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Preclinical Study of Tacrolimus Ointment for Prevention of Its Systemic Absorption in Atopic Dermatitis Model Mice According to Their Skin Conditions

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Tacrolimus (TL) is used topically for atopic dermatitis (AD) treatment. In AD, the skin shows various physiological alterations across individuals, body sites, and time-courses. Our previous study using rats showed that alterations in the skin barrier function and skin blood flow affected systemic absorption of topically applied TL. In this study, we performed an *in vivo* skin absorption study using NC/Nga mice repeatedly administered *Dermatophagoides farinae* extract as an AD animal model to assess skin barrier function and skin blood flow. We used three types of TL ointment: original TL ointment (Protopic® 0.1% ointment) and liquid paraffin-diluted TL ointment with or without adrenaline (0.5 $^{w}/_{w}$%) to suppress systemic absorption of TL. Skin barrier function correlated positively with the systemic absorption of TL in AD skin as well as in tape-stripped rat skin, and dilution of TL ointment suppressed TL absorption and showed high skin TL retention. Although skin blood flow affected TL absorption as the skin barrier was disrupted, the distinct relationship among skin blood flow, TL absorption, and the effect of combined-use of adrenaline was unclear. The relationship between blood and skin disposition of TL was biphasic—the ratio of blood to skin disposition increased rapidly at an inflection point. These results demonstrate that the dilution of TL ointment is useful for increasing treatment safety while retaining efficacy. The observed relationship between TL behavior and skin barrier function in AD model mice may also occur in AD patients and needs to be confirmed in further studies.

Key words —— tacrolimus, skin absorption, atopic dermatitis, transepidermal water loss, skin blood flow, NC/Nga mouse

Introduction

Tacrolimus (TL, FK506, molecular weight of 803.5), a calcineurin inhibitor has potent immunosuppressive effects in dermal immunocompetent cells: epidermal Langerhans cells, dermal dendritic cells, mast cells, and eosinophils.[1-3] The topical formulation of TL (Protopic® ointment) is used as a second-line treatment for atopic dermatitis (AD) unresponsive to, or uncontrolled by topical corticosteroids (TCIs)[4, 5]; a long-term continuous use of TCIs causes skin atrophy and vasodilation, and attenuates the therapeutic effect of TCIs; however, TL is administered instead of TCIs because it does not cause these side effects[6] and has a mechanism of action different from that of TCIs. AD, a chronic inflammatory skin disease accompanied by severe itching, is characterized by repeated exacerbation and remission of symptoms, such as dryness, erythema, edema, incrustation, or hemorrhage.

The efficacy of short- and long-term use of TL ointment in AD patients is widely known.[7-11] Local adverse effects, such as burning sensations and pruritus at the application site,[12] are temporary and improve as skin lesions heal.[10, 13, 14] In

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contrast, safety of long-term use of TL is controversial. Hui and others reported that the risk of T-cell lymphoma increased among TL users in a retrospective cohort study,[15] whereas several studies reported that the risk of lymphoma was not greater than its spontaneous occurrence rate.[16-18] Drug safety as well as efficacy should be evaluated along with an assessment of the relationship between pharmacokinetics and main and adverse effects since drug concentration at a site of action generally correlates with its pharmacological effect. However, so far, the quantitative relationship between the rate of systemic adverse effects of TL ointment and its concentration in blood has been unclear. With respect to safety, systemic absorption of TL should be minimized or prevented.

Several clinical studies on TL blood concentration and/or skin concentration in AD patients have been reported.[19-24] The tested skin in the clinical studies was described as "lesional skin" or was classified broadly into 'mild', 'moderate', and 'severe' based on the extent or severity of AD. Inflammatory responses, such as dysfunction of stratum corneum (SC) barrier,[25-27] alteration of skin blood flow,[28] vascular hyperpermeability,[29] and leakage of plasma components,[30] occur in AD skin. The inflammatory reaction is complex and the dynamic process can affect the pharmacokinetics of TL after application of TL ointment on AD skin. However, the relationship between physiological skin conditions and the pharmacokinetics of TL is unrevealed.

In our previous study,[31] we performed an *in vivo* skin absorption study of TL in rats with physiologically altered skins. We prepared several skin models with different physiological conditions in hairless rats in order to separate the complex inflammatory responses into fundamental processes and evaluate their relationships with TL absorption. The results showed that transepidermal water loss (TEWL) correlated positively with systemic absorption of TL, and in the case of skin without an SC layer, skin blood flow had a positive correlation with TL absorption. Based on the results, we devised an administration plan to minimize systemic absorption of TL: diluting TL ointment according to TEWL and combined use of adrenaline (Adr). Since the alterations in tested rat skin were artificially produced, the translatability of data from the artificially altered skin to human-AD skin is unknown. In order to investigate whether these relationships were observed even in AD skin exhibiting a complex alteration and whether systemic absorption of TL was suppressed by diluting the ointment and combining Adr and whether the ointments affected the skin disposition of TL, we performed *in vivo* skin absorption study using AD model mice.

AD model mice have been commonly used for elucidating AD pathogenesis,[32] drug discovery,[33] and evaluating pharmacotherapy including TL ointment.[34] In this study, we used NC/Nga mice that received repeated application of *Dermatophagoides farinae* extract (Dfe) on the ear and dorsal skin as an animal model for human AD. This model mice exhibit high serum IgE levels, Th2 and Th1 cytokine production, and clinical and histological symptoms observed in human AD.[35, 36] Thus, this preliminary study can provide fundamental information for clinical studies in AD patients and pharmacotherapeutic regimens of TL.

Materials and Methods

1. Materials

TL ointment (Protopic® ointment: 0.1%) was purchased from Astellas Pharma Inc (Tokyo).

Biostir® AD was purchased from Biostir Inc (Kobe). L-Adrenaline was purchased from Tokyo Chemical Industry Co, Ltd (Tokyo). Ascomycin was purchased from AG Scientific, Inc (San Diego, CA, USA). Novo-Heparin Injection 5000® (heparin sodium, 5000 units / 5 mL) was purchased from Mochida Pharmaceutical Co, Ltd (Tokyo). Sodium dodecyl sulfate (SDS), liquid paraffin, and formic acid were purchased from Wako Pure Chemical Industries (Osaka). Ammonium acetate was purchased from Sigma Aldrich Inc (Tokyo). All other chemicals were of reagent grade.

2. Animals

Eight- to ten-week-old, female, specific pathogen free NC/Nga mice were purchased from SLC, Inc (Shizuoka). The mice were housed under conventional conditions and controlled temperature with a 12-h light/dark cycle, and were fed ad libitum until *in vivo* percutaneous absorption experiments. Animal studies were performed according to the guidelines for animal use approved by the Institutional Animal Care and Use Committee of Josai University (approval number: H27044 – April 8, 2015).

3. Preparation of AD model mice by treatment with *Dermatophagoides farinae* extract

In order to induce AD-like lesion, a hydrophilic petrolatum ointment including *Dermatophagoides farinae* extract (Dfe ointment, Biostir® AD) was topically applied. The mice (8-10 weeks old, 20-25 g/body) were administered 5.0% isoflurane via inhalation for induction of anesthesia and were then continuously anesthetized using 2.0% isoflurane. The hair on their backs and behind their ears was shaved with a clipper and a shaver on the day before the first application of Dfe. The complete removal of the dorsal hair was conducted by a depilatory that also caused skin barrier disruption 2 h before the first application of Dfe. The Dfe ointment (100 mg) was applied on the dorsal skin and both surfaces of each ear evenly. From the second application of Dfe, the hair on the back and behind the ears was shaved with a shaver whenever it grew. The skin barrier was disrupted by the application of 150 μL of 4 $^{w}/_{v}$% SDS in distilled water 2 h before the Dfe treatment. Dfe ointment (100 mg) was applied on the same site. This procedure was repeated twice a week for three weeks (time course of Dfe-application: day 0, 3, 7, 10, 14, 17).

4. Evaluation of macroscopic features of Dfe-applied skin

Scoring of dermatitis was assessed macroscopically using the following scoring procedure as stated in the technical information of Biostir Inc (Protocol for Biostir AD ointment, http://www.biostir.com/english/pdf/Protocol-e.pdf, March 1, 2017). The development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate) and 3 (severe). Edema was evaluated by measuring ear thickness using a thickness gauge (Digimatic Indicator, Mitsutoyo, Tokyo). The other features were assessed by referring to scored pictures produced by Biostir Inc ("Merkmal of Symptom Score", http://www.biostir.com/english/pdf/Score-e.pdf, March 1, 2017). The AD score in each mouse was defined as the sum of the individual scores on the day of Dfe application and skin absorption study.

5. *In vivo* measurement of physiological skin condition

In order to evaluate SC barrier function, TEWL measurements on the mice dorsal skin were performed using a VapoMeter SWL4001JT (Delfin Technologies, Kuopio, Finland) before the skin barrier disruption by the 4% SDS application in the Dfe ointment treatment procedure and the administration of TL ointment in the *in vivo* absorption studies. The room condition was temperature controlled at 24-5℃ with 40-60% relative humidity during all of the experiments. In this study, we defined the term $TEWL_{intact}$ for the TEWL of mice intact skin and $TEWL_{AD}$ for the TEWL of AD mouse skin.

In order to measure skin blood flow, laser Doppler perfusion imaging scanning was performed on the dorsal skin and perfusion units (PU), which are arbitrary units, were calculated by means of a PeriScan PIM 3 (Perimed AB, Stockholm, Sweden). The measurements were done before the skin barrier disruption in the Dfe ointment treatment procedure and both before the administration of TL ointment and immediately following the administration at time 0, 30, 60, 90 and 120 min in the *in vivo* absorption studies.

6. Histological assessment of Dfe-treated and intact skins

One Dfe-treated mouse used for skin histological assessment was housed with the other Dfe-treated mice that were in the skin absorption study, and one intact mouse as control was housed alone in a different cage in order not to contaminate it with Dfe. On day 21 after the first application of Dfe ointment, the histological assessment Dfe-treated and intact mice were decapitated under isoflurane anesthesia. Their dorsal skins were removed, fixed in 10% neutral phosphate-buffered formalin (pH 7.4), embedded in paraffin, and sectioned. The sectioned skins were stained with hematoxylin and eosin (HE) to assess eosinophilic infiltration, and with toluidine blue to assess mast cell infiltration. The numbers of eosinophils and mast cells were counted in 10 areas (0.14 mm^2 total) of microscopic field (100 μm × 140 μm) at 400 × magnification and the mean values were calculated.

7. Preparation of TL ointment diluted with liquid paraffin and/or mixed with Adr

TL ointment was diluted to suppress TL absorption with the dilution ratio calculated using the equation, $(TEWL_{AD} - TEWL_{intact})/TEWL_{intact}$. TL ointment with liquid paraffin in an ointment jar was incubated (37℃, 10 min) and then mixed using Nanko Rentaro NR-50 (THINKY Co, Ltd) for 5 minutes (orbital velocity, 130 × g; rotation velocity, 60 × g). Adr was adequately mixed with TL ointment (Adr, 0.50 $^{w}/_{w}$%) using a stainless steel spatula on a glass dish before use.

8. *In vivo* skin absorption study of TL

In order to test TL ointment on skin types at two different levels of AD, TL treatment was started 21 or 29 days after initiation of Dfe treatment. On day 21 or 29, the hair of the dorsal skin was removed using a shaver with the mice under isoflurane anesthesia. The application site of TL ointment was marked with a marker pen. The dosage of the TL ointment was 10 mg/cm^2 and an application area was 0.79 cm^2. The strengths of the TL ointment were 0.1 $^{w}/_{w}$% or different values according to $TEWL_{AD}$ values. The three types of ointment used in this study were 0.1 $^{w}/_{w}$ TL ointment (nine subjects at day 21 and six subjects at day 29), paraffin-diluted TL ointment (seven sub-

jects at day 21) and Adr-mixed TL ointment with dilution (three subjects at day 21). Two hours after topical application of TL, the mice were beheaded and the whole blood was collected. Furthermore, in order to measure the skin concentration of TL, liquid-liquid extraction was performed without the day 29-subjects. The residual ointment on the dorsal skin was wiped out carefully with a swab, and the dorsal skin of an ointment-applied area was dissected with micro-dissecting scissors. After weighing the skin specimens, each specimen was minced thoroughly with micro-dissecting scissors in a 1.5 mL micro tube with 1 mL of acetonitrile previously added, acting as extracting solvent, and was incubated for 18 h on a shaker. The skin samples were centrifuged (16,000 × g, 25℃, 5 min) and the supernatant diluted 10-fold with an internal standard solution. The mixtures were injected (10 μL) into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

9. Sample preparation for whole blood

To a 0.30 mL of blood sample, 0.45 mL of a mixed solution of methanol and 0.1 M zinc sulfate solution (7 : 3, $^{v}/_{v}$) were added and vortexed for 10 s. Subsequently, 0.75 mL of the internal standard solution (ascomycin in acetonitrile, 10 ng/mL) was added and vortexed for 30 s. After centrifugation of this mixture at 16,000 × g for 5 min, 1.35 mL of the supernatant evaporated at 45℃ under vacuum (5.1 Torr) with 800 rpm in a SpeedVac 2010 (Thermo Fisher Scientific Inc, Yokohama). The residue was re-dissolved in 70 μL of the mobile phase and centrifuged at 16,000 × g for 5 min. The supernatant (10 μL) obtained from the reconstituted solution was injected into a LC-MS/MS system.

10. LC-MS/MS conditions

TL blood and skin concentration measurements were performed using LC-MS/MS analysis with a Prominence modular high-performance liquid chromatograph (Shimadzu, Kyoto) coupled to an API4000 (AB SCIEX, Tokyo). A Hypersil GOLD CN column (3 μm, 2.1 × 150 mm, Thermo Fisher Scientific, Yokohama) fitted with a Hypersil GOLD CN guard column (3 μm, 2.1 × 10 mm, Thermo Scientific, Yokohama) and a guard cartridge (2.1 × 4.6 mm, Thermo Fisher Scientific, Yokohama), was held at a temperature of 30℃. The mobile phase consisted of 0.1% formic acid and 2 mM ammonium acetate in a mixed solution of acetonitrile and water (65:35, $^{v}/_{v}$) and was isocratically eluted at a flow rate of 0.20 mL/min. The injection volume of each sample was 10 μL. Mass spectra were detected by preforming electrospray ionization in positive ion mode. For the MS/MS analysis, the parent ions and daughter ions were selected at 822.5 m/z ($[M^{+}NH_3]^{+}$ ion) and 769.5 m/z for TL, and at 810.5 m/z ($[M^{+}NH_3]^{+}$ ion) and 757.4 m/z for ascomycin, respectively.

11. Data analysis

The area under the PU versus time curve (AUC_{PU}, 0-2 h) was calculated using the trapezoid rule.

Multiple and simple regression analyses were performed using *fitlm* function with Matlab_R2015b (The MathWorks, Inc, USA). All the other statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc, San Diego, USA). A one-tailed test was indicated and two-tailed was not indicated. The Kolmogorov-Smirnov test, Student's *t*-test, Welch's *t*-test, Man-Whitney's *U*-test, one-way analysis of variance, Tukey-Kramer's test, Dunnett's test, Kruscal-Wallis one-way analysis of variance, Dunn's test,

two-way analysis of variance, and Bonferroni's test were used. Correlations were computed by the Pearson test.

Results

1. Development of AD model mice and the time course of changes in skin conditions and severities

Twenty-six AD mice were developed: nineteen mice used for absorption studies on day 21, six mice on day 29, and one mouse used for histological assessment on day 21. In order to confirm the development of AD, the dorsal skins dissected from a Dfe-treated or intact mouse were histologically assessed. The results of cell counting in microscopic fields at 400 × magnification showed that compared to the intact skin, the Dfe-treated skin showed significant infiltration of eosinophils (Mean cell count/mm^2 ± SEM, 12.70 ± 2.33 (Dfe-treated), 5.70 ± 0.63 (intact), corrected-t (10.32) = 2.90, P = 0.02, Welch's t-test). There was no difference in the number of mast cells between the skins (Mean cell count ± SEM, 8.20 ± 1.21 (Dfe-treated), 7.30 ± 0.86 (intact), t (18) = 0.61, P = 0.55, Unpaired t-test). Microscopic visualization at 100 × magnification in the Dfe-treated skin showed parakeratosis, hyperkeratosis, and epidermal hyperplasia that are also observed in AD patients with chronic eczema (**Fig 1A**, right). Macroscopic visualization showed hemorrhage due to itching, drying, and melanin pigmentation in the Dfe-treated skin (**Fig 1B**, right). These findings confirm the development of AD model mice via repeated Dfe application on the ear and dorsal skin of NC/Nga mice in this study.

Dfe application led to a sustainable increase in dermatitis score from day 0 to day 21, after which, the score deceased to the final observation

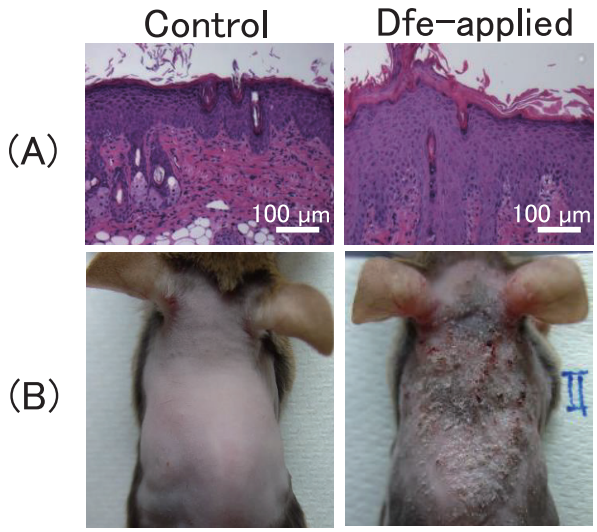

Fig 1 Development of atopic dermatitis (AD) mice by repeatedly Dfe-treatment
Microscopic view of a biopsy dorsal skin specimen at 100 × magnification (A). Gross appearance by digital camera (B). Intact skin (left) and Dfe-applied skin (right) at day 21.

on day 29 (**Fig 2A**). Ear thickness persistently increased from day 0 to day 21 and ear swelling was maintained through day 29 (**Fig 2B**), indicating that edema occurred even in the dorsal skin. The level of TEWL in the dorsal skin increased until day 17, slightly decreased by day 21 and by day 29, was not significantly different compared to the initial level (day 0) (**Fig 2C**). The medium value of PU in dorsal skin tended to increase until day 10, decrease to the initial level at days 14 and 17, and again increase at day 21 (**Fig 2D**); however, the values varied widely. The PU value on day 29 returned to the initial level found on day 0. Comparing day 21 with day 29, the dermatitis score and TEWL values had significant differences, and the PU values at day 21 were widely distributed, yet not as significant as ear thickness. Therefore, we assessed the relationship between the parameters (TEWL and PU) and the systemic absorption of TL through dorsal skin in AD mice, described in the following section.

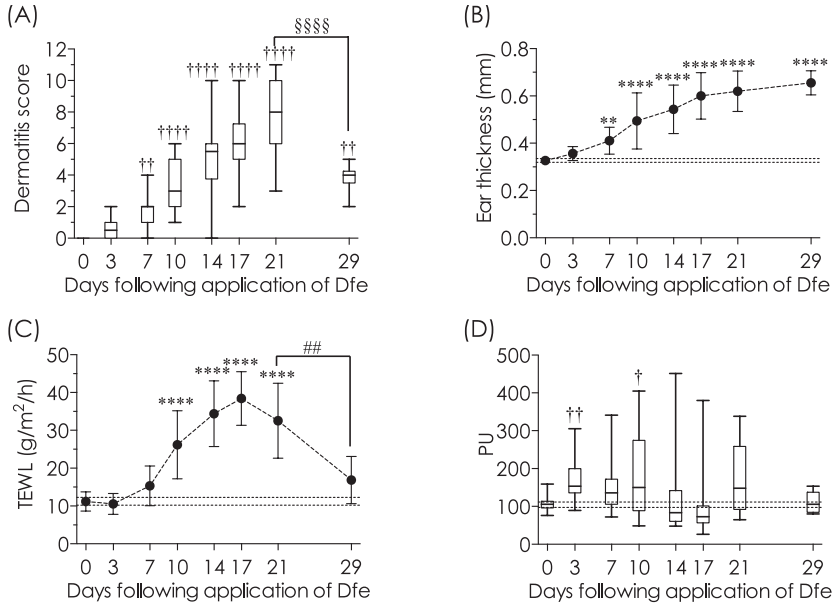

Fig 2 Assessment of skin condition during preparation of atopic dermatitis (AD) model mice

Skin condition was assessed by dermatitis score (A), ear thickness (B), transepidermal water loss (TEWL) (C) and area under perfusion unit (PU) versus time curve (AUC_{PU}) (D). Box plots showing dermatitis scores (A) and PU (D) representing median and interquartile ranges; whiskers represent the highest and lowest values. Ear thickness and TEWL shows mean ± SD. Ear thickness is expressed as mean of both ears. The dashed lines in (B and C) and (D) show the 95% confidence interval of mean and median, respectively. †$P < 0.05$, ††$P < 0.01$, ††††$P < 0.001$, the Dunn's test following the Kruskal-Wallis test; §§§§$P < 0.001$, the Mann-Whitney U-test. **$P < 0.01$, ****$P < 0.001$, the Dunnett's test following ANOVA; ##$P < 0.01$, unpaired t-test.

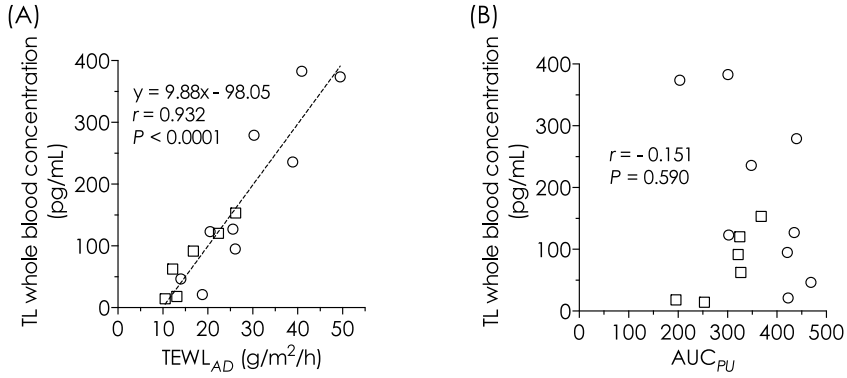

Fig 3 Relationship between skin condition and systemic absorption of tacrolimus (TL) in different time-courses of atopic dermatitis (AD)

Scatter plot showing transepidermal water loss (TEWL) in AD ($TEWL_{AD}$) values (A) or area under perfusion unit (PU) versus time curve (AUC_{PU}) values (B) and TL whole blood concentration 2 h following application of TL ointment on dorsal skin in 15 AD mice classified by time-course as day 21 (○) and day 29 (□) following first application of *Dermatophagoides farinae* (Dfe). The dashed line in (A) shows the regression curve for all data. The r and P values were calculated using the Pearson test.

2. Systemic absorption of TL through dorsal skin in AD mice

The 0.1% TL ointment was applied to the dorsal skin in AD mice (0.79 cm^2, 8 mg/body). AUC_{PU} was calculated from the PU-time profile (data not shown) during the *in vivo* absorption study, and showed no significant difference between day 21 and 29 (Mean ± SEM, 371 ± 29 (day 21), 298 ± 25 (day 29), corrected-t (12.88) = 1.88, $P = 0.083$, Welch's t-test). The whole blood concentration of TL 2 h after application correlated positively ($r = 0.932$, $P < 0.001$) with $TEWL_{AD}$ (**Fig 3A**), but not with AUC_{PU} ($r = -0.151$, $P = 0.59$) (**Fig 3B**). These results sug-

gested that the SC barrier affected systemic absorption of TL through the skin of AD mice and could exhibit a linear relationship at different stages of AD.

3. Effect of administration of liquid paraffin-diluted TL ointment mixed with or without Adr on systemic absorption of TL through AD mice dorsal skin

Because of the linear relationship between TEWL and systemic absorption of TL as described in the previous section, we applied the TL ointment diluted with liquid paraffin on AD mice at day 21 and examined whether the whole blood concentration of TL could be predicted. Furthermore, in order to evaluate the effect of Adr, we mixed Adr into the diluted TL ointment and applied the TL ointment on the AD mice dorsal skin with severe barrier disruption on day 21.

TL ointment was diluted to achieve less TL absorption compared to the estimated TL whole blood concentration. The estimated value was calculated by the following regression curve equation for the AD mice classified by time-course as day 21, represented as a circle (◯) in **Fig 3A**: TL whole blood concentration = 10.702 × $TEWL_{AD}$ – 127.42. The data of the 29th day AD mice were not included for computing the equation and the 21th day AD mice were only used in further experiments. Using the equation, we could target the TL concentration to 'zero' based on **Fig 4A** if TL was not absorbed through intact skin, *ie*, the dilution ratio was defined as $TEWL_{AD}/TEWL_{intact}$. However, in the present study, we used the dilution ratio defined as $(TEWL_{AD} - TEWL_{intact})/TEWL_{intact}$ based on **Fig 4B**, in order to quantitatively assess whether the observed and predicted values of TL systemic absorption were consistent. The $TEWL_{intact}$ used for the calculation was the mean values of $TEWL_{intact}$ of each mouse at day 0. As shown in **Table 1**, two mean $TEWL_{intact}$ values were used for computing the dilution ratio, because six of twenty-six mice additionally participated in this experiment after

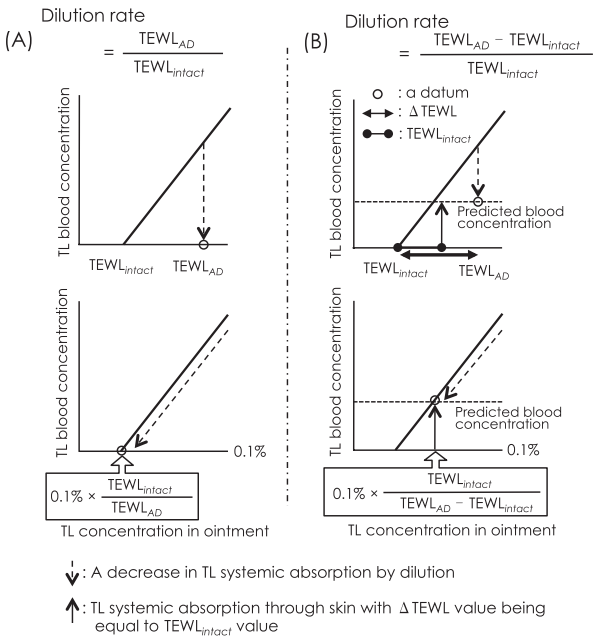

Fig 4 Schematic diagram of a decrease in tacrolimus (TL) systemic absorption by dilution of TL ointment using two types of dilution ratios to achieve TL concentration 'zero' (A), or the predicted TL concentration (B) according to the linear relationship between transepidermal water loss (TEWL) in AD ($TEWL_{AD}$) and TL systemic absorption

Table 1 Dilution ratio according to the TEWL values of each AD mice skin and the TL concentration in the liquid paraffin-diluted TL ointment with (+) or without (–) Adr

Adr	$TEWL_{AD}$ ($g/m^2/h$)	Dilution ratio	$TEWL_{AD}$ before TL application ($g/m^2/h$)	TL concentration in the ointment diluted ($^{w}/_{w}\%$)
–	39.6	[1] 2.39	36.9	0.042
–	46.8	[1] 3.01	44.9	0.033
–	40.9	[1] 2.50	43.6	0.040
–	31.2	[1] 1.67	33.2	0.060
–	23.7	[2] 1.15	22.8	0.087
–	25.3	[2] 1.29	23.6	0.078
–	26.5	[2] 1.40	26.9	0.071
+	45.2	[1] 2.87	44.1	0.035
+	37.6	[1] 2.22	35.7	0.045
+	40.1	[1] 2.43	41.9	0.041

Dilution rates were calculated using the following two different values.
[1] $TEWL_{intact}$ = 11.68 ± 2.05 (mean ± SD)
[2] $TEWL_{intact}$ = 11.05 ± 2.37 (mean ± SD)

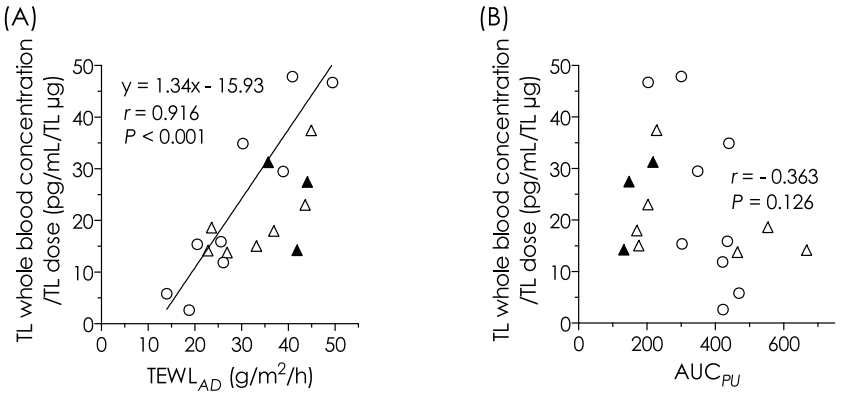

Fig 5 Relationship between skin barrier function and systemic absorption of tacrolimus (TL), and between skin blood flow and systemic absorption of TL

Scatter plot showing transepidermal water loss (TEWL) in atopic dermatitis (AD) ($TEWL_{AD}$) values (A) or area under perfusion unit (PU) versus time curve (AUC_{PU}) values (B) and TL whole blood concentration 2 h following application of TL ointment on dorsal skin in 19 AD mice classified by applied ointment types as original (○), dilution (△) and dilution plus adrenaline (Adr) (▲). The dashed line in (A) shows the regression curve for all data. The *r* and *P* values in (A) and (B) were calculated using the Pearson test for all data.

the permeation study on the other mice had been done and the mean $TEWL_{intact}$ value was recalculated by adding the six $TEWL_{intact}$ values. $TEWL_{AD}$ for dilution and $TEWL_{AD}$ before TL application varied slightly because of the time for ointment dilution (**Table 1**). The difference was not significant (t (9) = 0.54, P = 0.60, Paired t-test) and was therefore ignored.

Figure 5A shows the relationship between $TEWL_{AD}$ and the whole blood concentration of TL, presented as a value divided by each TL dose. The solid line shows the regression curve from the 0.1 $^{w}/_{w}$% TL application data. The application of the diluted TL ointment with or without Adr resulted in TL blood concentration values on or below the regression curve. Some observed values were much lower than each value predicted by the curve, showing that diluting TL ointment suppressed the systemic absorption of TL. Use of Adr resulted in lower AUC_{PU} values than those in the absence of Adr (Mean ± SEM, 165 ± 26 (Adr-use, (n = 3)), 380 ± 42 (non-use, (n = 16)), t (17) = 2.14, P = 0.024, Unpaired t-test, one-tailed), while a similar shift in TL concentration was observed in the diluted ointment with or without Adr (**Fig 5A**). AUC_{PU} and systemic absorption of TL were still not correlated for all data (**Fig 5B**, r = – 0.363, P = 0.126). Thus, we could not conclude that the skin blood flow reduction caused by Adr suppressed TL absorption.

In our previous study using rats,[31] skin blood flow correlated positively with systemic absorption of TL through fully SC-stripped skin. This suggests that a severely disrupted SC barrier results in TL skin permeation and that skin blood flow affects TL uptake into the blood stream. Therefore, we assessed the combined effect of SC barrier and skin blood flow on TL absorption using a multiple linear regression analysis. The analysis was performed to predict TL blood concentration as a response variable based on two explanatory valuables: $TEWL_{AD}$ values, and AUC_{PU} multiplied by $TEWL_{AD}$-squared. Multiplying by $TEWL_{AD}$-squared as a coefficient with exponential growth, the influence of AUC_{PU} on TL absorption is strengthened as $TEWL_{AD}$ values increases. **Table 2** shows that a significant regression equation was found (F (2, 16) = 19.6, P < 0.000), with a freedom-adjusted coefficient of determination (R_{adj}-squared) of 0.673. Predicted

Table 2 Multiple regression analysis for whole blood concentration of TL

variable	B	β	t value	*P* value	*R* of single regression
TEWLAD	0.538	0.433	2.524	0.023	0.744
$(TEWL_{AD})^2 \times AUC_{AD}$	0.000	0.503	2.933	0.010	0.771
Intercept	-10.836		-1.870	0.080	
R^2_{adj}	0.673				

R = correlation coefficient, R^2 = coefficient of determination, R^2_{adj} = adjusted R^2, B = partial regression coefficient; β = standardized partial regression coefficient

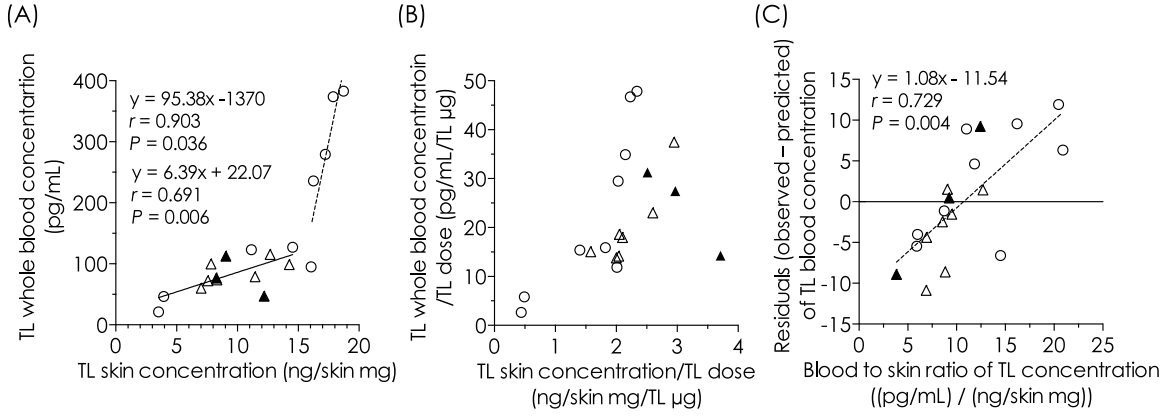

Fig 6 Alteration of tacrolimus (TL) disposition in skin and blood among different types of TL ointments

(A), Scatter plot showing TL skin concentration and TL whole blood concentration 2 h following application of TL ointment on dorsal skin in 19 atopic dermatitis (AD) mice classified by applied ointment types as original (○), dilution (△) and dilution plus adrenaline (Adr) (▲). The solid line and dashed line show the regression curve for lower TL skin concentration and higher one at 15 (ng/skin mg) as inflection point, respectively. The *r* and *P* values were calculated using the Pearson test. (B), both functions represent normalized values by dividing with TL dose. (C), Residuals of observed and predicted TL blood concentration in multiple regression analysis in **Table 2** as a function of blood to skin ratio of TL concentration. The dashed line shows the regression curve. The *r* and *P* values were calculated using the Pearson test.

blood concentrations of TL were equal to –10.836 + 0.538 ($TEWL_{AD}$) + 4.929 × 10^5 ($(TEWL_{AD})^2$ × AUC_{PU}). Both parameters are significant predictors of TL blood concentration and had little collinearity (VIF = 1.620, tolerance = 0.617). The R_{adj}-squared (0.673) in the multiple regression analysis was higher than that (0.527) in the single regression based on $TEWL_{AD}$ as one variable, suggesting that skin blood perfusion could affect systemic absorption of TL through skin with severe SC-barrier disruption.

4. Disposition of TL in skin and blood after administration of liquid paraffin-diluted TL ointment mixed with or without Adr

After the whole blood sampling in skin absorption study, the dorsal skin treated with TL ointment was dissected, and then TL concentration in the skin was measured. **Figure 6A** shows that TL concentration in skin was related to TL blood concentration and the relation was diphasic with an inflection point around TL skin concentration of 15 ng/skin mg where TL blood concentration increased rapidly. Dividing each datum by each applied dose, the positional relation of data was changed (**Fig 6B**). In the groups of diluted TL ointment with or without Adr, the TL blood concentration and TL skin concentration tended to be low and high, respectively, as compared with that in the group of original TL ointment. This result shows that both dilution groups had low TL transitivity into blood and high TL retention in the skin. **Figure 6C** shows the relationship between

the blood to skin ratio of TL concentration and the residual value that was calculated subtracting the TL blood concentration predicted in the multiple regression analysis in the previous section from the observed concentration. The data sets have a positive correlation ($r = 0.729$, $P = 0.004$), suggesting the down-gaps of observed TL blood concentration to the regression curve in **Fig 6A** could result from skin retention as well as skin blood flow.

Discussion

In the present study, an *in vivo* skin absorption study was performed using AD model mice with measurements of TEWL and PU as physiological skin parameters in order to examine the factors influencing the systemic and skin absorption of TL. There were three findings from our study: TEWL linearly correlated with the systemic absorption of TL through dorsal skin, the effect of skin blood flow on TL absorption increased based on the degree of TEWL, and the relationship between skin and blood concentrations was biphasic.

The linear relationship between TEWL and topical absorption of drugs has been reviewed in *in vivo* human and animal experiments using healthy and damaged skin.[37] Aalto-Korte and others[38] reported the linear relationship using hydrocortisone in AD patients. The result of our absorption experiment using TL in AD mouse was consistent with the previous studies. In addition, we reported the linear relationship at a different time-course of AD developmental model, suggesting that TEWL measurement can predict systemic absorption of TL in various stages of AD. Diluting TL ointment according to $TEWL_{AD}$ values resulted in lower values than predicted, and TL blood concentration was not controlled precisely; however, the ointment dilution was useful in suppressing the systemic absorption of TL. We must also consider whether the diluted TL ointment has a therapeutic effect on AD. Oranje and others studied the treatment effect of TL ointment under occlusion at different dilutions using AD model mice.[39] Daily treatment with 0.01%, 0.03%, or 0.1% TL ointment for 2 weeks, showed delayed progression of barrier loss and prevention of skin thickening and epidermal hyperplasia; however, this study did not perform TEWL measurements. Some clinical studies in AD patients have reported a TEWL value of approximately 15 to 40 ($g/m^2/h$) in lesional skin of AD patients; this value is 2- to 4-fold greater than that reported for the skin of healthy volunteers (5 to 10 ($g/m^2/h$)).[26, 40-42] Therefore, assuming that skin barrier and TL absorption is linearly related and TL absorption can be controlled by the diluted ointment based on the relationship in AD patients, at least about 2- to 4-fold dilution of TL ointment could prevent TL absorption using the equation in **Fig 4A** since TL is rarely or not absorbed through intact skin. 0.03% TL ointment, a commercial product for pediatric patients, whose efficacy has been confirmed in adult AD patients,[19] also can be an option for prevention of systemic absorption of TL. As skin barrier heals, twice-daily application of 0.1% TL ointment is recommended as a proactive treatment for subclinical inflammation.[43]

There have been few studies reported regarding the effect of skin blood flow on skin absorption of drugs compared to the effect of skin barriers because the permeation of drugs through SC is commonly understood to be a rate-limiting step of systemic absorption. In the present study, we could not reveal whether the use of Adr to suppress blood flow affected TL absorption in AD

model mice. Although skin barrier was disrupted in AD model mice, SC was observed in the skin biopsy of an AD mouse (**Fig 1A**). Therefore, the rate-limiting step of systemic absorption in the AD mice in the present study was not uptake of TL into blood but the permeation of TL through SC. Note that the AUC_{PU} value multiplied by $TEWL_{AD}$-squared was significant as an explanatory variable in multiple regression analysis for TL blood concentration. The rate-limiting step of TL absorption could be shifted to uptake into blood stream from SC permeation because of severe skin barrier disruption.

Interestingly, the relationship between skin and blood concentration of TL was biphasic; the blood concentration rapidly increased as skin concentration increased beyond a certain value. The ear thickness increased persistently with Dfe application (**Fig 2B**), indicating that edema, which is caused by vascular hyperpermeability and leakage of plasma components, occurred in the dorsal skin. Since TL binds with high affinity to skin components and plasma proteins,[44] at first the concentration of unbound TL is low. However, if the saturation of TL-protein binding occurs, the concentration of unbound TL rapidly increases and then subsequently systemic absorption of TL could be enhanced. This may result in the biphasic relationship. Thus, a low ratio of blood to skin TL concentration can be expected with the dilution of TL ointment in addition to suppressing systemic absorption of TL (the first phase in **Fig 6A**).

Maibach and others have concluded that *in vivo* hairless mouse data are not a predictive of human skin *in vitro* permeability because of the lack of statistically significant correlation between hairless mouse skin and human skin.[45] As mentioned in the results section, however, the AD mice induced by Dfe application exhibited the representative physiological and histological features of human-AD. Thus, the interpretation of a relationship between skin condition and TL behavior in this AD mouse model could be expanded into AD patients. Finally, based on our results and discussion so far, we suggest an administration design for TL ointment that is tailored to various skin conditions in order to prevent systemic absorption of TL. TL absorption depends strongly on SC barrier function. Even if dysfunction of the SC barrier develops and a loss of barrier function occurs, TL ointment could be applied due to prevention of its systemic absorption by diluting according TEWL. Furthermore, TL exhibits skin barrier recovery as well as an anti-inflammatory effect.[46, 47] From both safe and efficacy, the diluted TL ointment is possibly expected as an option for the eroded surface to which TL ointment cannot be applied. In the case of severely SC-disrupted skin exhibiting redness or warmth, the combination use of vasoconstrictor also could be beneficial in preventing excessive vascular uptake of TL.

In conclusion, topical pharmacotherapy is recommended to be prescribed along with a quantitative assessment of skin condition, in order to predict drug disposition, which will provide more safe and effective treatments for skin disease. We expect that a further investigation into the relationship between TEWL and TL absorption in AD patients, and an assessment of the therapeutic effect of diluted TL ointment will yield critical insights that help facilitate individual dosage design of TL ointment.

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Conflicts of Interest

The authors declare no conflicts of interest.

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