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Original Article

Topical calcineurin inhibitors compromise stratum corneum integrity, epidermal permeability and antimicrobial barrier function

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Abstract

Background: Topical calcineurin inhibitors (TCIs) such as pimecrolimus and tacrolimus have recently been used for dermatologic diseases including atopic dermatitis instead of topical glucocorticoids, because they display comparable efficacy, but less-frequent side effects. Although even short-term topical glucocorticoid compromise epidermal permeability barrier homeostasis, the effects of TCI on barrier function have not yet been reported. However, viral infections such as eczema herpeticum and molluscum contagiosum, which could indicate an impaired skin barrier, continue to occur with TCI use in atopic dermatitis.

Objectives: We determined here whether TCIs disrupt epidermal permeability barrier and antimicrobial function, and whether these effects can be prevented.

Methods and results: In normal humans, topical pimecrolimus and tacrolimus applied twice-daily for 5 days, delay barrier recovery without an increase in basal transepidermal water loss was observed. Co-application of physiologic lipid mixture (PLM) containing an equimolar ratio of ceramides, cholesterol and free fatty acids normalized barrier homeostasis in the face of topical TCIs. In hairless mice, 4 days of TCI treatment also disrupted barrier function significantly. TCIs-treated epidermis showed the decrease of epidermal lipid content, lamellar body number and secretion, and lipid synthesis-related enzymes such as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, serine-palmitoyl transferase and fatty acid synthase, implying decreased lipid synthesis. TCIs also suppressed expression of IL-1α and antimicrobial peptides, CRAMP and mouse β-defensin 3. However, these TCI-induced abnormalities can be overridden by topical replacement with PLM.

Conclusions: Our results demonstrate that TCIs induce negative effects on the skin barrier including permeability and antimicrobial functions, which are mediated by decreasing epidermal lipid synthesis, lamellar body secretion and antimicrobial peptides expression through suppression of cytokine such as IL-1α, therefore co-treatment with PLM would be helpful to overcome these negative effects.

Key words: antimicrobial peptides – epidermal permeability barrier – pimecrolimus – physiologic lipid mixture – tacrolimus

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Introduction

Recently developed topical calcineurin inhibitors (TCIs), including tacrolimus and pimecrolimus, provide an alternative to topical glucocorticoids (GCs) for the topical treatment of atopic dermatitis and other many inflammatory disease due to anti-inflammatory efficacy of comparable to low-to-mid-potency topical GCs (1) and rare cutaneous side effects (2,3). While prolonged therapy with topical GCs can provoke cutaneous side effects, such as skin atrophy and increased skin infections (4), and potent topical GC can compromise barrier function after just 2–3 days of therapy (5). In spite of their putative, improved safety profile in comparison to GC, there are lingering concerns

about the use of immunosuppressive agents in patients already at increased risk for cutaneous viral infections such as eczema herpeticum, herpes simplex and molluscum contagiosum (6–9). Moreover, the risk of conversion from localized herpes simplex infection to eczema herpeticum is not negligible in patients treated with topical tacrolimus (10).

When permeability barrier function is damaged by external insults, such as organic solvents or tape stripping, barrier homeostasis is restored via accelerated preformed lamellar bodies (LBs) secretion (11), signalled by changes in the epidermal Ca^{2+} gradient, new lipid synthesis, and later, an increase in DNA synthesis (12–14). Stratum corneum (SC) lipids composed of an approximately equimolar mixture of cholesterol, free fatty acids and ceramides form SC intercellular membrane bilayers that mediate permeability barrier function (13). Synthesis of these lipids after barrier disruption increases in parallel with increased activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (HMG-CoA), fatty acid synthase (FAS) and serine-palmitoyl transferase (SPT), the rate-limiting enzymes for cholesterol, free fatty acids and ceramides, respectively (15). LB contains polar SC lipid precursors such as glucosylceramide, cholesterol, glycerophospholipids and sphingomyelin, and the hydrolytic enzymes that convert them to non-polar SC lipids (14). Therefore, LB secretion and epidermal lipid synthesis perform important key roles in the restoration of normal barrier function after barrier disruption.

Antimicrobial peptides (AMPs) play an important role as antimicrobial effectors on the skin surface, as well as distal outposts of the innate immune system. In humans, two main AMP families are β-defensins (hBD1, 2, 3 and 4) and cathelicidins (LL-37). Murine homologues of hBD2 and LL-37 are mouse β-defensin 3 (mBD3) and cathelin-related antimicrobial peptide (CRAMP). Furthermore, hBD2 and cathelicidins are synthesized and stored in LB, and correlated with permeability barrier function (16–18).

Atopic dermatitis exhibits phenotype-dependent abnormalities in both permeability and antimicrobial barrier function (19). Because the permeability and antimicrobial barriers are co-regulated and interdependent (20–22), increased microbial pathogenesis could be linked to the compromised permeability barrier in atopic dermatitis.

Previous studies have shown that chronic use of topical GCs negatively impacts epidermal permeability barrier function both by decreasing epidermal proliferation and differentiation (23), and by inhibiting synthesis of the three key epidermal barrier lipids (cholesterol, fatty acids and ceramides) (5), which mediate barrier function. Moreover, topical GCs down-regulate the expression of both hBD2 and LL-37 (18). Yet, the GC-induced permeability and antimicrobial defects can both be largely overcome with topical mixtures of these three physiological lipids (5,18). Since epidermal permeability and antimicrobial functions are intrinsically linked, we hypothesized here that the increased risk of infection in TCI-treated patients correlates with abnormal permeability barrier function, as occurs with topical GCs. We evaluated the mechanisms for the TCI-induced barrier abnormality, and methods of overcome TCI's negative effects.

Materials and methods

Human study

Eight normal volunteers (21–29 years, four males and four females) for the tacrolimus study and ten normal volunteers (20–40 years, six males and four females) for the pimecrolimus study were recruited. The medical ethical committee of Yonsei University Wonju College of Medicine approved all described studies. This study was conducted according to Declaration of Helsinki Principles. Participants gave written informed consent. The subjects of each study applied either 0.03% tacrolimus ointment (Astellas Pharma Korea Inc, Seoul, Korea) or 1% pimecrolimus cream (Elidel®, Novartis AG, Basel, Switzerland) on one side of the volar forearm and petrolatum or hydrobase as a control base, respectively on the contralateral side twice daily for 5 days. At 24 h after the last application, the barrier recovery rate was assessed 3 h after acute barrier disruption with tape stripping with D-Squame tape (CuDerm, Dallas, TX, USA) by measuring transepidermal water loss (TEWL) using Tewameter TM 210 (Courage & Khazaka, Cologne, Germany) (24).

To elucidate whether a physiologic lipid mixture (PLM) (Triceram®, Osmotics Corp., Denver, CO, USA) could improve TCIs-induced barrier impairment in humans, eight volunteers for tacrolimus study (21–29 years, four males and four females) and six for pimecrolimus (21–26 years, four males and two females) without any skin diseases were treated with either 0.03% tacrolimus or 1% pimecrolimus cream on both forearms twice daily for 5 days. At 24 h after the last application, PLM was applied on one forearm and petrolatum as a control base on the other immediately after tape stripping. Basal TEWL and barrier recovery rate at 3 and 6 h were measured.

Animal study

Female hairless mice (Skh1/Hr) were housed in the animal laboratory of Yonsei University Wonju College of Medicine. About 0.03% tacrolimus (Sigma, St Louis, MO, USA) with vehicle (propylene glycol:ethanol 7:3 v/v) or vehicle alone was applied to the backs of mice twice daily for 4 days ($n = 5$ in each group). At 24 h after last application, basal TEWL and SC integrity which was determined by TEWL after acute barrier disruption with tape stripping were measured. Barrier recovery rate was determined at 3 and 6 h after tape stripping. Skin specimens were taken

from all hairless mice and processed for nile red staining, electron microscopic examination, immunohistochemical staining and quantitative real time RT-PCR assays.

To assess the effect of PLM on impaired skin barrier by TCIs in mice, hairless mice were divided into three groups; petrolatum as a control base alone, pimecrolimus with petrolatum, and pimecrolimus with PLM (n = 6, 6 and 7, respectively). PLM or petrolatum was applied 20 min after 1% pimecrolimus (Elidel®, Novartis A.G.) treatment twice daily for 4 days. At 24 h after the last application, skin specimens from hairless mice were taken for nile red staining, electron microscopic examination, immunohistochemical staining and quantitative real time RT-PCR assays.

Nile red staining

Nile red staining to visualize SC intercellular and epidermal lipids was performed with frozen section. The nile red dye, 9-diethylamino-5H-benzo[a]phenoxazine-5-one (Sigma), is an excellent vital stain for the detection of intracellular lipid droplets by fluorescence microscopy because it interacts and fluoresces in the presence of phospholipids, cholesterol, cholesteryl esters and triacylglycerols (25).

Electron microscopy and quantitative EM analysis

Samples for electron microscopy (EM) were processed using both 0.5% ruthenium tetroxide and 2% aqueous osmium tetroxide postfixation. To exclude subjective bias in these morphologic studies, we quantitated the number and secretion of both corneodesmosome (CD) and lamellar body (LB) in EM pictures using an objective method as described previously (26). Five EM pictures taken at the same magnification (×20 000) were analysed and compared between each groups.

Real time RT-PCR

Isolation of epidermis

Skin samples excised from the treated area were immediately placed epidermis side downward on Petri dishes. Subcutaneous fat was removed with a scalpel, and then skin samples were placed epidermis side up onto 10 ml of 10 mM EDTA in PBS, then incubated at 37°C for 35 min to separate epidermis from dermis. Epidermis was scraped off with a scalpel and total RNA was extracted.

Total RNA preparation and cDNA synthesis

Total RNA was extracted using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol Reagent; Gibco BRL). RNA concentration was determined by UV spectrometer at 260 nm. Aliquots (1.0 μg) of RNA from each sample were reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (MML-V RTase, Promega). Briefly, RNA samples were incubated at 80°C for 5 min with molecular biology grade water. After incubation on ice, primer extension and reverse transcription were performed by adding 1× RT-buffer, 2 mM deoxynucleotide triphosphates (dNTPs), 0.2 pM random hexamer primer (Promega) and MML-V RTase (2.5 units/μl) in 20 μl reaction volumes. Samples were then incubated at 42°C for 45 min before storage at -20°C.

Quantitative PCR analysis of gene expression

Expression of specific mRNAs was quantified using a Rotor-Gene™ 3000 (Corbett Life Science, Sydney, Australia). Briefly, 10 μl PCR reactions were set up containing: Quanti-Tect probe PCR Master mix (Qiagen) in a 2× solution, 8 mM manganese chloride, 200 μM dNTPs, 1.25 units HotstartTaq polymerase, and 0.5 pM/μl each probes and primers. About 60 ng of cDNA were used per reaction. All reactions used glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, provided as an optimized control probe labelled 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 labelled 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 pimecrolimus-treated samples from their respective base control (or vehicle)-treated samples. The resulting ΔΔCt values were then used to calculate fold change in gene expression as $2^{\Delta\Delta Ct}$. All reactions were done in triplicate and results are expressed as the mean of values from three separate experiments. Samples were amplified using the primers and probes under the following conditions: 95°C for 15 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

Primers and probes for PCR reactions

Primer and probe sequences for real-time RT-PCR analyses were as follows:

HMG-CoA reductase primers (forward: 5′-CCGAATTG-TATGTGG CACTG-3′, reverse: 5′-GGTGCACGTTCCTTGA AGAT-3′, Operon Biotechnologies) and HMG-CoA probe (5′-CTTGATGGCAGCC TTGGCAG-3′, Operon Biotechnologies); FAS primers (forward: 5′-CTGAAGAGCCTGGAA-GATCG-3′, reverse: 5′-TGTCACGTTGCC ATGGTACT-3′, Operon Biotechnologies) and FAS probe (5′-TGAGCTTTGC TG CCGTGTCC-3′, Operon Biotechnologies); SPT primer (forward: 5′-GAGAGATGCTGAAGCGGAAC-3′, reverse: 5′-TGGTATGAGCTGCTGACAGG-3′, Operon Biotechnologies) and SPT probe (5′-TGGGATTTCCTGCTACCCCG-3′, Operon Biotechnologies); CRAMP primers (forward: 5′-

TGAGCCCCAAGGGGACGAGG-3′, reverse: 5′-GCCGGGTT CAGGGTGACTGC-3′, Operon Biotechnologies) and CRAM P probe(5′-GTGGCAAGGCAGAGCGG CAGCT-3′, Operon Biotechnologies); mBD3 primers (forward: 5′-GGCTTCAGT CATGAGGATCCATTACC-3′, reverse: 5′-TGCACCGATTC-CAGCATCTGCC-3′, Operon Biotechnologies) and mBD3 probe (5′-TGGTGGCTGCTGTCTCCACCTGCAGCT-3′, Operon Biotechnologies) as an external negative control; and GAPDH as an internal standard and the housekeeping gene used to derive ΔCt values (forward primer 5′-TGCGACTTCAACAG-CAACTC-3′, reverse primer 5′-ATGTAGGCCATGAGG TCCAC-3′, Operon Biotechnologies) and GAPDH probe (5′-TCTTCCACCTTCGATGCCGG-3′, Operon Biotechnologies).

Immunohistochemical staining

Skin specimens were fixed in 10% formalin solution and embedded in paraffin. Sections of 5 μm thickness were processed for immunohistochemical staining of proliferation cell nuclear antigen (PCNA), IL-1α, mBD3 and CRAMP. We used primary antibodies for PCNA, IL-1α, mBD3 and CRAMP (Santa Cruz, CA, USA). After de-paraffinization, sections were rehydrated sequentially with 100%, 90% and 70% ethanol and incubated for 5 min in 3% H_2O_2 in Tris-buffered saline (TBS) to inactivate endogenous peroxidases. Samples were then blocked for 10 min with blocking serum solution (DAKO, Carpinteria, CA, USA) and incubated overnight at 4°C with primary antibodies. After several washes in TBS, they were incubated for 30 min with secondary biotinylated antibodies. Antigen was detected with the avidin–biotin complex system (Vector, Burlingame, CA, USA), according to the manufacturer's instructions by using 3,3′-diaminobenzidine tetrahydrochloride as substrate. Then they were examined under a light microscope.

To quantify the density of IL-1α, three pictures for each section were taken at a 100× magnification, and were labelled. An investigator without knowledge of the origin of the specimen ranked the pictures in order of the intensity of immunostaining (highest intensity: first rank, in descending order in each). Mann–Whitney test was used for statistics. A representative picture for each group was chosen for publication.

Binding of the PCNA primary antibody was detected by ABC peroxidase from Vector, utilizing diaminobenzidine as the substrate (Vector, Burlingame, CA, USA). For the PCNA experiments, stained nuclei were counted as positive and the number of positive cells per length of basal epidermis was calculated.

Statistical analyses

All data are expressed as mean ± SEM. Statistical analyses were performed using paired and unpaired Student's *t*-tests or Mann–Whitney test for assaying density of immunohistochemical staining.

Results

TCIs disrupt epidermal permeability barrier homeostasis, and this delayed barrier recovery is restored by co-treatment with a three-component mixture of SC lipids in TCI-treated human skin

We first assessed whether TCIs alter permeability barrier function in normal human skin, even after short-term applications. While applications of either tacrolimus or pimecrolimus twice-daily for 5 days changed neither skin appearance nor basal TEWL in comparison to control base (data not shown), both topical pimecrolimus and tacrolimus significantly delayed the kinetics of barrier recovery 3 h after acute barrier disruption by repeated tape stripping (Fig. 1a,b).

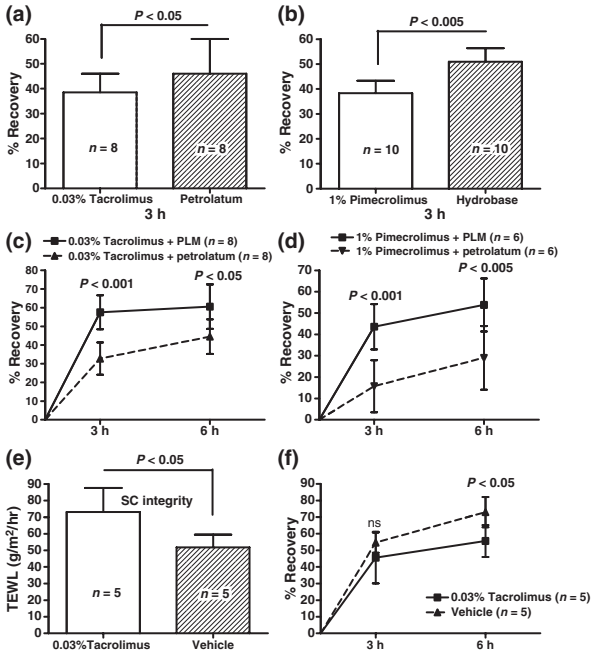

Figure 1. Topical calcineurin inhibitors (TCIs) disrupt epidermal permeability barrier homeostasis in normal human and mice skin, which delayed barrier recovery, is restored by co-treatment with physiologic lipid mixture (PLM) of SC lipids in TCI-treated human skin. In human, either 0.03% tacrolimus ointment or 1% pimecrolimus cream was applied on one volar forearm, while petrolatum or hydrobase was applied as a control base to equivalent contralateral sites of normal human subjects twice-daily for 5 days. At 24 h after the last application, barrier recovery was measured 3 h after tape stripping of the treated skin sites (a, b). PLM or petrolatum was applied on either tacrolimus- or pimecrolimus-treated human forearms immediately after acute barrier disruption with tape stripping. Barrier recovery rates were measured at 3 and 6 h (c, d). In hairless mice, their flanks were treated with topical 0.03% tacrolimus or vehicle (PG:ethanol = 7:3 v/v) twice daily for 4 days. SC integrity (e) and barrier recovery rate at 3 and 6 h after tape stripping (f) were assessed. Statistical analyses in this and all subsequent experiments were performed using a paired or unpaired Student's *t*-test. Results in this and subsequent experiments are expressed as mean ± SEM.

We next determined whether the three-component mixture of cholesterol, free fatty acids and ceramides improves skin barrier recovery in TCI-treated human skin, as shown in the previous work by Kao et al. (5). The PLM was co-applied to skin sites that were being co-treated with either pimecrolimus or tacrolimus, immediately after barrier disruption by tape stripping, while petrolatum as a control base was applied to TCI-treated human sites on the opposite forearm. PLM normalized the barrier recovery kinetics at 3 and 6 h after acute barrier disruption in both pimecrolimus- and tacrolimus-treated skin compared to petrolatum-treated controls (Fig. 1c,d).

TCIs also impair epidermal permeability barrier homeostasis in mice

To assess whether hairless mice would provide a suitable model to elucidate pathomechanisms for the TCI-induced barrier abnormalities shown in human skin, we next examined effects of TCIs on hairless mouse skin, treated twice a day for 4 days with 0.03% tacrolimus or vehicle (PG:ethanol = 7:3 v/v) as control. As in humans, basal TEWL did not show differences between tacrolimus- and vehicle-treated sites (data not shown). But SC integrity was compromised in comparison to vehicle-treated skin (Fig. 1e), and permeability barrier recovery was significantly delayed in tacrolimus-treated group (Fig. 1f). The correspondent results between murine and human study suggests that hairless mice can serve as a suitable model for the mechanistic studies of the skin barrier in TCI-treated skin.

Negative effects of TCIs on permeability barrier function result from decreased epidermal lipid production

We next assessed whether the tacrolimus-induced abnormality in permeability barrier function reflects altered epidermal lipid production by several different assays. First, nile red fluorescence staining of frozen sections of mouse epidermis demonstrated a global decrease in the lipid content of epidermis in unfixed biopsies after either tacrolimus or pimecrolimus treatment (Figs 2a,b and 5a), indicating a markedly reduction in lipid content in TCI versus vehicle-treatment skin. We assayed the mRNA expression of three key enzymes of epidermal lipid synthesis; i.e. 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA), SPT and FAS to assess whether decreased mRNA levels for these three key enzymes leads to the TCI-induced reduction in epidermal lipid synthesis. Topical tacrolimus treatment decreased all mRNA levels for all three of these enzymes (Fig. 2c), and a similar result was shown with pimecrolimus even though different set of experiment design (Fig. 5b).

To assess whether the TCI-induced reduction in lipid content results in abnormalities of LB production and/or secretion, LB morphology and quantification from EM studies were evaluated. Tacrolimus-treated skin revealed immature (largely empty) organelle contents and a significant decrease in the density of LBs (≈50% decrease), in comparison to vehicle-treated skin (Fig. 2d–f). Pimecrolimus-treated skin showed the similar changes, including a decreased density of LB and largely empty organelle contents (Fig. 5c,d).

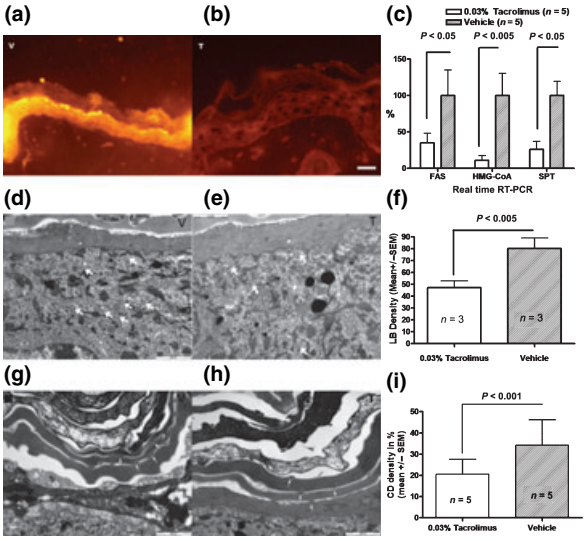

Figure 2. Topical tacrolimus impairs barrier homeostasis and SC integrity not only by inhibiting epidermal lipid synthesis and lamellar body (LB) formation, but also by decreasing corneodesmosome (CD) density. Skin biopsy samples were taken from the skin treated with topical 0.03% tacrolimus (T) or vehicle (PG:ethanol = 7:3 v/v) (V) twice daily for 4 days, and then stained with fluorescent nile red to visualize the overall epidermal lipid content (a, b), and assayed real time RT-PCR for mRNA level of epidermal lipid synthesis-related enzymes (c), and processed for electron microscopy (EM) to analysis LBs (large white arrow, d–f) and CDs (white arrow, g–i). Mag bars = 2 mm in Fig. a,b; 2 μm in Fig. d,e,g, h (FAS: fatty acid synthase, HMG-CoA: HMG-CoA reductase, SPT: serine-palmitoyl transferase).

Lastly, we performed PCNA stain to make sure that above negative effects of tacrolimus were not due to its toxic effect. The number of PCNA positive cells was not different between tacrolimus- and vehicle-treated skins (data not shown). These results demonstrate that TCIs lead to a decreased generation of both epidermal lipids and LB, and a decline in permeability barrier recovery kinetics.

TCIs also disrupt SC integrity through a decrease in CD

Intercorneocyte cohesion is critical not only for SC integrity, but also for the epidermal permeability barrier (14,27). This function is mediated largely by CD, unique intercellular junctions, modified from epidermal desmosomes (28). Individual CDs appeared prominently foreshortened and fragmented (Fig. 2g,h) and its density decreased by about

40% (Fig. 2i) in the tacrolimus-treated group, compared to the vehicle-treated group. EM of pimecrolimus-treated mice yielded similar results (data not shown). Thus, TCIs impair SC integrity/cohesion and barrier homeostasis not only by inhibiting lipid synthesis, but also by decreasing CD density.

TCIs decrease AMPs expression

Prior studies have shown that the expression of mBD3 and CRAMP are changed in parallel with permeability barrier status (21). Since we show above that TCIs decrease LB secretion in murine skin, we next assessed whether the expression of mBD3 and CRAMP decreases in murine epidermis after TCI treatment. While vehicle-treated skin revealed that expression of both mBD3 and CRAMP (mouse homologues of hBD2 and LL-37, respectively) largely localized to the outer epidermis (Fig. 3a,c), tacrolimus-treated skin showed a marked decrease in immunostaining for both AMPs in the outer epidermis (Fig. 3b,d). Additionally, we measured the mRNA level of mBD3 and CRAMP. Topical tacrolimus treatment significantly decreased mRNA levels for mBD3 and CRAMP (Fig. 3e). These results suggest that TCIs could also disrupt, at least in part, the antimicrobial barrier of the SC.

TCIs suppress epidermal expression of IL-1α

When acute barrier disruption occurs, the release and production of pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α and GM-CSF (29,30), signal a restoration of impaired barrier function (31,32). Since TCIs suppress expression of several pro-inflammatory cytokines, we next assessed IL-1α expression in tacrolimus-treated murine skin by immunohistochemical staining. Tacrolimus-treated skin sites showed much lower expression of IL-1α than did vehicle-treated murine skin (Fig. 4a,b), confirmed by semi-quantitative analysis of randomly obtained, coded (investigator-blinded) micrographs (see Materials and methods) (Fig. 4c). These results provide one mechanism whereby TCIs could cause negative effects on permeability barrier function; i.e. by suppression of IL-1α-mediated stimulation of epidermal lipid synthesis.

Co-treatment with a three-component mixture of physiologic lipids compensates for defective permeability and antimicrobial barrier homeostasis in TCI-treated murine skin

It is forementioned that a three-component mixture of topical physiologic lipids overrides TCI-induced abnormalities in permeability barrier function and SC integrity in human (Fig. 1c,d). To confirm the above results in detail, we next assessed whether co-treatment with PLM increased LB production and epidermal lipid content, assessed by nile red staining, EM analysis, and mRNA level of lipid synthesis

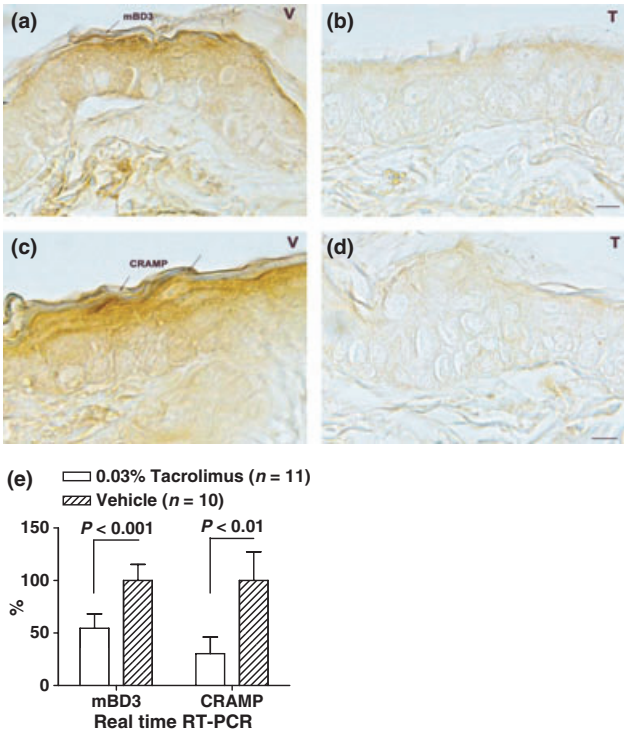

Figure 3. Topical tacrolimus decreases expression of antimicrobial peptides such as mBD3 and CRAMP at the mRNA level in murine epidermis. Biopsy specimens from 0.03% tacrolimus (T) or vehicle (V) (PG:ethanol = 7:3 v/v) -treated skin sites after 4 days of twice-daily applications were immunostained with for mBD3 (a, b) and CRAMP (c, d), and assayed by real time RT-PCR for RNA level of mBD3 and CRAMP in murine epidermis (e). Mag bars = 2 mm.

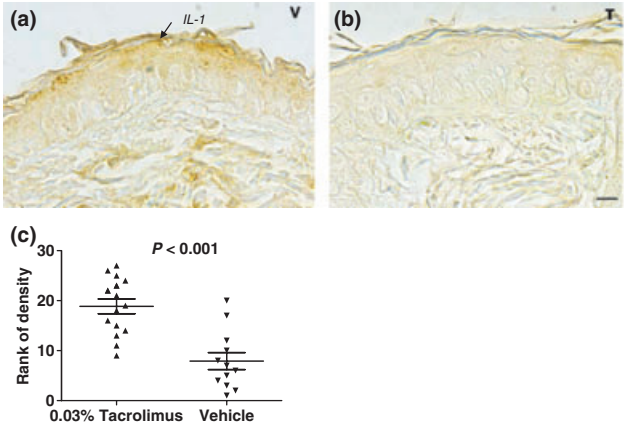

Figure 4. Topical tacrolimus suppresses expression of IL-1α in murine epidermis. Biopsy specimens from 0.03% tacrolimus (T) or vehicle (V) (PG:ethanol = 7:3 v/v) -treated skin sites after 4 days of twice-daily applications were immunostained with IL-1α (a, b) (tacrolimus: $n = 5$; vehicle: $n = 4$). Ranking (semi-quantitative) analysis (c) of randomly obtained, coded (investigator-blinded) micrographs was estimated by the intensity of staining signal (see Materials and methods). Mag bars = 2 mm.

rate-related enzymes in pimecrolimus-treated murine skin. Experimental mice were divided into three subgroup: pimecrolimus+PLM, pimecrolimus+petrolatum and petrolatum as a control base alone (as a normal control). PLM or petrolatum was applied 20 min after pimecrolimus treatment twice daily for 4 days. PLM co-treated epidermis restored nile red fluorescence in comparison to pimecrolimus plus petrolatum-treated epidermis (Fig. 5a). In accordance with above data, co-treatment with PLM also increased mRNA level of epidermal lipid synthesis-related enzymes, HMG-CoA reductase, FAS and SPT (Fig. 5b), which were decreased in TCIs-treated murine skin (Fig. 2c). Moreover, PLM co-treated skin showed a marked increase in normal shaped LB density in comparison to petrolatum co-treated skin (Fig. 5c,d). These results show that barrier replacement therapy with PLM improves the TCI-induced skin barrier defect, reinforcing the model that TCIs affect epidermal permeability barrier function by decreasing epidermal lipid synthesis. Also co-treatment with PLM increase CD length compared to pimecrolimus with petrolatum (data not shown). These results mean that PLM could improve not only epidermal permeability but also SC integrity of impaired murine skin by TCIs.

Additionally, we measured mRNA levels of AMPs and performed immunostaining to assess whether co-treatment with PLM overcome TCI's negative effect on the antimicrobial barrier function. Co-treatment with PLM increased not only mRNA levels of CRAMP and mBD3 but also protein expression of AMPs on the immunostaining in TCIs-treated murine skin (Fig. 6).

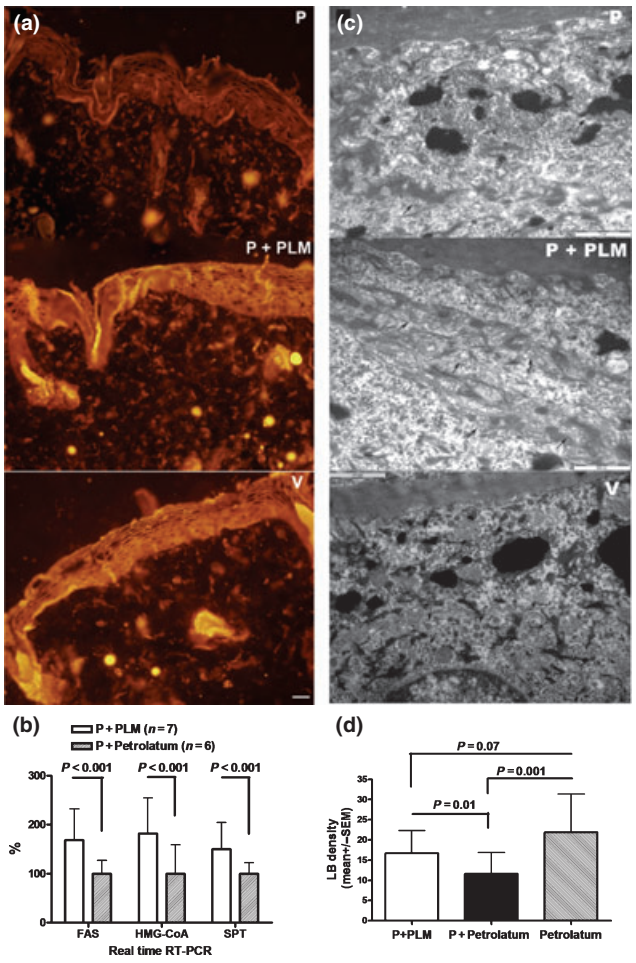

Figure 5. Physiologic lipid mixture (PLM) restores SC intercellular lipids and epidermal lipid synthesis, and the production of lamellar body (LB) in murine epidermis treated by topical pimecrolimus. Hairless mice were divided into three groups; petrolatum as a control base alone (V), pimecrolimus with petrolatum (P), and pimecrolimus with PLM (P+PLM) (n = 6, 6, and 7, respectively). PLM or petrolatum was applied 20 min after 1% pimecrolimus treatment twice daily for 4 days. At 24 h after the last application, skin specimens were taken for nile red staining (a), quantitative real time RT-PCR assays for mRNA of lipid synthetic enzymes (b), electron microscopic examination to analyse LB secretion and production (black arrows) (c, d). (FAS: fatty acid synthase, HMG-CoA: HMG-CoA reductase, SPT: serine-palmitoyl transferase) Mag bars = 2 mm in Fig. a; 2 μm in Fig. c.

Discussion

Because TCIs such as tacrolimus and pimecrolimus are potent immunosuppressant and are prescribed mainly for patient with AD, many dermatologists are concerned about the vulnerability to cutaneous infection of patients. In fact, there were some reports associated with cutaneous viral infections after TCIs treatment, including potentially life-threatening episodes of eczema herpeticum (6,8,33), although it seems that the incidence of bacterial skin infections such as *Staphylococcous aureus* does not increase (34,35). In addition, since AD patients at greater risk for viral dissemination exhibit severely impaired barrier function (36), and since permeability barrier and antimicrobial function are co-regulated and interdependent (20,21), we wanted to know here whether TCI treatment also impairs permeability barrier and antimicrobial function.

First, we demonstrated that short-term TCIs treatment does not increase basal TEWL either in human or murine skin, a very similar result to previous observations with topical GC (5). However, a significant delay in barrier recovery after acute barrier disruption was observed both in pimecrolimus and tacrolimus groups, another similar result with topical GC (5). The barrier defect could not be ascribed to nonspecific toxicity, because epidermal mitogenesis did not change, and with the exception of abnormal LB, there was no evidence of cytotoxicity in TCI-treated skin. The similarities of the functional abnormalities produced by topical GCs and TCIs suggested to us that similar pathogenic mechanisms could be operative. GCs compromise barrier function by decreasing epidermal lipid synthesis. Hence, we examined here whether the TCI-induced delay in barrier recovery is

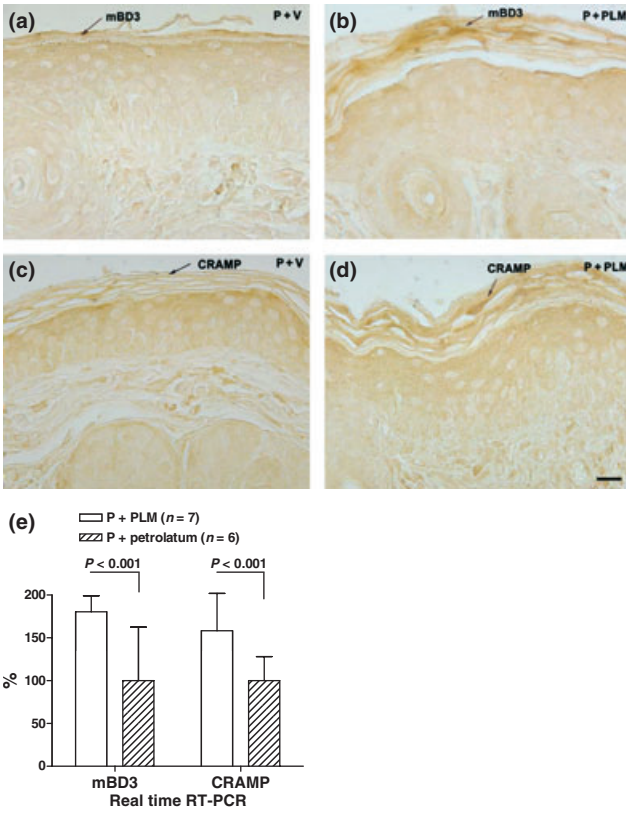

Figure 6. Co-treatment with physiologic lipid mixture (PLM) increases the expression of mRNA and protein of antimicrobial peptides in the topical pimecrolimus-treated epidermis. PLM or petrolatum as a control base was applied 20 min after 1% pimecrolimus treatment twice daily for 4 days on the mice skin. At 24 h after the last application, skin specimens were taken and immunostained with mBD3 (a, b) and CRAMP (c, d), and assayed by real time RT-PCR for mRNA level of mBD3 and CRAMP in murine epidermis (e). (P+V: pimecrolimus plus petrolatum, P+PLM: pimecrolimus plus PLM). Mag bars = 2 mm.

also due to inhibition of lipid production. Using the nile red stain, an EM study for LB density and real time RT-PCR for mRNA of the rate-limiting enzymes of epidermal lipid synthesis, we showed that TCIs decrease epidermal lipid production, LB secretion, and key enzymes of epidermal lipid synthesis (HMG-CoA, FAS and SPT). The decline in epidermal lipid production could be further attributed to an antecedent decrease of the mRNA of their rate-limiting enzymes (13–15). Therefore, the TCI-induced permeability barrier impairment is due to a decrease in LB production and epidermal lipid synthesis due to decreased transcription of their rate limiting enzymes.

We also explored one mechanism that could explain the decrease in epidermal lipid synthesis in TCI-treated skin. TCIs display anti-inflammatory effects through inhibition of many cytokines including IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, TNF-α and GM-CSF (37–39). When epidermal permeability barrier is disrupted, several primary cytokines, including TNF-α, IL-1α, IL-1ra, IL-1β, IL-6, IL-8 and GM-CSF, are up-regulated prior to increased epidermal lipid synthesis (29,30). This increased cytokine expression could restore barrier function by up-regulating keratinocyte differentiation and/or proliferation (40,41). However, cytokine knockout models (TNF-α or IL-1α) suggest that cytokine/growth factors could also regulate epidermal lipid synthesis (42), and IL-1α stimulates epidermal lipid synthesis and normalizes LB structure in aged human skin (31,32). Transcription of several epidermal lipid synthetic enzymes is, in turn, regulated by sterol regulatory element-binding proteins (SREBPs), which modulate cholesterol and fatty acid synthesis in response to changes in the cellular concentration of these lipids in epidermis (43). Some of inflammatory cytokines, including TNF and IL-6, have been shown to activate SREBP (32,44). These findings suggest that cytokines may regulate lipid synthesis through activation of SREBP (32,44). Our data show that TCIs suppress the expression of IL-1α, a cytokine having an important role of epidermal lipid enzyme modulation and repairing impaired permeability barrier. We propose that the inhibitory effects of TCIs on expression of IL-1α and perhaps other cytokines could alter epidermal lipid synthesis leading to a barrier abnormality. Like TCIs, GC may also act on the same mechanism regarding inhibition of the enzyme expression. But, it is still unclear how pro-inflammatory cytokines affect epidermal lipid synthesis-related enzymes at the transcriptional level. Further experiments are needed to assess the molecular mechanism.

Our data showed that TCIs decreased LB secretion and production. We focused on mBD3 and CRAMP, because both are sequestered in LB (16,17), and likely secreted into the SC extracellular domains along with LB derived lipids (20). Results from immunohistochemistry and real time RT-PCR in the present study demonstrated that mBD3 and CRAMP expression in the epidermis are markedly down-regulated at both the protein and mRNA levels after TCIs application for 4 days, which is correlated with decreased LB secretion. AMPs could be influenced by several cytokines such as IL-1 or barrier permeability (21,45). Therefore, the suppressive effect of TCI on IL-1α would directly or indirectly impact AMP expression and LBs formation in parallel by several mechanisms, suggesting that TCIs could impair both permeability barrier and antimicrobial barrier.

To overcome these negative effects of TCI on the skin barrier, we applied PLM already adopted in previous topical GC study (5). Optimal lipid mixture (cholesterol, ceramide and fatty acid 3:1:1) has been considered a good treatment to accelerate barrier repair after disruption by tape stripping (mechanical), acetone or solvent (chemical) and GC (46,47). Co-administration of PLM increased not only epidermal lipid content, LB secretion and epidermal lipids synthesis-related enzymes but also AMPs level in

TCIs-treated murine skin in this research. Through being transported across the SC into the stratum granulosum cells and being mixed with the endogenous pool of lipids (13), physiological lipids could supplement the lipids or lipid precursors used as like a fuel for barrier recovery after acute barrier disruption (such as tape stripping) in TCI-treated skin, which has a weak ability to synthesis epidermal lipids. Barrier replacement therapy thus could prevent TCI-induced impairment of the permeability and antimicrobial barrier. Yet, it has been not known whether the additional treatment of lipid mixture affects the penetration and/or bioavailability of TCIs.

Even though many parts of this report were performed with tacrolimus, the results described in this study could sufficiently represent the negative effects of TCIs on the epidermal permeability barrier, based on following points: (a) The corresponding results from human study using both tacrolimus and pimecrolimus and some of mouse study were shown. Particularly, mouse studies with pimecrolimus (Figs 5 and 6) somewhat showed the correspondence between tacrolimus and pimecrolimus. (b) Tacrolimus and pimecrolimus are known to have the same action mechanism and very similar pharmacokinetics (except for lipophilicity and permeation) due to their similar molecular structures. There are only two chemical structure differences (1,48). (c) We think the negative effects in this report could be mediated by calcineurin inhibition itself.

Because of difficulties in obtaining human tissue and overall similarities, hairless mouse model is often used as a model for the study of epidermal permeability barrier. However, hairless mouse skin is more permeable to water and its SC thickness is less half than that of human SC, therefore they accordingly have poorer barrier function (49). Thus, it should be careful to translate the findings in hairless mouse models to human skin.

In conclusion, our results demonstrate that TCIs-induce negative effects on the skin barrier including permeability and antimicrobial functions, which are mediated by decreasing epidermal lipid synthesis, LB secretion and AMP expression through suppression of cytokine such as IL-1α, therefore co-treatment with PLM would be helpful to overcome these negative effects.

Conflict of interest

Peter M. Elias is a co-inventor of physiologic lipid mixture (TriCeram®, Osmotics Corporation, Denver, CO, USA), a patented technology, and an officer of Ceragenix Pharmaceuticals, the licensee of this technology. Other authors state no conflict of interest.

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