidine. Two independent blind observers evaluated serial sections. For quantitative analysis, the stained cells were counted in three consecutive microscopic fields (×400).

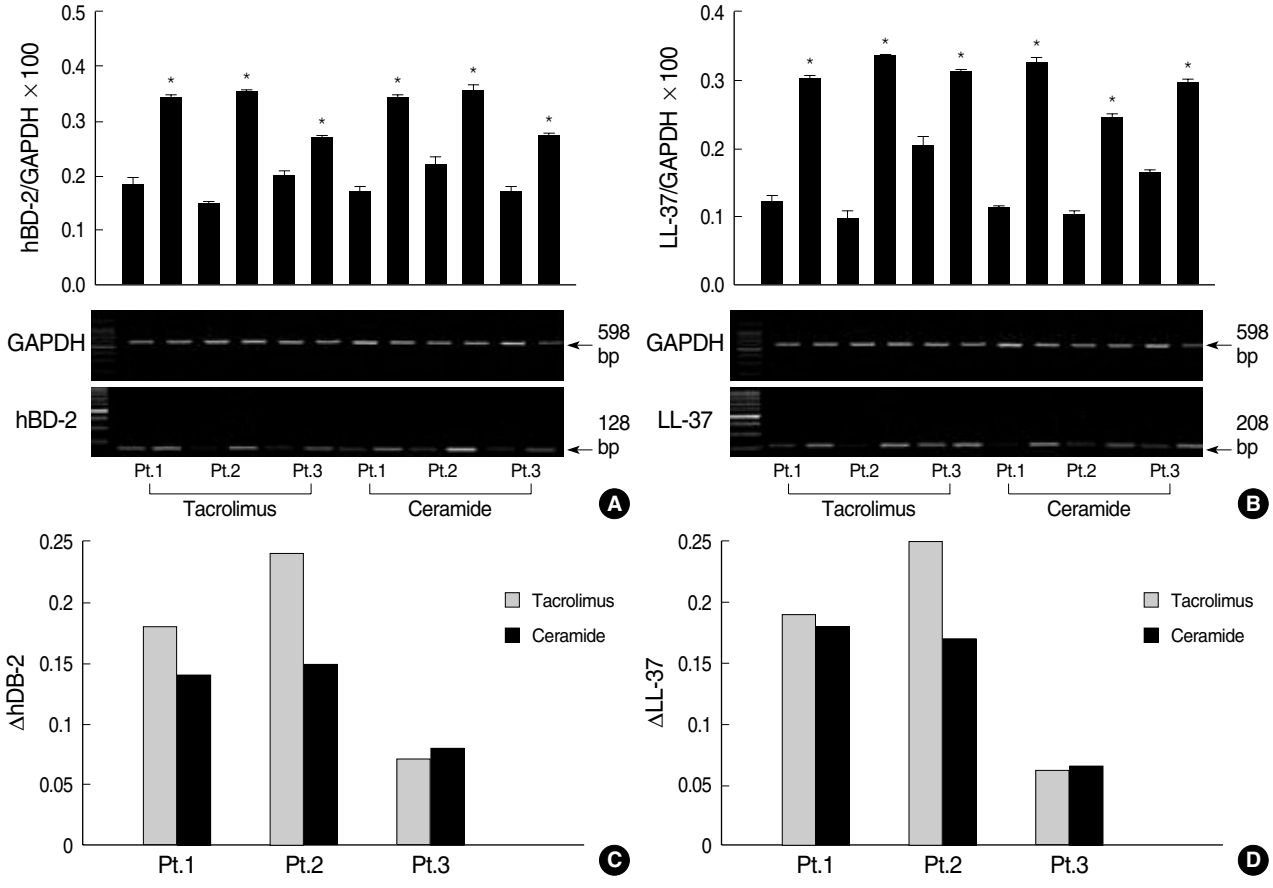


Fig. 1. RT-PCR for expression of hBD-2 and LL-37 before and after application of tacrolimus and ceramide-dominant emollient. (A) Increased expression of hBD after application of tacrolimus and ceramide-dominant emollient. (B) Increased expression of LL-37 after application of tacrolimus and ceramide-dominant emollient (*$P$<0.05). (C) No statistical difference of hBD-2 between tacrolimus-applied and emollient-applied skin. (D) No statistical difference of LL-37 between tacrolimus-applied and emollient-applied skin. Pt., patient.

### Statistical analysis

Statistical analysis was conducted using Wilkoxon T test. Statistical differences in mRNA expression or protein staining of hBD-2 and LL-37 between groups were determined, with significant differences conferred when $P \leq 0.05$.

## RESULTS

### TEWL before and after application of tacrolimus and ceramide-dominant emollient

For comparison of TEWL levels between tacrolimus-applied and emollient-applied skin in atopic patient, measurements were performed 5 times for each site, repeatedly. The mean TEWL levels decreased on the both sites. The mean changes of TEWL were 17.7 g/hm$^2$ (14-24 g/hm$^2$) for tacrolimus-applied lesions and 15 g/hm$^2$ (13-16 g/hm$^2$) for emollient-applied lesions, but there is no statistical difference between tacrolimus-applied and emollient-applied skin (Table 2).

### Expression of LL-37 and hBD-2 before and after application of tacrolimus and ceramide-dominant emollient using RT-PCR method

The expressions of hBD-2 and LL-37 mRNA were increased on the both sites after application of either tacrolimus or ceramide-dominant emollient. The mean changes of AMPs, however, showed no statistical difference between tacrolimus-applied and emollient-applied skin (Fig. 1).

### Expression of LL-37 and hBD-2 before and after application of tacrolimus and ceramide-dominant emollient using Western blotting

The expressions of hBD-2 and LL-37 proteins were increased on the both sites after application either tacrolimus or ceramide-dominant emollient. The mean changes of AMPs, however, showed no statistical difference between tacrolimus-applied and emollient-applied skin (Fig. 2).

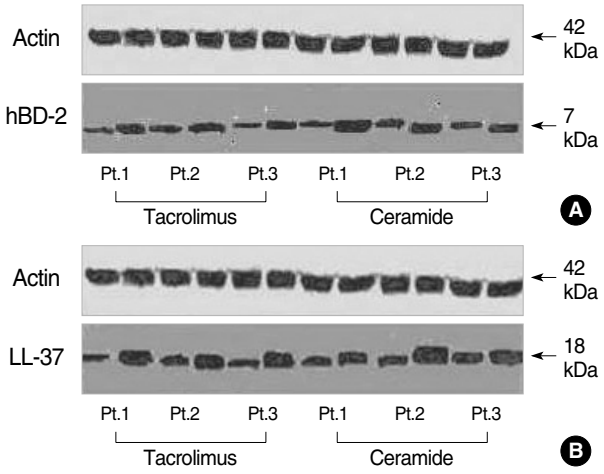


Fig. 2. Western blotting for expression of hBD-2 and LL-37 before and after application of tacrolimus and ceramide-dominant emollient. (A) Increased expression of hBD after application of tacrolimus and ceramide-dominant emollient. (B) Increased expression of LL-37 after application of tacrolimus and ceramide-dominant emollient.

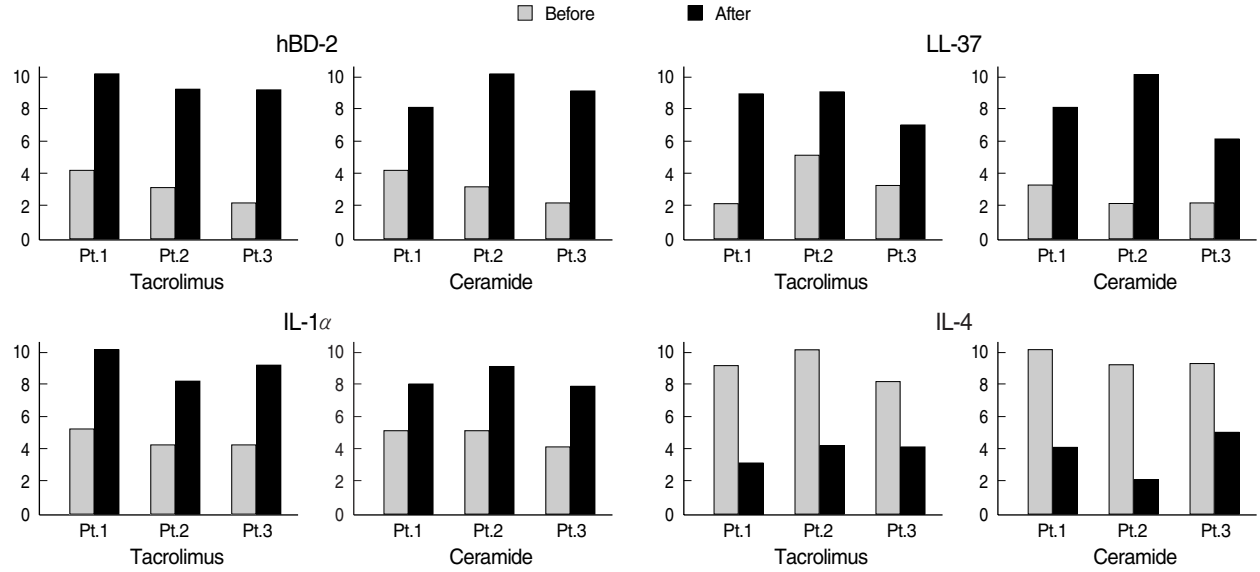


Fig. 3. Immunohistochemical staining for hBD-2 and LL-37 in section from patients with atopic dermatitis. Expression of hBD-2, LL-37, IL-1$\alpha$ and IL-4 receptors before and after tacrolimus and ceramide treatments as assessed by immunohistochemistry. Y axis: numbers of stained cells in microscopic fields ( × 400).

### IHC for LL-37, hBD-2, IL-1$\alpha$, and IL-4 before and after application of tacrolimus and ceramide-dominant emollient

The intensity of staining of LL-37, hBD-2 were significantly stronger in the epidermis after application on the both sites. And the intensity of staining of IL-4 was slightly weaker in the papillary dermis after application (Fig. 3).

## DISCUSSION

Although both patients with AD and psoriasis have altered skin barrier function, patients with psoriasis are more resistant to skin infection; about 30 percent of patients with AD have bacterial or viral infections of the skin, as compared with only 7 percent of patients with psoriasis (14, 15). In psoriatic skin, keratinocytes are rapidly proliferating and differentiating, making epidermis thickened. So, quantitatively, abundant antimicrobial peptides are expressed in the psoriatic epidermis (15, 16). A deficiency in the expressions of AMPs in AD may account for the susceptibility of AD patients to skin infection, such as *Staphylococcus aureus* (9). The inability to increase AMPs may be due to suppression by Th2 cytokines that are elevated in AD. In fact, in vitro keratinocytes in culture could be shown to lose that ability to increase hBD-2 expression when exposed to IL-4 or IL-13 (17, 18). Enhanced Th2 cell activity is a hallmark of acute AD (17). Increased production of IL-4, IL-5, and IL-13 by Th2 cells, in turn, inhibit Th1 cytokine production, including IFN-$\gamma$ and IL-18, which are two beneficial mediators of AMPs. IL-4, IL-13 have also direct inhibitory effects for AMPs expression (19, 20).

When emollients or moisturizers are used in the treatment of AD and other inflammatory dermatoses, the intended aim is limited to an improvement in skin hydration and mitigation of the xerosis. No reduction in inflammatory disease activity is expected, and such activity has been documented only infrequently (21). However, Chamlin et al. (22) reported that ceramide-dominant, barrier repair lipids alleviate childhood atopic dermatitis, and attribute the improvement seen in their patients to a normalization of barrier function. The normalized barrier function, however, dampened the cytokine cascade that initiates and sustains AD. It implied that emollients are helpful in not only skin hydration but also ceasing to cytokine cascade leading to inflammation through normalization of permeability barrier function.

Various immunological parameters are affected in patients with AD: the relative and absolute numbers of circulating T cells are reduced; lymphocyte response to mitogens are impaired; the cytotoxic potential of several cell types, such as monocytes and natural killer cells, is decreased; and chemotatic responsiveness is reduced (17, 20). There are few published data on the effect of tacrolimus ointment on these parameters, although Nghiem et al. (23) have shown that topical calcineurin inhibitors specifically target key immunological mechanisms that have been implicated in the pathogenesis of AD, and act primarily on T cells in lesional skin. Failure of AMPs to up-regulate appropriately in AD is attributed currently to excess Th2 cytokines (9). Tacrolimus suppresses Th2 activity and increases AMPs in AD. Although Kis et al. (24) showed no effect on the IL-1$\alpha$, IL-8, and TNF-$\alpha$ production by tacrolimus in normal human keratinocytes, it is regarded as being fundamental immunologic differences between normal and atopic conditions.

While levels of the IL-1$\alpha$ generally increase in inflammatory dermatoses, they showed paradoxical decline in AD epidermis (25). Recently, Elias et al. (4) proposed the hypothesis that increase in serine protease activity would generate active forms of IL-1, which is a primary contributor to inflammation in AD. However, the results in this study support the opposite conclusion about the cytokine milieu of AD; elevated skin production of Th2 cytokines and low levels of proinflammatory cytokines, such as, TNF-$\alpha$, IFN-$\gamma$, and IL-1. And it is also reported that IL-13 and IL-4 inhibited TNF-$\alpha$ and IFN-$\gamma$-induced hBD-3 production (20).

The antimicrobial barrier is intimately linked to the permeability barrier, and as with water egress, pathogen ingress occurs through the extracellular domains (7). We investigated whether improvement of antimicrobial defense by tacrolimus could simultaneously restored permeability barrier, and the study yielded the expected results. There are several reports supporting the hypothesis that the permeability and antimicrobial barriers of the skin may not be discrete, but rather co-regulated and even inter-dependent (7, 11). The intimate link between these two functions is likely originated from the co-assembly of AMP with barrier lipid precursors within nascent lamellar body (7, 8). The metabolic response of AMP parallels the metabolic response of the lipid to acute disruption of permeability barrier. When the permeability barrier is perturbed by solvent treatment or tape stripping, a sequential, lipid-synthetic response ensues in the underlying epidermis that rapidly restores normal permeability barrier homeostasis (26). Permeability disruption not only removes lipid, but it also initiates a concomitant and rapid loss of AMP from stratum corneum (4). According to the 'outside-inside' hypothesis, the barrier abnormality first provokes and sustains AD through activation of an epidermis-initiated cytokine cascade, followed by recruitment of the specific immunologic response (3).

This study showed that ceramide-replacement improved not only permeability barrier function but also antimicrobial barrier in AD; meanwhile tacrolimus-application improved permeability barrier in addition to antimicrobial barrier. In summary, the restoration of permeability barrier leads the recovery of antimicrobial barrier, and the recovery of antimicrobial barrier leads the restoration of permeability barrier. Through the present study, we could support the theory about interrelationship between antimicrobial barrier and permeability barrier in AD patient. Yet, further studies about

the intermediate process linking antimicrobial barrier to permeability barrier (e.g., inflammatory cells, cytokines, skin pH, filaggrin, serine protease) are required.

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