Interleukin-1α Stimulation Restores Epidermal Permeability and Antimicrobial Barriers Compromised by Topical Tacrolimus

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Directed by Professor Eung Ho Choi

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Table of Contents

List of Figures

List of Tables

ABSTRACT

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Background: Currently tacrolimus has been widely used for many dermatologic diseases including atopic dermatitis, vitiligo, and psoriasis. Tacrolimus has anti-inflammatory and immunosuppressive effects comparable to glucocorticoids but with fewer side effects. Our previous study showed that barrier recovery was delayed after acute barrier disruption in skin treated by topical calcineurin inhibitors (TCIs), including tacrolimus. In that study, the epidermis of hairless mice treated with tacrolimus showed the decrease of number and secretion of lamellar body (LB), lipid

synthesis-related enzymes, the expression of antimicrobial peptides (AMPs) and interleukin 1α (IL-1α). IL-1α is an important cytokine in improving barrier function, LB production, and lipid synthesis in keratinocytes.

Objectives: We aimed to evaluate whether IL-1α stimulation would restore the barrier dysfunction observed in tacrolimus-treated skin.

Methods and Results: In humans, topical tacrolimus was applied twice daily for five days, followed by the individual application of topical imiquimod cream and control cream from immediately after tape stripping until acute barrier disruption. Topical imiquimod accelerated barrier recovery compared to the control. In hairless mice, topical tacrolimus was applied twice a day and topical imiquimod was done concurrently once a day on one flank and a control cream on the other flank for four days. Topical imiquimod improved epidermal permeability barrier homeostasis compared to the control. Imiquimod-treated epidermis displayed an increase in LB number and lipid synthesis-related enzymes such as HMG-CoA reductase, serine palmitoyl transferase, and fatty acid synthases. Imiquimod also increased the expression of AMPs (CRAMP, mBD3) and IL-1α. Furthermore, intracutaneous injection of IL-1α restored permeability barrier recovery. In IL-1 type 1 receptor knockout (KO) mice, topical imiquimod failed to restore permeability barrier recovery after tacrolimus treatment.

Conclusion: IL-1α stimulation induced positive effects on epidermal permeability and antimicrobial barrier functions in tacrolimus-treated skin. These positive effects were mediated by an increase in epidermal lipid synthesis, LB production, and AMP

expression. These findings have clinically important implication that an IL-1α inducer such as imiquimod could prevent barrier dysfunction in tacrolimus-treated skin

Key Words: IL-1α, imiquimod, topical tacrolimus, skin barrier, antimicrobial peptide

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I. Introduction

Skin has a major role in keeping an intact barrier from the external environment and the organism[1]. Permeability barrier is localized to the outermost, anucleated layers of the epidermis, the stratum corneum, and it is mediated primarily by extracellular, nonpolar, lipid-enriched lamellar membranes that are impermeable to water[1]. A various insults, including mechanical trauma, produced by tape stripping, or contact with either solvents or detergents, can injure the stratum corneum, resulting in acute perturbations of cutaneous permeability barrier function. Disruption of the permeability barrier stimulates a vigorous homeostatic repair

response in the underlying viable epidermis, thereby leading to the rapid restoration of permeability barrier function[2]. Preformed lamellar bodies from cells of the outer stratum granulosum rapidly secreted[3]. This secretory response is followed by an increase in the mRNA levels and activity of key enzymes such as 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, fatty acid synthase and serine palmityl transfeases, which results in a marked increase in epidermal cholesterol, fatty acid, and ceramide synthesis[4]. This increased lipid synthesis provides the key lipids-cholesterol, phospholids, and glucosylceramides that are required for the formation of new lamellar bodies. Human epidermis expresses two major families of AMPs, the β-defensin and cathelicidin[5]. Although both are expressed at low levels in unperturbed skin, higher levels occur in healing wounds and in inflammatory dermatoses such as psoriasis[6]. β-defensins and catelicidins exhibit potent, overlapping antimicrobial activity against a variety of gram-negative and gram-positive bacteria, yeasts, and viruses[7]. While all four β-defensins are expressed in epidermis, hBD2 and hBD3 predominate in the outer epidermis and stratum corneum. hBD2 has been further shown to translocate from the endoplasmic reticulum to lamellar body following interleukin-1α stimulation and then to further localize to stratum corneum membrane domains in inflammatory dermatoses. With regard to further potential links between different protective functions, IL-1α release is an inevitable accompaniment of external perturbations of the stratum corneum[8].

Cushing's syndrome and prolonged topical or systemic treatment with glucocorticosteroids (GC) produce a variety of well-recognized cutaneous abnormalities including cutaneous atophy, increased skin fragility, and increased

risk of infection. GC therapy also perturbs epidermal differentiation, resulting in a decrease in keratohyalin granule formation, as well as a reduced expression of various protein markers of epidermal differntation[9]. Moreover, prolonged GC therapy increases basal TEWL, indicating a defect in permeability barrier function[10]. The decrease in barrier function associated with prolonged GC therapy has been associated with a decrease in the thickness of the stratum corneum, a reduction in stratum corneum lipids, and a decrease in the number of lamellar bodies in stratum granulosum cells[11]. In addition to negative effects on barrier homeostasis, topical GC exert negative effects on both the integrity and cohesion of the stratum corneum. The decrease in stratum corneum integrity and adhesion is clinically significant, because it would increase the susceptibility of the skin to injury form relatively minor insults such as those that occur with exposure to solvents, detergents, or mechanical forces[12]. The basis for the GC-induced abnormality in stratum corneum integrity and cohesion appears to be a reduction in the number of corneodesmosomes in the straum corneum[12].

Tacrolimus, an inhibitor of phosphatase calcineurin, has been emerged as an effective and safe topical therapeutic agents for atopic dermatitis[13 14]. Tacrolimus is the first topical immune suppressant that is not a derivative of hydrocortisone, the key component in dermatological treatment for nearly 50 years[15]. Tacrolimus does not provoke glucocorticoid-related side effects such as skin atrophy and telangiectasiae. However, tacrolimus does not prevent viral skin infections such as eczema herpeticum which can be life-threatening, while it reduces the incidence of bacterial skin infections compared to other treatments[16-17]. We previously showed

that tacrolimus negatively affected skin barrier function including permeability and antimicrobial functions, which are mediated by the down-regulation of epidermal lipid synthesis, lamellar body (LB) secretion, and the epidermal expressions of mBD3 and CRAMP, two major epidermal antimicrobial peptides. Topical tacrolimus also suppressed the expression of interleukin 1α (IL-1α), suggesting that it acts on skin barrier function[18].

IL-1α is a proinflammatory and immunomodulatory cytokine which plays an important role in inflammatory diseases of the skin, including bacterial infections, bullous diseases, UV damage and especially psoriasis[19]. IL-1 significantly regulated 388 genes, including genes associated with proteolysis, adhesion, signal transduction, proliferation, and epidermal differentiation[19]. In keratinocyte, IL-1α is stored intracellularly, but can be quickly released in case of epidermal infection or injury. Released IL-1 serves as a paracrine signal to fibroblast to produce prostaglandins and collagenase, to endothelial cells to express selectins, and guides the chemotaxis of lymphocytes toward the site of injury[20]. The IL-1 released from keratinocyte also serves as an autocrine signal to the surrounding, undamaged keratinocytes, stimulating them to become activated. Activated keratinocytes become migratory and hyperproliferative to produce growth factors and cytokines that regulate inflammatory and wound healing processes. When added to cultured human keratinocytes, IL-1α stimulates the synthesis of epidermal lipids, the expression of CCL20, and the production of a potent bacteriostatic agents[21].

Imiquimod, a nucleoside analogue of the imidazoquinoline family, has shown efficacy against many tumor entities. The major biologic effect of imiquimod are

mediated through agonistic activity towards toll-like receptors 7 and 8, and consecutively, activation of nuclear factor-kappa B. Imiquimod induces the gene products include the pro-inflammatory cykokines IFN- α, TNF- α, IL-1α, IL-2, IL-6, IL-8, IL-12, granulocyte-colony stimulating factor and granulocyte macrophage-colony stimulating factor, as well as chemokine such as CCL3, CCL4, and CCL2[22-23]. In general, imiquimod leads to marked elevation of numerous gene products involved in the regulation of innate immune function[24]. Additional effects attributable to the TLR-dependent activation of NF-κb by imiquimod include enhanced expression of the opioid growth factor receptor as well as changes in the epidermal barrier function[25].

Since IL-1α production and the epidermal permeability barrier are closely linked, we hypothesized that decreased IL-1α production observed in tacrolimus-treated skin would be attributed to abnormal skin barrier function.

II. Materials & Methods

1. Imiquimod application and functional study

For the human study, fifteen volunteers (20-50 years old) without skin disease were recruited. This study was conducted according to the Declaration of Helsinki Principles. The medical ethical committee of Institutional Review Board (IRB) of Yonsei University Wonju College of Medicine approved all described studies. All participants granted written informed consent. Subjects applied 0.03 % tacrolimus cream (Protopic®, Fujisawa Healthcare, Deerfield, IL, USA) on both sides of the volar surface of the forearms twice daily for five days. Twenty-four hours after the final application, 2.5 % imiquimod cream, which was made by mixing 5 % imiquimod cream (Aldara®, 3M Health Care, St Paul, MN, USA) with Cetaphil® cream (Galderma, Les Templiers, France), was applied on one forearm, and plain Cetaphil® cream was applied to the other forearm as a control cream immediately after tape stripping (TS). Basal transepidermal water loss (TEWL) and barrier recovery rate values were measured six hours after acute barrier disruption by TS using Tewameter TM 210 (Courage and Khazaka, Cologne, Germany)[26-28]. The baseline value of TEWL of normal human skin is 7.2±0.48 g/m^2 per hour. The measurement conditions at room temperature ranged between 20 ° and 23 ℃ with a relative humidity between 55 % and 58 %.

In the animal study, female hairless mice (Skh1/Hr) were housed in the animal laboratory of Yonsei University Wonju College of Medicine. Transgenic animals knocked out for the IL-1α functional (Type 1) receptor and wild-type age-matched controls were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Yonsei University Wonju Campus Institutional Animal Care and Use Committee (IACUC) approved this animal experiment. We subdivided the mice into two groups. One group (n=10) represented mice treated with the combination of tacrolimus and imiquimod on one flank, and tacrolimus alone on the other flank. The other group (n=10) of mice was treated with only imiquimod on one flank and a control on the other flank. In the former group, both flanks of the hairless mice were treated with 0.03% tacrolimus (Protopic®) twice daily. Then 2.5 % imiquimod was applied once daily on one flank and a control cream (Cetaphil®) was applied on the other flank for four days. In the latter group, only 2.5 % imiquimod was applied once daily on one flank and a control (Cetaphil®) on the other flank for four days. Twenty-four hours after the last application, basal TEWL and SC integrity, which was determined by TEWL after stripping with D-Squame tape (CuDerm, Dallas, TX, USA), were measured. The barrier recovery rate was determined six hours after TS. Skin specimens were taken from all the hairless mice and processed by electron microscopy (EM), immunohistochemical staining of IL-1α, mBD3 and CRAMP, real time RT-PCR for mRNAs of mBD3, CRAMP, and epidermal lipid synthesis related enzymes.

2. IL-1α intracutaneous administration

Flanks of hairless mice (n=6) were treated with topical 0.03 % tacrolimus or petrolatum twice daily for four days. Twenty-four hours after the final application, IL-1α (Sigma-Aldrich, Inc. St. Louis, MO, USA) (50 ng in 100 μL PBS) (n=6) or PBS (100 μL) (n=6) was injected intracutaneously into the flank of the mice. Tape stripping was performed five minutes after IL-1α or PBS injection and the barrier recovery rate was measured after six hours. The dose of IL-1α (50 ng) was chosen based on previous experiment[25].

3. Electron microscopy (EM) and quantitative analysis

Samples for EM were processed using 2 % aqueous osmium tetroxide postfixation, as described previously[29]. In order to exclude subjective bias in these morphologic studies, we quantitated both corneodesmosome length and lamellar body number in EM pictures using a previously described objective method[29]. Five EM pictures taken at the same magnification (20,000x) were analyzed and compared between the 2.5 % imiquimod cream and control cream-treated groups.

4. Assay for epidermal lipid synthesis-related rate limiting enzymes

To evaluate the effect of imiquimod on epidermal lipid synthesis in tacrolimus-treated skin, full-thickness murine skin samples were obtained from mice. For the quantitative analysis of 3-hidroxy-3-methylglutaryl-CoA reductase, serine palmitoyl transferase, and fatty acid synthase activity, respective mRNAs were measured using real time RT-PCR.

5. Real time reverse transcription (RT)-PCR

Isolation of the epidermis

Skin samples that were excised from the treated area were immediately placed with the epidermis side down on petri-dishes. Subcutaneous fat was removed with a scalpel, and then the skin samples were placed epidermis side up in 10 ml of 10 mM EDTA pH 8.0 in PBS, at an incubation of 37 ℃ for 35 minutes in order to separate the epidermis from the dermis. The epidermis was finally scraped off with a scalpel and total RNA was extracted[30].

Total RNA preparation and cDNA synthesis

Total RNA was extracted using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol Reagent; Gibco BRL, Grand Island, NY, USA). RNA

concentration was determined by a U.V. spectrometer at 260nm. Aliquots (1.0 ug) of RNA from each sample were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (MML-V RTase, Promega, San Luis Obispro, CA, USA). Briefly, RNA samples were incubated at 80 ℃ for five minutes with molecular biology grade water. After incubation on ice, primer extension and reverse transcription was performed by adding 1X RT-buffer, 2 mM deoxynucleotide triphosphates (dNTPs), 0.2 pM random hexamer primer (Promega, CA, USA), and MML-V RTase (2.5units/ul) in 20 ul reaction volumes. Samples were then incubated at 42℃ for 45 minutes before storage at -20℃[31].

6. Quantitative PCR analysis of gene expression

The expression of specific mRNAs was quantified using a Rotor-Gene™ 3000 (Corbett Life Science, Sydney, Australia). Briefly, 10 ul PCR reactions were set up containing Quantitect probe PCR Master mix (Qiagen, Hilden, Germany) in a 2X solution, 8 mM manganese chloride, 200 uM deoxynucleotide triphosphates (dNTPs), 1.25 units HotstartTaq polymerase, and 0.5 pM/ul each of probes and primers. About 60 ng of cDNA were used per reaction. All reactions used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, provided as an optimized control probe labeled with TAMRA (Operon Biotechnologies, Cologne, Germany), enabling data to be expressed in relation to an internal reference to allow for differences in sampling. All fluorogenic probes for genes of interest were labeled with 6-carboxyfluorescein (6-FAM). 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 used to determine ΔCt values (Ct of target gene−Ct of housekeeping gene) as raw data for gene expression. Fold change in gene expression was determined by subtracting ΔCt values for imiquimod-treated samples from their respective control cream-treated samples. The resulting ΔCt values were then used to calculate fold change in gene expression as $2^{-\Delta\Delta Ct}$. All reactions were performed in triplicate and the results are expressed as the mean of values from three separate experiments. Samples were amplified using primers and probes under the following conditions: 95 ℃ for 15 minutes followed by 45 cycles of 95 ℃ for 15 seconds and 60 ℃ for 1 minute[31-32].

7. Immunohistochemical staining

Skin specimens were fixed in 10 % formalin solution and embedded in paraffin. Sections of 5 ㎛ thickness were cut and stained with primary antibodies for IL-1α (SantaCruz, CA, USA), mBD3 (SantaCruz, CA, USA), and CRAMP (SantaCruz, CA, USA). Briefly, after de-paraffinization, the sections were rehydrated sequentially with 100 %, 90 %, and 70 % ethanol and incubated for five minutes in 3 % H_2O_2 in Tris-buffered saline (TBS) to inactivate endogenous peroxidases. Samples were then blocked for 10 minutes with blocking serum solution (DAKO, Carpinteria, CA, USA) and incubated overnight at 4 ℃ with a primary antibody. After several washes in TBS, samples were incubated for 30 minutes with a secondary biotinylated antibody. The

antigen was visualized with the avidin-biotin complex system (Vector, Burlingame, CA, USA), according to the manufacturer's instructions, by using 3,3'-diaminobenzidine tetrahydrochloride as the substrate. Samples were examined under a light microscope[31, 33].

8. Statistical analyses

All data are expressed as mean ± SEM. Statistical analyses were performed using paired and unpaired students' t-tests. $P<0.05$ was considered statistically significant.

Table 1. Oligonucleotide primers and probe sequences for real-time RT-PCR

Target gene	**Oligonucleotides**	Sequence
GAPDH	Forward primer	5'-TGCGACTTCAACAGCAA CTC-3'
	Reverse primer	5'-ATGTAGGCCA TGAGGTCCAC-3'
	Probe	5'-TCTTCCACCTTCGATGCCGG-3'
HMG-CoA	Forward primer	5'-CCGAATTGTATGTGGCACTG-3'
	Reverse primer	5'-GGTGCACGTTCCTTGAAGAT-3'
	Probe	5'-CTTGATGGCAGCC TTGGCAG-3'
FAS	Forward primer	5'-CTGAAGAGCCTGGAAGATCG-3'
	Reverse primer	5'-TGTCACGTTGCC ATGGTACT-3'
	Probe	5'-TGAGCTTTGCTG CCGTGTCC-3'
SPT	Forward primer	5'-GAGAGATGCTGAAGCGGAAC-3'
	Reverse primer	5'-TGGTATGAGCTGCTGACAGG-3'
	Probe	5'-TGGGATTTCCTGCTACCCCG-3'

GAPDH, glyceraldehydes-3-phosphate-dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; FAS, fatty acid synthases; SPT, serine palmitoyl transferase

III. Results

1. Topical imiquimod restored epidermal permeability barrier recovery that had been inhibited by tacrolimus treatment in human and murine skin

Our previous report showed that tacrolimus disrupted epidermal permeability barrier homeostasis and decreases IL-1α in murine epidermis[31]. In this study, we first assessed whether topical imiquimod, an IL-1α activator, restored permeability barrier function in tacrolimus-treated skin. Imiquimod restored permeability barrier recovery in human skin (Figure 1a). We next assessed the effects of imiquimod on tacrolimus-treated murine skin. Imiquimod significantly restored permeability barrier recovery. However, imiquimod did not affect barrier recovery in control mice that had not been treated with tacrolimus (Figure 1b).

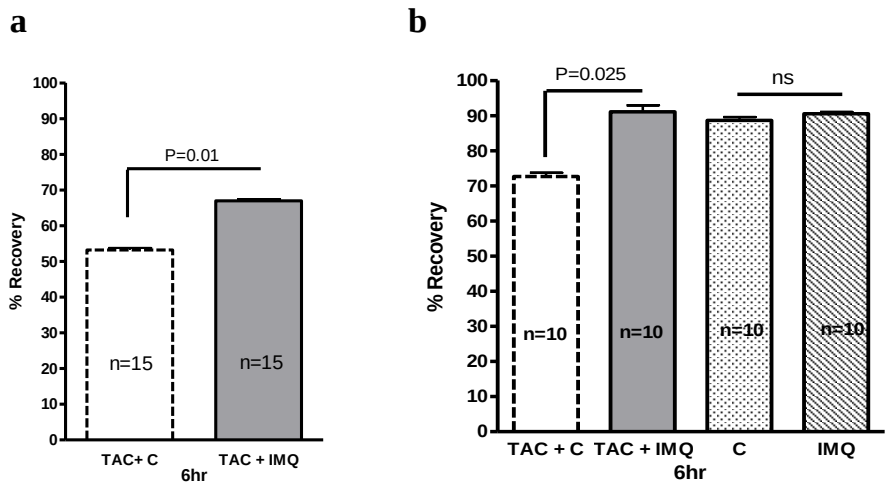


Figure 1. Topical imiquimod restored epidermal permeability barrier delayed by tacrolimus treatment in human and murine skin.

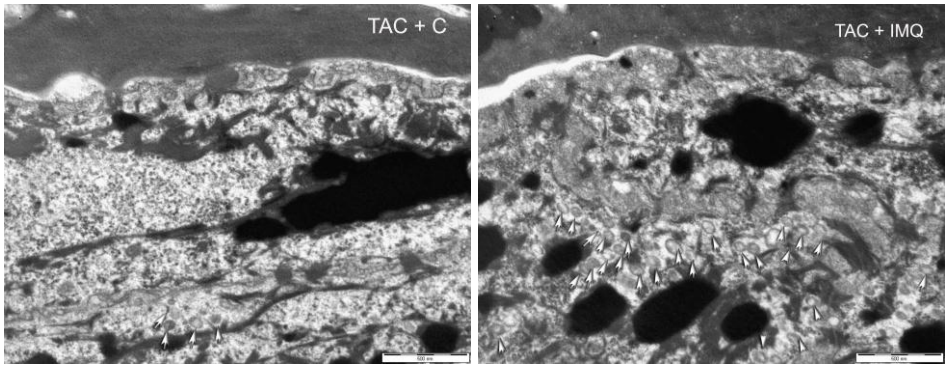
In humans, topical tacrolimus was applied on both forearms twice a day for five days. Immediately after acute barrier disruption, imiquimod was applied on one forearm, and the control on the other forearm. Barrier recovery rates were measured after six hours. Imiquimod restored the delayed barrier recovery induced by tacrolimus in human skin (n=15) (a). In the animal study, one group consisted of flanks of mice treated with tacrolimus twice daily and then imiquimod once daily on one flank with a control cream on the other flank for four days. The other group was treated the same way minus tacrolimus application. As seen in human, the barrier recovery rates improved in tacrolimus and imiquimod-treated skin (n=10) (b). The values represent mean ± SEM.

2. Topical imiquimod stimulated epidermal lipid production that had been decreased by tacrolimus treatment in murine skin

Using an LB counting and lipid synthesis-related enzyme assay, we examined whether imiquimod would reverse tacrolimus-induced barrier abnormalities by promoting epidermal lipid production. Murine epidermis treated with imiquimod exhibited an increased number (density) of LBs in comparison to control sites treated with an inactive cream (Figure 2a). Quantitative analyses of randomly obtained and coded EM pictures by a blinded investigator also indicated a significant increase in LB density in imiquimod-treated murine skin (Figure 2b).

We examined whether the imiquimod-induced increase in LB production is, in turn, attributed to activated epidermal lipid synthesis. The activities of rate-limiting enzymes for three key epidermal lipids such as cholesterol, ceramides, and free fatty acids that mediate barrier function are normally high in epidermal keratinocytes[2]. Previous study showed that three key enzymes required for epidermal lipid synthesis, 3-hydroxy-3-methylglutaryl-CoA(HMG-CoA) reductase, serine-palmitoyl transferase (SPT), and fatty acid synthase (FAS), decreased after tacrolimus treatment compared to controls[31]. We found that the mRNA expression of HMG-CoA reductase, SPT, and FAS was measured, finally confirmed that the mRNA levels for these three key enzymes increased after imiquimod treatment (Figure 3).

a



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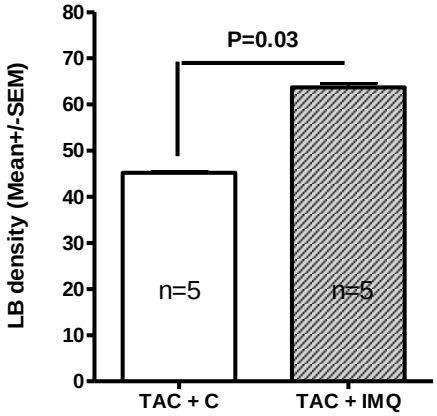


Figure 2. Topical imiquimod increased the density and content of LBs in tacrolimus-treated murine skin.

Both flanks of hairless mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for four days. Biopsy samples were taken from tacrolimus-treated skin with or without imiquimod and processed for EM to analyze LB concentration. Epidermis treated with imiquimod shows an increased number (density) of LBs (white arrows) in comparison

to the control (a). Quantitative analysis of randomly obtained and coded EM pictures showed a significant increase in LB density in imiquimod-treated murine skin (b). The values represent mean ± SEM (n=5 in each group, bar=2μm).

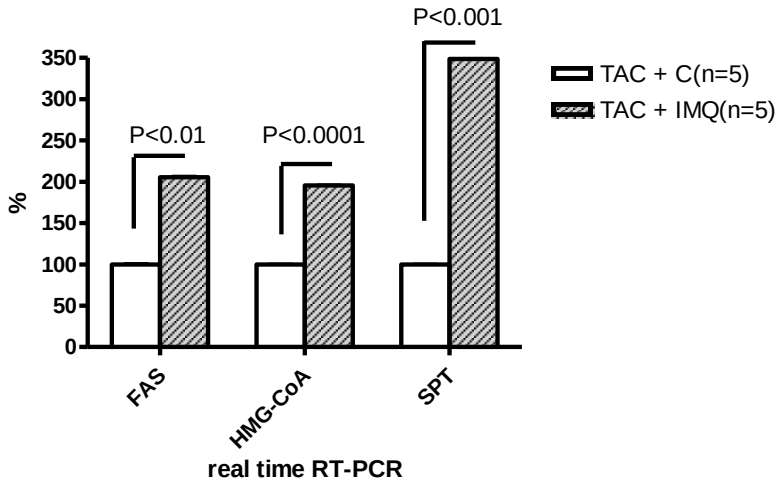


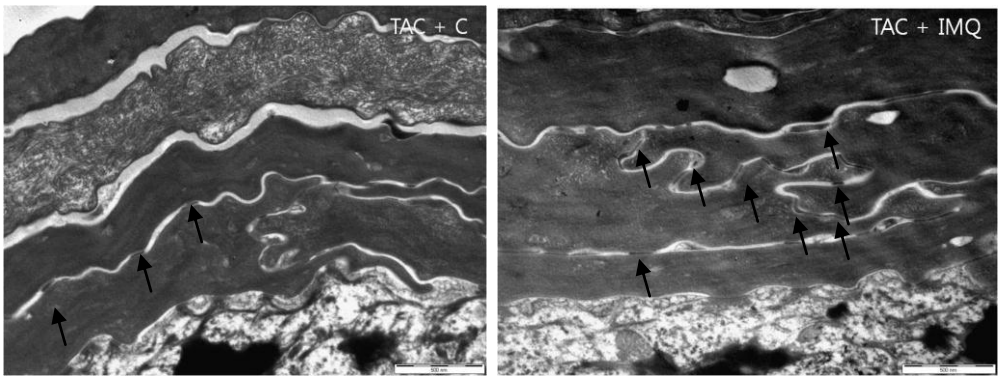
Figure 3. Topical imiquimod increased epidermal lipid synthesis-related enzymes in tacrolimus-treated murine skin.

Both flanks of hairless mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for four days. Biopsy samples were taken from tacrolimus-treated skin with or without imiquimod and assayed with quantitative RT-PCR to assess the mRNA levels of epidermal lipid synthesis related enzymes. mRNA levels in murine epidermis treated with imiquimod increased compared to the control. The values represent mean ± SEM. (FAS: fatty acid synthases, HMG-CoA: 3-hidroxy-3-methylglutaryl-CoA reducatase, SPT: serine-palmitoyl transferase, each group n=5)

3. Topical imiquimod improved SC integrity by restoring corneodosmosomes that had been decreased by tacrolimus treatment in murine skin

Intercorneocyte adhesion, which is mediated largely by corneodesmosomes (CD), a unique intercellular junction modified from epidermal desmosomes [34-35], is important not only for SC integrity, but also for the maintenance of the epidermal permeability barrier. CD density was measured in the lower SC by quantitative EM analysis using a previously described method[29]. We observed that CD density increased in the imiquimod-treated group compared to the control group (Figure 4), indicating that imiquimod improved SC integrity in tacrolimus-treated skin by restoring CD density.

a



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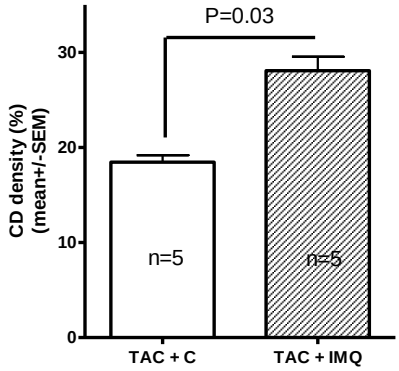

Figure 4. Topical imiquimod restored the corneodesmosome (CD) density decreased in the tacrolimus-treated murine skin.

Both flanks of hairless mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for four days. Biopsy samples were taken from the imiquimod co-applied skin and control skin, and processed for EM (a). CD (black arrows) density was quantitated as described in material and methods. EM images of the murine epidermis co-applied with imiquimod showed longer CDs compared to the control (p=0.03) (b). The values represent mean ± SEM; n=5 in each group, bar=2μm.

4. Topical imiquimod augmented the epidermal expression of IL-1α that had been diminished by tacrolimus treatment in murine skin

IL-1α is an important cytokine for improving permeability barrier function, LB structure, and lipid synthesis in human keratinocytes[25]. In our previous study, we observed that topical calcineurin inhibitors suppressed the epidermal expression of IL-1α[31]. Based on these findings, we measured IL-1α expression using immunohistochemical staining in murine skin co-applied with imiquimod, which means that tacrolimus was applied and followed by imiquimod. Skin sites co-applied with imiquimod showed a much greater expression of IL-1α than the control sites (Figure 5), suggesting that the positive effect of imiquimod on the permeability barrier of tacrolimus-treated epidermis possibly resulted from the augmentation of IL-1α.

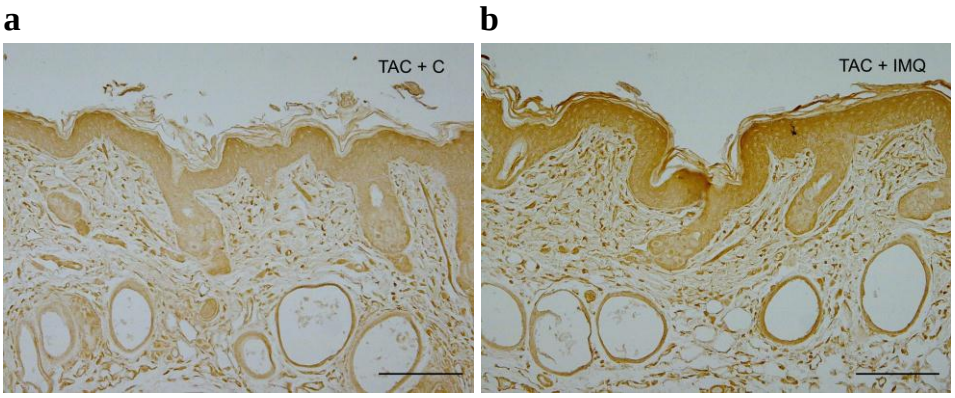

Figure 5. Topical imiquimod increased the epidermal expression of IL-1α decreased by tacrolimus in murine skin.

Both flanks of hairless mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for four days. Biopsy samples were taken from tacrolimus-treated skin with or without imiquimod. Biopsy specimens for the IL-1α immunohistochemical stain were taken from imiquimod or control sites. Imiquimod-treated skin (a) showed more intense IL-1α expression in immunohistochemical stain compared to the control (b). Bar=100 μm.

5. Intracutaneous IL-1α injection restored epidermal permeability barrier recovery that had been inhibited by tacrolimus treatment in murine skin

To further assess the importance of IL-1α on tacrolimus-induced barrier disruption, we compared the barrier recovery rates between intracutaneous IL-1α and vehicle injection in tacrolimus-treated mice and controls.

Barrier recovery kinetics accelerated significantly in IL-1α and tacrolimus-treated mice. This indicated that IL-1α restored the damages induced by tacrolimus. However, the barrier recovery rate was not restored significantly in normal IL-1α level mice injected with IL-1α alone (Figure 6). This result might suggest that IL-1α plays an important role in restoring barrier function impaired by tacrolimus treatment.

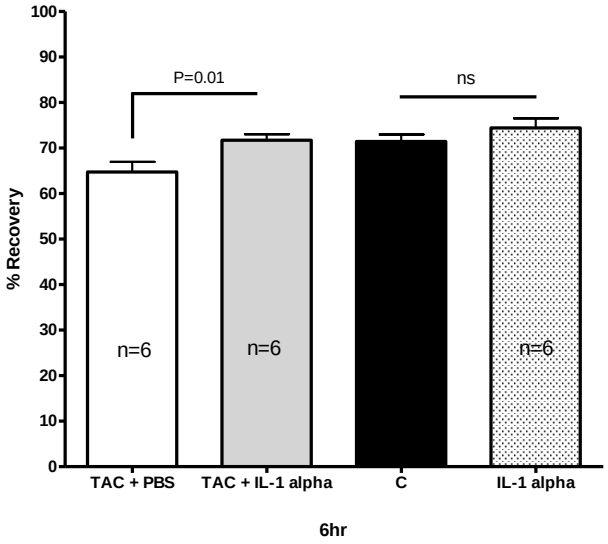

Figure 6. Intracutaneously injected IL-1α restores the delayed permeability barrier recovery induced by tacrolimus in murine skin.

IL-1α and the vehicle (PBS) were injected intracutaneously into the flanks of tacrolimus-treated mice at five minutes prior to tape stripping. In addition, the vehicle was injected intracutaneously into petrolatum-treated mice as a normal control. IL-1α-injected mice exhibited an improvement in barrier recovery compared to the vehicle (n=6).

6. In transgenic IL-1 receptor knockout (KO) mice, topical imiquimod did not restore epidermal permeability barrier recovery that had been inhibited by tacrolimus treatment

To assess whether IL-1α signaling plays a key role in permeability barrier abnormality induced by tacrolimus, permeability barrier recovery rates were compared between IL-1 type 1 receptor KO mice and wild-type mice. IL-1 receptor type 1 KO mice exhibited no significant difference in barrier recovery between imiquimod and control cream-treated sites. In contrast, wild-type mice showed that imiquimod restored permeability barrier recovery delayed by treatment with topical tacrolimus (Figure 7). These findings highlights the role of IL-1α stimulation in restoring barrier function impaired by tacrolimus treatment.

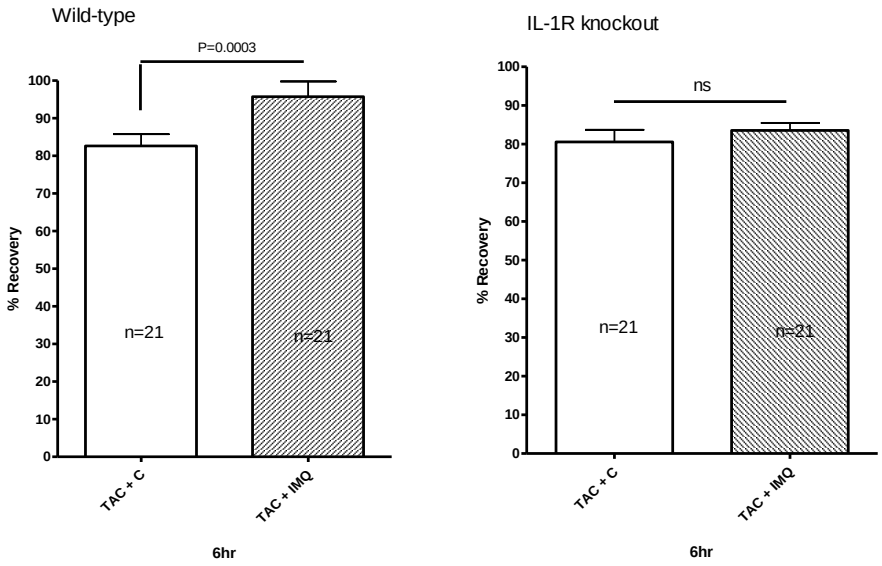

Figure 7. IL-1 receptor KO mice model support evidence that IL-1α signaling mediated the permeability barrier homeostasis inhibited by tacrolimus.

Both flanks of IL-1R type 1 knockout and wild mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for four days. The barrier recovery rates were measured six hours after tape stripping. Imiquimod restored permeability barrier recovery in wild-type mice (n=21). IL-1R type 1 knockout mice exhibited no significant difference in permeability barrier homeostasis between imiquimod and the control (n=21). The numbers represent mean ± SEM.

7. Topical imiquimod restored the expression of mBD3 and CRAMP, two major epiderml antimicrobial peptides that were decreased by tacrolimus treatment in murine skin

The epidermal expressions of mBD3 and CRAMP changed according to permeability barrier status because of their co-localization in the LB[5, 36-37]. Since topical calcineurin inhibitors (TCIs) suppressed the epidermal expression of mBD3 and CRAMP[31], we assessed whether the expression of mBD3 and CRAMP recovers on tacrolimus-treated murine epidermis with the co-application of imiquimod. In immunohistochemical staining, imiquimod co-applied skin showed more intense mBD3 and CRAMP expression compared to the control skin (Figure 8a, b). The mRNA levels of mBD3 and CRAMP of the tacrolimus-treated murine epidermis with the co-application of imiquimod also increased compared to the control skin (Figure 8c).

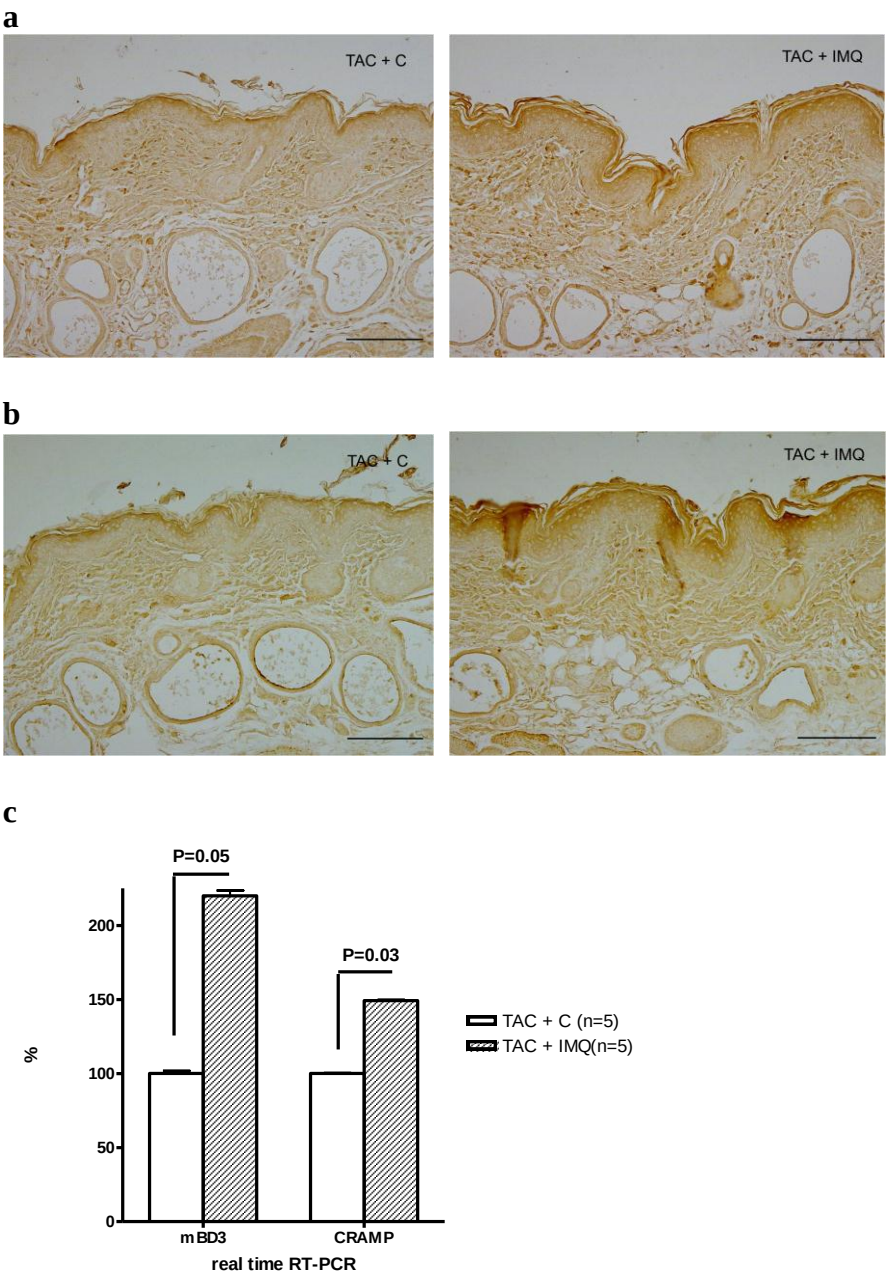

Figure 8. Imiquimod restored the expression of mBD3 and CRAMP that was decreased by tacrolimus in murine epidermis.

Both flanks of hairless mice were treated with tacrolimus twice daily and then imiquimod once daily on one flank, and a control on the other flank for four days. Biopsy samples were taken from tacrolimus-treated skin with or without imiquimod. Biopsy specimens taken from imiquimod or the control groups were processed with immunohistochemical staining for mBD3 and CRAMP, and assayed for real time RT-PCR to assess mRNA levels in the epidermis. Imiquimod-treated skin showed more intense staining for mBD3 and CRAMP expression compared to the control (a:mBD3, b:CRAMP). mRNA levels for mBD3 and CRAMP in imiquimod-treated murine epidermis increased compared to the control (n=5) (c). The numbers represent mean ± SEM. Bar=100 μm.

IV. Discussion

Topical calcineurin inhibitors such as tacrolimus and pimecrolimus are topical immune suppressants that have fewer side effects than topical glucocorticoids, which frequently cause HPA axis suppression, skin atrophy, telangiectasiae, and secondary skin infection[17,38]. TCIs are widely used to treat not only atopic dermatitis, but also other dermatoses including vitiligo and psoriasis[39]. TCIs decrease the incidence of bacterial skin infections such as *Staphylococcous aureus* compared to vehicle[15-17, 40], but do not prevent viral skin infections including potentially life-threatening cases of eczema herpeticum[41-42]. We have recently shown that topical tacrolimus treatment negatively impacts epidermal permeability barrier function and antimicrobial peptide expression in normal skin[31]. Tacrolimus-treated epidermis exhibits delayed barrier recovery in both human and murine skin. Tacrolimus decreases epidermal lipid production, as evidenced by fewer LBs and the reduced activity of lipid synthesis related enzymes. Tacrolimus also suppresses the expressions of mBD3, CRAMP, and IL-1α, suggesting a mechanism for its negative impact on skin barrier function[31].

Emerging evidences showed that topical tacrolimus treatment suppresses cytokine and co-stimulatory molecule expression in epidermal cells[43]. By analyzing its immunosuppressive action mechanisms *in vivo*, they demonstrated that topical tacrolimus suppresses mRNA expression of both primary (IL-1 and TNF-α) and secondary (GM-CSF and MIP-2) epidermal cytokines during the early and late stages of primary contact hypersensitivity responses in mice[44-48]. Rosacea, an inflammatory

skin disease has unknown etiology, which symptoms are exacerbated by the factors triggering innate immune responses such as the release of cathelicidin antimicrobial peptides. Yamasaki et al. showed that individuals with rosacea express abnormally high levels of cathelicidin and serine protease activity in their facial skin[24]. This finding may be related with effectiveness of tacrolimus in rosacea patients through a decrease of AMPs.

Cytokines such as IL-1, IL-6, and TNF-α play a crucial role in signaling the repair response after barrier disruption. Injuries to the epidermis stimulate the secretion of IL-1, IL-6, TNF-α, and other cytokines, which play a crucial role in signaling the repair response after barrier disruption[49]. Cytokine treatment after barrier disruption accelerates barrier repair, perhaps by enhancing epidermal lipid synthesis and the production of LB. Animal models knocked out for cytokines or their receptors displayed delayed barrier repair compared to the wild-type models[50-51]. Imiquimod, a nucleoside analogue of the imidazoquinoline family, has major biological effects through agonistic activity on toll-like receptors 7 and 8, and consecutively, activation of nuclear factor-kappa B, which enhances the induction of proinflammatory cytokines such as IL-1α, IL-6, and TNF-α with other mediators activating antigen presenting cells along with other components of innate immunity. It also stimulates a profound T helper-weighted cellular response[22-23, 52-57].

Based on these results, we first assessed whether the activation of IL-1α by an application of topical imiquimod could restore barrier recovery down-regulated by tacrolimus. We demonstrated that barrier recovery was restored with an application of 2.5% imiquimod cream to the tacrolimus-treated human and murine skin compared to

a control cream (Cetaphil®, Galderma, France). Topical imiquimod failed to potentiate barrier recovery in the normal control. This result demonstrates that the compensation of IL-1α levels decreased by topical tacrolimus restores barrier homeostasis down-regulated by tacrolimus treatment. However, topical imiquimod had no effect on normal skin having a normal level of IL-1α. The other cytokine levels including IL-6 and TNF-α induced by imiquimod treatment, were not checked within this study. We found IL-1α suppression in tacrolimus-treated skin in our previous study and focused on IL-1α stimulation for the recovery of impaired barrier homeostasis in tacrolimus treated skin[31]. The role of other cytokines except IL-1 in tacrolimus-treated skin would be clarified in the future.

In our preliminary study, we observed no differences in barrier recovery between normal mice skin and Cetaphil-applied mice skin. Cetaphil® cream improved stratum corneum hydration but did not affect TEWL[58]. We used 2.5% imiquimod cream instead of 5% imiquimod cream because adverse reactions including erythema and mild weeping were seen in 5% imiquimod-treated mice. The role of IL-1α signaling in tacrolimus-induced permeability barrier dysfunction was assessed by the intracutaneous injection of IL-1α. We observed that intracutaneously injected IL-1α significantly improved barrier recovery in tacrolimus-treated murine skin when compared to controls. Barland et al. reported similar results that topical imiquimod accelerated barrier recovery after acute insults to aged BALB/c mice skin. These results were correlated with increased IL-1α production in the epidermis following topical imiquimod administration. Intracutaneous injection of IL-1α also accelerated barrier recovery in aged mice. The improvement in barrier recovery in young mice

was not as pronounced as it was in aged mice[25]. The definite importance of IL-1α signaling for barrier homeostasis diminished by TCIs was supported by the observation that transgenic mice with knockout of the IL-1 receptor type 1 demonstrated no significant difference in barrier recovery rate between imiquimod and control cream-treated skin, while the permeability barrier recovery rate was improved in imiquimod-treated skin compared to the control in wild-type mice. Therefore, we concluded that imiquimod improved barrier homeostasis affected by tacrolimus, which was derived from increased IL-1α levels in the epidermis.

IL-1 is a proinflammatory and immunomodulatory cytokine that plays a key role in inflammatory diseases of the skin[59-61]. In keratinocytes, IL-1α is stored intracellularly, but can be quickly released in the case of epidermal infection or injury. Released IL-1 serves as a paracrine signal to fibroblast and endothelial cells and guides the chemotaxis of lymphocytes toward the site of injury[19-20, 62]. IL-1 also serves as an autocrine signal to surrounding, undamaged keratinocytes, stimulating them to become activated. Activated keratinocytes are migratory, hyperproliferative, and produce growth factors and cytokines that function in inflammatory and wound healing processes[63,64]. Barrier recovery of the epidermis is linked with an increase of lipid synthesis and the increased production of potentially regulatory cytokines including IL-1α. IL-1α administration results in increased lipid synthesis in cultured human keratinocytes[25]. Aged mice with knockouts of the IL-1α receptor type I develop more profound barrier deficits than age-matched wild-type mice[65]. In the present study, we demonstrated that topical imiquimod applied to tacrolimus-treated skin increased the expression of IL-1α and induced epidermal lipid production via

lipid synthesis-related enzymes, which in turn enhanced LB production. These findings may indicate that IL-1α plays a key role in barrier abnormalities caused by topical tacrolimus treatment, and that the stimulation of IL-1α in tacrolimus-treated epidermis induces positive effects on the skin barrier.

IL-1α is also related to antimicrobial functions[8, 66-70]. There are several antimicrobial genes induced by IL-1α. One such protein, CCL-20, has greater antibacterial potency against *Escherichia coli* and *Staphylococcus aureus* than beta-defensins. β-defensins, the most important defensins for host protection against microbes, display a broad spectrum of antimicrobial activity and are most effectively induced by IL-1α in keratinocytes[8,71-75]. Yano et al. observed the induction of β-defensin expression and lipocalin 2 protein with a bacteriostatic function in IL-1α-treated keratinocytes. All of these genes are induced by IL-1α in keratinocytes, implying a correlation of the antimicrobial effects and IL-1α[76-77]. We found that imiquimod up-regulated the mRNA levels of mBD3 and CRAMP in the epidermis, suggesting that imiquimod-induced IL-1α plays an antimicrobial role in tacrolimus-treated skin through the induction of AMP.

V. Conclusion

Topical calcineurin inhibitors including tacrolimus and pimecrolimus delayed barrier recovery after acute barrier disruption when they were applied normal skin. Decrease of IL-1α expression was considered a main cause of barrier abnormalities due to TCI. From our study, IL-1α stimulation induced positive effects on epidermal permeability and antimicrobial barrier functions in tacrolimus-treated skin. These positive effects were mediated by an increase in epidermal lipid synthesis, LB production, and AMP expression. These findings have clinically important implication that an IL-1α inducer such as imiquimod could prevent barrier dysfunction in tacrolimus-treated skin

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Abstract in Korean (국문요약)

국소 타크로리무스 도포에 의해 손상된 피부장벽의 회복을 촉진시키는 Interleukin-1 α 의 효과

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국소 칼씨뉴린억제제는 현재 아토피피부염, 백반증, 건선을 비롯한 많은 피부질환에 처방되는 항염증성 면역억제제로서 이 중 하나인 국소 타크로리무스는 피부 위축이나 모세혈관 확장증 등의 부작용이 드물고 세균감염의 빈도가 스테로이드에 비해 적다고 알려져 있다. 타크로리무스는 국소 스테로이드 치료에 비해 부작용이 적어 스테로이드 대체제로 각광받으며 피부과 임상에서 많이 사용되고 있으나, 아토피피부염등의 만성피부질환 환자에서의 장기간 사용이 늘어나고 있어 타크로리무스 자체에 대한 피부 부작용도 최근 연구되고 있다. 이전 연구에서 국소 타크로리무스를

도포한 피부에서 정상피부에 비해 급성 피부장벽 손상 후 피부장벽의 회복이 지연되었고, 무모마우스를 이용한 실험에서는 국소 타크로리무스를 도포한 피부에서 정상피부에 비해 층판소체, 표피지질합성효소, 표피항균펩타이드, Interleukin 1α(IL-1α)의 발현이 감소됨을 확인하였다. 한편, IL-1α는 세균감염, 물집질환, 자외선 손상된 피부에서 중요한 역할을 하는 전염증성 사이토카인으로 각질형성세포를 활성화 시킨다. IL-1α에 의해 활성화된 각질형성세포는 성장인자 및 사이토카인을 분비하며 증식되게 되고 이것들은 상처치유과정에 관여하게 된다. 이전의 보고에 따르면 IL-1α를 배양각질세포에 처리하였을 때 표피지질 및 항균물질이 생성되었으며, 노화 마우스에서도 IL-1α 유도물질인 국소 이미퀴모드 도포 후에 피부장벽기능이 향상되었음이 밝혀진바 있다. 따라서 IL-1α의 자극이 국소 타크로리무스 도포에 의해 손상되는 표피투과장벽 및 항균장벽의 회복에 영향을 줄 수 있는지 알아보기 위해 연구를 시행하였다.

사람의 양쪽 전완부에 5 일간 타크로리무스를 도포하고, 테이프스트립핑으로 피부장벽을 손상시킨 직후 IL-1α 유도물질인 이미퀴모드 크림과 기제를 각각 도포한 결과 피부장벽 회복이 이미퀴모드 크림을 도포한 부위에서 증가하였다. 무모마우스의 양쪽 등에 타크로리무스를 바르고, 한쪽 등은 이미퀴모드, 반대편 등은 기제를 도포한 후 기능적 평가와 생검을 통한 면역조직화학염색, 전자현미경검사, real time RT-PCR 을 시행하였다. 기초 경표피 수분손실량 (Basal TEWL)은 두 군간에 차이가 없었으며, 급성 피부장벽 손상 후 피부장벽의 회복은

이미퀴모드를 도포한 치료부위에서 더 빠른 호전을 보였다. 전자현미경상 층판소체 농도는 이미퀴모드를 도포한 부위에서 증가되었고, 표피지질합성관여효소와 mBD3, CRAMP 같은 항균 펩타이드 또한 증가되었다. 무모마우스 등에 타크로리무스를 도포하고 직접 IL-1α 피하 주사를 시행한 후에 피부장벽 손상후 경표피 수분손실량을 측정 결과 IL-1α 피하 주사를 한 부위에서 PBS 를 주사한 대조군에 비해 더 빠른 피부장벽회복 능력을 보였다. IL-1α knock-out 마우스와 Wild type 마우스의 실험에서는 각 그룹의 양쪽 등에 타크로리무스를 도포하고 한쪽 등에는 국소 이미퀴모드 크림을, 다른 쪽에는 기제를 도포한 후 테이프스트리핑 후의 경표피 수분손실량을 측정한 결과 Wild type 마우스에 비하여 IL-1α knock-out 마우스에서 피부장벽 회복의 지연이 관찰되었다.

결론적으로 타크로리무스 국소도포에 의해 저하되는 표피투과장벽 기능 및 항균장벽 기능 저하의 발생기전에 IL-1α의 감소가 중요한 역할을 함을 확인할 수 있었으며, 임상적으로는 향후 장기간 타크로리무스를 도포하는 아토피피부염 등 피부질환에서 IL-1α생성을 촉진하는 치료약제 개발에도 긍정적인 역할을 할 것이다.

핵심 되는 말 : IL-1α, 이미퀴모드, 타크로리무스, 표피 투과장벽, 항균펩티드

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