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Comparison of the efficacy of tacrolimus and cyclosporine A in a murine model of dinitrofluorobenzene-induced atopic dermatitis

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ABSTRACT

Tacrolimus (FK506) and cyclosporine A (Cys A) are immunosuppressive drugs used in the treatment of inflammatory diseases and for preventing rejection of allogeneic transplants. Tacrolimus forms a complex with FK506 binding protein (FKBP), and Cys A forms a complex with cyclophilin. These tacrolimus–FKBP and Cys A–cyclophilin complexes interact with calcineurin (CaN), thereby suppressing activation of T cells. In contrast, steroidal anti-inflammatory drugs suppress the immune system mainly via inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the activating protein-1 (AP-1) pathway. Previously, we reported that tacrolimus, but not dexamethasone, reduced scratching behavior in a murine model of atopic dermatitis. To elucidate the mechanism involved in the inhibition of scratching behavior, we used a mouse model of allergic dermatitis to compare the characteristics of tacrolimus and Cys A treatment. We found that Cys A suppressed scratching behavior induced by application of 2,4-dinitrofluorobenzene, as did tacrolimus. In addition, both drugs attenuated increases in vascular permeability and scratching behavior induced by passive cutaneous anaphylaxis. These results indicate that inhibition of the CaN pathway plays an important role in tacrolimus- and Cys A-induced inhibition of scratching behavior in mice. Furthermore, we observed that CaN inhibitors suppressed mast cell-dependent allergic reaction.

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1. Introduction

Atopic dermatitis is frequently refractory to treatment. Therapy for inflammatory eczema and pruritus involves the use of steroid and tacrolimus ointments for inflammation, and antihistamines and antiallergic drugs for pruritus (Furie et al., 2004). Pruritus is the hallmark of atopic dermatitis, and chronic and severe pruritus can have a deleterious impact on patient quality of life. Furthermore, scratching damages the skin barrier and worsens inflammation of skin, resulting in more itching (Wahlgren, 1999). Although antihistamines are frequently used to suppress pruritus in atopic dermatitis, little objective evidence exists to demonstrate their effectiveness (Klein and Clark, 1999). However, tacrolimus did improve the clinical score in an assessment of pruritus (Ruzicka et al., 1997; Boguniewicz et al., 1998).

In 2008, cyclosporine A (Cys A) was introduced in Japan for the treatment of severe atopic dermatitis. Cys A is cyclic peptide of 11 amino acids derived from the fungus *Tolypocladium inflatum* Gams (Pritchard, 2005), and is a representative immunosuppressive drug for preventing rejection of transplanted liver, kidney, heart, and lung (Frei, 1999). Oral administration of Cys A is an effective treatment for immunological diseases such as psoriasis, Behcet's syndrome, and

nephrosis syndrome. In addition, it was reported that Cys A significantly reduced pruritus intensity, eczema score, and the total number of blood eosinophils (Wahlgren et al., 1990).

By forming a complex with FK506 binding protein (FKBP), tacrolimus suppresses activation of T cells; Cys A achieves the same result by forming a complex with cyclophilin. Because the complexes interact with and inhibit calcineurin (CaN), activation of T cells is suppressed (Liu et al., 1991). In contrast, steroidal anti-inflammatory drugs are frequently used for treatment of allergic diseases. Glucocorticoids suppress immune inflammation by interacting with various transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and activating protein-1 (AP-1), which activate transcription of genes for inflammatory cytokines and enzymes.

Previously, we reported that, in mice, repeated treatment with 2,4-dinitrofluorobenzene (DNFB) at a concentration of 0.15%, dissolved in acetone, induced eczematous changes of the skin, including marked infiltration of neutrophils and eosinophils and hypertrophy of epidermis (Nagai et al., 1997), and caused a persistent increase in scratching behavior (Inagaki et al., 2006). Tacrolimus suppressed the scratching behavior induced by the application of DNFB solution, but dexamethasone did not (Inagaki et al., 2006). Our findings suggested that tacrolimus was not dependent on an intermediary of the I κ B/NF- κ B pathway in its suppression of scratching behavior in the mouse model. However, the mechanism responsible for tacrolimus-induced suppression of scratching behavior remains unknown.

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In the present study, to elucidate the mechanism responsible for tacrolimus-induced inhibition of scratching behavior, we used a mouse model of atopic dermatitis to compare the effects of tacrolimus with those of Cys A.

2. Materials and methods

2.1. Animals

Seven-week-old male BALB/c mice and male ICR mice were purchased from SLC, Inc. (Hamamatsu, Japan). Mice were housed in an air-conditioned animal room at a temperature of $22 \pm 1^\circ\text{C}$ and a humidity of $60 \pm 5\%$. Experiments were undertaken in adherence with the guidelines established by the Animal Experiment Committee of Gifu Pharmaceutical University. The Animal Experiment Committee of our university approved the experimental protocols employed.

2.2. Antigens and antibody

2,4-Dinitrofluorobenzene (DNFB, Sigma Chemical Co., St. Louis, MO, USA) was used to induce cutaneous reaction. DNFB was dissolved in acetone at a concentration of 0.15%. Dinitrophenyl-conjugated *Ascaris suum* extract (DNP-Asc) and DNP-conjugated bovine serum albumin (DNP-BSA) were prepared using the method of Eisen et al. (1954). Aluminum hydroxide gel (alum) was used as an adjuvant for sensitization of DNP-Asc. DNP-Asc 10 μg in the presence of alum 1 mg (DNP-Asc/alum) was injected intraperitoneally for sensitization. Mouse monoclonal IgE antibody against DNP residue (anti-DNP IgE mAb) was prepared by culturing a cell line, EC1, as reported previously (Sakurai et al., 1994). The culture supernatant of EC1 was stored at -80°C . The IgE content of the preparation, as estimated by enzyme-linked immunosorbent assay (ELISA), was 1.5 $\mu\text{g}/\text{ml}$.

2.3. Drugs

Tacrolimus (Astellas Pharma Inc., Tokyo, Japan) was dissolved in ethanol at concentrations of 0.01, 0.1, and 1%, and applied topically to both sides of each ear lobe in a total volume of 100 μl . Cyclosporine A (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in 0.5% carboxymethyl cellulose sodium (Nacalai Tesque, Kyoto, Japan) solution, and administered orally at doses of 1, 3, 10, 30, and 100 mg/kg, or topically at a concentration of 0.1%. Dexamethasone (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in ethanol at a concentration of 0.01%.

In an experiment on passive cutaneous anaphylaxis (PCA), tacrolimus at doses of 3 and 10 mg/kg, cyclosporine A at doses of 10 and 30 mg/kg, and diphenhydramine (DPH, Sigma Chemical Co., St. Louis, MO, USA) at a dose of 20 mg/kg, were dissolved in 0.5% carboxymethyl cellulose sodium and administered orally 1 h before eliciting the reaction.

2.4. Induction of cutaneous reaction and scratching behavior by application of DNFB

Dermatitis and scratching behavior were induced by repeated treatment of each surface of both ears with DNFB solution in BALB/c mice, as previously described (Inagaki et al., 2010). Briefly, 2 weeks after sensitization with DNP-Asc/alum, a total volume of 100 μl of 0.15% DNFB acetone solution was topically applied 5 times every 48 h to both sides of each ear lobe of the mice. Two weeks after the fifth application, the mice received a similar final challenge. Control mice were similarly treated with acetone in the absence of DNFB (vehicle). Each drug was treated one time or for 10 days before the final DNFB application.

2.5. Evaluation of cutaneous reaction

Cutaneous reaction was evaluated by ear swelling induced by the final challenge with DNFB. Ear thickness was measured using a dial thickness gauge (Ozaki MFG Co., Ltd., Tokyo, Japan) and was expressed as the increase in ear thickness from the pre-challenge value.

2.6. Evaluation of scratching behavior

Scratching behavior was measured using the MicroAct system (Neuroscience, Tokyo, Japan), by which scratching behavior is automatically detected and objectively analyzed (Inagaki et al., 2002, 2003). Briefly, a small magnet (1 mm in diameter, 3 mm long, coated with Teflon) was inserted subcutaneously into both hind paws under ether anesthesia, at least 1 week before the start of the experiment. Mice with inserted magnets were placed in an observation chamber (11 cm in diameter, 18 cm high) that was surrounded by a round coil. The electric current induced in the coil by the movement of magnets attached to the mouse hind paws was amplified and recorded. Then, characteristic waves corresponding to scratching behaviors were detected by using a computer. Parameters for the detection of consecutive scratching behavior were upper limit, 0.90 V; lower limit, 0.03 V; event gap, 0.05 s; maximum frequency, 20.0 Hz; minimum frequency, 3.0 Hz; and minimum duration, 0.43 s. The results of scratching behavior were expressed as both the incidence of consecutive scratching behavior and total duration of scratching in the period from 0 to 2 h after DNFB challenge.

2.7. Detection of mRNA expression

Effects of drugs on mRNA expression in ears of the mice were evaluated by real time reverse transcriptase-polymerase chain reaction (RT-PCR). In brief, total RNA was extracted from excised ears of the mice using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan). cDNA was prepared from the RNA by reverse transcription (Super-script II, Invitrogen Corporation, CA, USA) and then amplified by PCR iCycler. PCR products were analyzed by iCycler iQ Real time Detection System. Primers employed and the conditions are listed in Supplemental Table 1. Expression of cytokines was corrected by each expression of β -actin.

2.8. Histological analysis

Right ears of the mice were excised before corresponding timing to the last DNFB challenge, fixed in 4% paraformaldehyde, and embedded in paraffin. Sections (3 μm) were stained with acidic toluidine blue (pH 4.1), and number of mast cells and ratio of degranulated mast cells in 3 sights chosen high-power field at random was counted.

2.9. Passive cutaneous anaphylaxis (PCA)

Male ICR mice were passively sensitized by intradermal injection of anti-DNP IgE mAb at a volume of 20 μl into clipped back skin. Twenty-four hours after the sensitization, PCA was elicited by intravenous injection of 0.25 ml of 0.5% Evans blue saline solution containing 0.1 mg/ml of DNP-BSA. Immediately after antigen challenge, mice were placed in a chamber for observation of scratching behavior for 1 h, as mentioned above. After evaluation of scratching behavior, mice were sacrificed by cervical dislocation, and the back skin that was stained by extravasated Evans blue was removed to measure the increase in vascular permeability. Quantification of extravasated dye was performed using a previously described method (Inagaki et al., 1984). Briefly, the back skin from a mouse was dissolved in 0.7 ml of 1 N KOH solution at 37°C overnight, and 9.3 ml of a mixture of 0.6 N phosphoric acid and acetone (5:13) was added.

After vigorous shaking, precipitates were filtered off and the amount of dye was measured colorimetrically at 620 nm.

2.10. Statistical analysis

The data were expressed as the mean values plus standard error. The statistical significance of the difference between 2 values was evaluated by the Student or Welch *t*-test using StatLight (Yukms Co., Ltd., Tokyo, Japan). Comparison of data from the tacrolimus, Cys A, and control groups was performed using the Dunnett comparison test. A *P* value less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Application-timing for tacrolimus

Tacrolimus at a concentration of 0.1% was applied topically to both sides of each ear 1, 2, 4, 8, and 12 h before the final DNFB challenge. At all time points, tacrolimus significantly reduced the number of scratch attempts (Fig. 1A) and duration of scratching (Fig. 1B) induced by the final challenge. The most potent inhibition of scratching behavior was observed when tacrolimus was given 8 h before challenge. Ear swelling at 6 h after the final DNFB challenge was significantly suppressed by tacrolimus given 8 h before challenge (Fig. 1C). Ear swelling at 24 h was suppressed by tacrolimus at all time points, and the suppression by tacrolimus given 8 h before challenge was the most potent (Fig. 1D).

3.2. Concentration-dependency of tacrolimus

The effects of tacrolimus concentrations ranging from 0.01% to 1% on ear swelling and scratching behavior elicited by DNFB were

examined. The drug was applied 8 h before the final challenge. Tacrolimus concentration-dependently suppressed the incidence and duration of scratching behavior (Fig. 2A, B), as well as ear swelling, at 6 and 24 h after the final challenge (Fig. 2C, D). This suppression was significant at concentrations of 0.1% and 1%.

3.3. Administration-timing for cyclosporine A (Cys A)

Cys A at a dose of 30 mg/kg was administered orally 1, 2, 4, 8, and 12 h before the final DNFB challenge. At all time points, oral administration of Cys A significantly reduced scratch number (Fig. 3A) and scratching time (Fig. 3B) induced by the final challenge. The inhibition of scratching behavior was most potent when Cys A was administered 4 h before the challenge. Cys A suppressed ear swelling at 6 h after the final challenge, but the suppression was significant only when the drug was administered 1 or 2 h before the challenge (Fig. 3C). Cys A did not affect ear swelling at 24 h after the challenge (Fig. 3D). Tacrolimus at a concentration of 0.1% applied topically 1 h before challenge significantly suppressed both scratching behavior and ear swelling (Fig. 3A–D).

3.4. Dose-dependency for Cys A

Cys A suppressed ear swelling most potently when given 1 h before the challenge. Furthermore, scratching behavior was also significantly suppressed by the drug when given 1 h before challenge. Therefore, Cys A was administered orally at doses of 1, 3, 10, 30, and 100 mg/kg 1 h before the final DNFB challenge. Cys A suppressed both the incidence and duration of scratching behavior (Fig. 4A, B) in a nearly dose-dependent fashion, and suppression at a dose of 30 mg/kg was significant. Cys A dose-dependently decreased ear swelling at 6 h after the DNFB challenge, and suppression at doses of 30 and 100 mg/

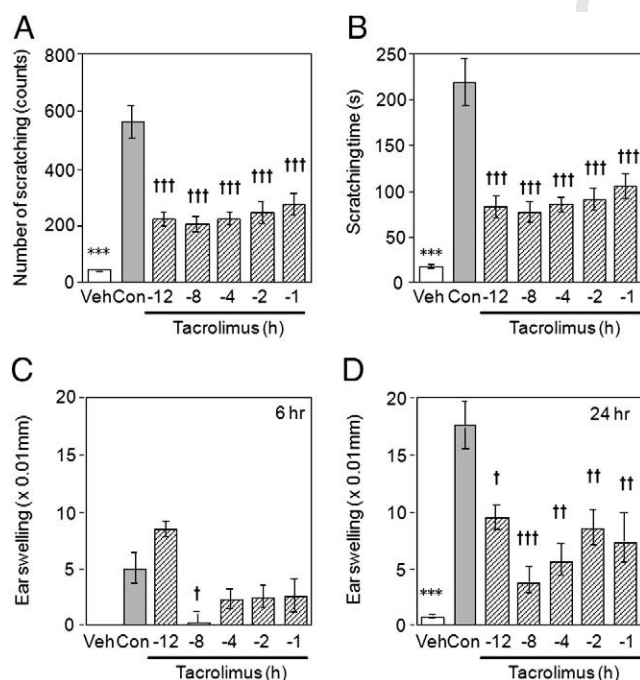


Fig. 1. Time-course study of the effect of tacrolimus on ear swelling and scratching behavior in BALB/c mice. Tacrolimus at a concentration of 0.1% was applied topically to both sides of each ear 1, 2, 4, 8, and 12 h before the final DNFB application. The number of scratch attempts (A) and duration of scratching (B) were measured from 0 to 2 h after the last DNFB challenge. Ear swelling was calculated as the increase in ear thickness from the time point immediately before the last challenge to 6 h (C) and 24 h (D). Each value represents the mean $\pm$ S.E.M. of 7 mice. Veh, vehicle; Con, control. ****P* < 0.001 vs. Con (Welch *t*-test); †, ††, †††*P* < 0.05, 0.01, 0.001 vs. Con (Dunnett test).

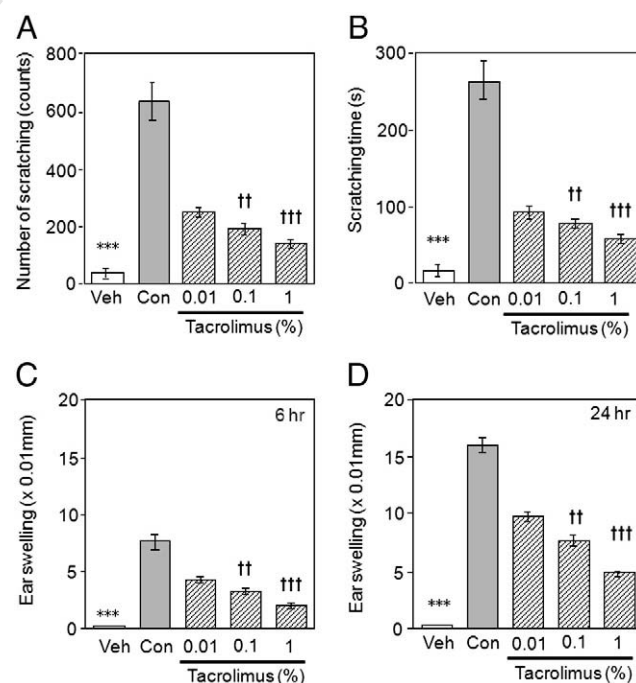


Fig. 2. Dose-response study of the effect of tacrolimus on ear swelling and scratching behavior in BALB/c mice. Tacrolimus at a concentration of 0.01, 0.1, and 1.0% was administered topically to both sides of each ear 1 h before the final DNFB application. Each value represents the mean $\pm$ S.E.M. of 7 mice. Veh, vehicle; Con, control. ****P* < 0.001 vs. Con (Welch *t*-test); †, ††, †††*P* < 0.01, 0.001 vs. Con (Dunnett test). The number of scratch attempts (A) and scratching duration (B) were measured for 0 to 2 h after the last DNFB challenge. Ear swelling was calculated as the increase in ear thickness from the time point immediately before the last challenge to 6 h (C) and 24 h (D).

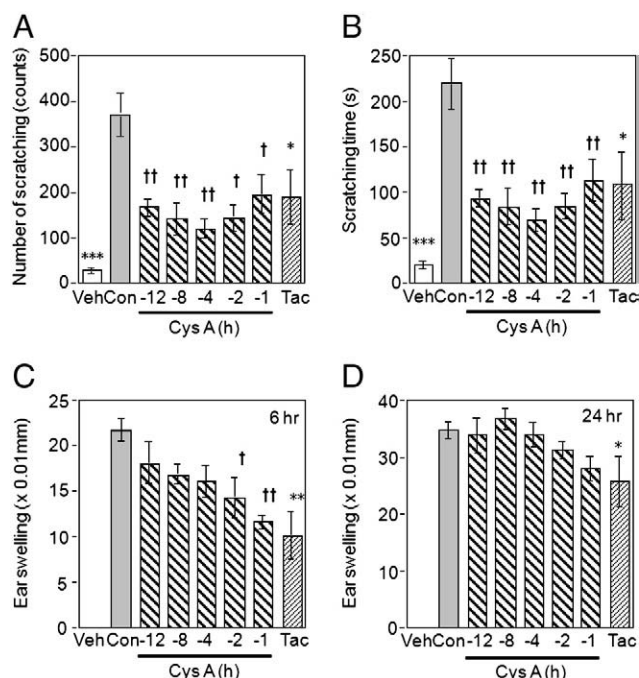


Fig. 3. Time-course study of the effect of cyclosporine A on ear swelling and scratching behavior in BALB/c mice. Cyclosporine A (Cys A) at a dose of 30 mg/kg was administered orally 1, 2, 4, 8, and 12 h before the final DNFB application. Tacrolimus (Tac) at a concentration of 0.1% was applied topically to both sides of each ear 1 h before the final DNFB application. Each value represents the mean $\pm$ S.E.M. of 6 to 8 mice. Veh, vehicle; Con, control. *, ** P <0.05, 0.01 vs. Con (Student or Welch t -test). †, †† P <0.05, 0.01 vs. Con (Dunnett test). The number of scratch attempts (A) and duration of scratching (B) were measured from 0 to 2 h after the last DNFB challenge. Ear swelling was calculated as the increase in ear thickness from the time point immediately before the last challenge to 6 h (C) and 24 h (D).

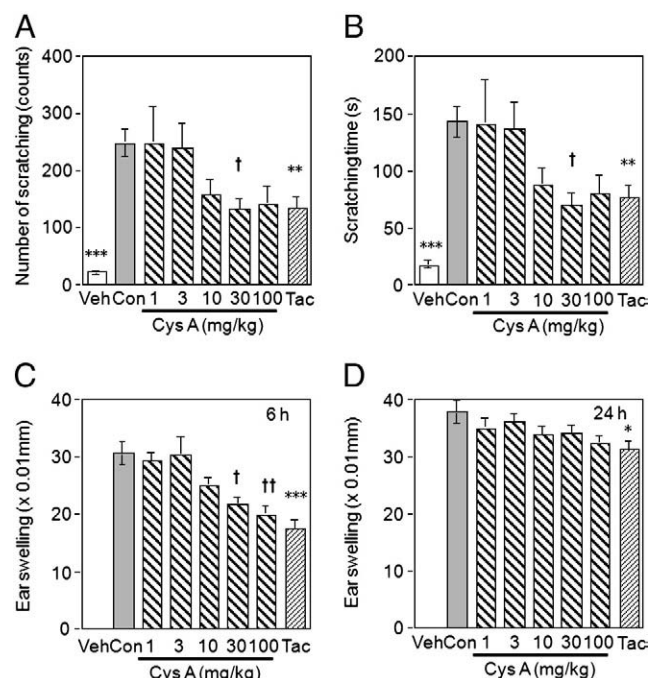


Fig. 4. Dose-response study of the effect of cyclosporine on ear swelling and scratching behavior in BALB/c mice. Cyclosporine A (Cys A) at a dose of 1, 3, 10, 30, and 100 mg/kg was administered orally 1 h before the final DNFB application. Tacrolimus (Tac) at a concentration of 0.1% was applied topically to both sides of each ear 1 h before the final DNFB application. Each value represents the mean $\pm$ S.E.M. of 6 to 8 mice. Veh, vehicle; Con, control. *, **, *** P <0.05, 0.01, 0.001 vs. Con (Student or Welch t -test). †, †† P <0.05, 0.01 vs. Con (Dunnett test). The number of scratch attempts (A) and duration of scratching (B) were measured from 0 to 2 h after the last DNFB challenge. Ear swelling was calculated as the increase in ear thickness from the time point immediately before the last challenge to 6 h (C) and 24 h (D).

kg was significant (Fig. 4C). Ear swelling at 24 h after the challenge was not affected by the drug (Fig. 4D). Topical tacrolimus at a concentration of 0.1% applied 1 h before challenge significantly suppressed both scratching behavior and ear swelling (Fig. 4A–D).

3.5. Repeated application of tacrolimus and cyclosporine A

Tacrolimus at concentration of 0.1%, Cys A at a dose of 30 mg/kg and dexamethasone at a concentration of 0.05% were treated for 10 days before the final DNFB challenge. Ear swelling 6 h (Supplementary Fig. 1A) and 24 h (Supplementary Fig. 1B) after the challenge was inhibited by repeated treatment of the drugs as well as single treatment. Scratching behavior was suppressed by repeated treatment of tacrolimus and Cys A, but not by dexamethasone (Supplementary Fig. 1C, D).

IL-4, IL-13, IFN- γ and IL-31 mRNA expressions in the ears of mice treated with the drugs were detected by real time RT-PCR (Supplementary Fig. 2). In this model, both Th2 cytokines (IL-4 and IL-13) and Th1 cytokine (IFN- γ), and IL-31 which related with pruritus were up-regulated by DNFB painting. Tacrolimus, Cys A and dexamethasone suppressed expression of Th1 and Th2 cytokines, but dexamethasone failed to suppress up-regulation of IL-31 expression.

Topical treatments of Cys A at a concentration of 0.1% once and for 10 days failed to suppress scratching behavior (Supplementary Fig. 3).

3.6. Effects of tacrolimus and Cys A on passive cutaneous anaphylaxis (PCA)

The effects of tacrolimus and Cys A on scratching behavior and the increase in vascular permeability associated with PCA were examined. Tacrolimus at doses of 3 and 10 mg/kg, Cys A at doses of 10 and 30 mg/kg, and diphenhydramine at 20 mg/kg (positive control) were

administered orally 1 h before elicitation of PCA. Tacrolimus and Cys A dose-dependently suppressed scratching behavior and dye leakage associated with PCA (Fig. 5). Suppression by 3 and 10 mg/kg of tacrolimus and 30 mg/kg of Cys A was significant. Diphenhydramine also significantly suppressed scratching behavior and dye leakage (Fig. 5C).

3.7. Histological analysis

Right ears of the mice which were applied with tacrolimus topically and Cys A orally once were excised right before the last DNFB challenge, and sliced ear sections were stained by toluidine blue. Number of mast cells and ratio of degranulated mast cells were counted in 3 sights chosen high-power field at random. Tacrolimus and Cys A did not affect on the mast cells number, and tend to inhibit the degranulation (Supplementary Table 2).

4. Discussion

In our previous report, tacrolimus and dexamethasone inhibited inflammation in back skin induced by repeated hapten treatment; tacrolimus simultaneously suppressed scratching behavior associated with the hapten treatment, but dexamethasone did not (Inagaki et al., 2006; Supplementary Fig. 3). In the present study, we investigated the characteristics of tacrolimus-induced suppression of scratching behavior by comparing tacrolimus to another calcineurin (CaN) inhibitor, cyclosporine A (Cys A).

First, the optimal concentration and timing of administration of tacrolimus were studied. Tacrolimus suppressed scratching behavior and ear swelling for a prolonged period, and was most effective when

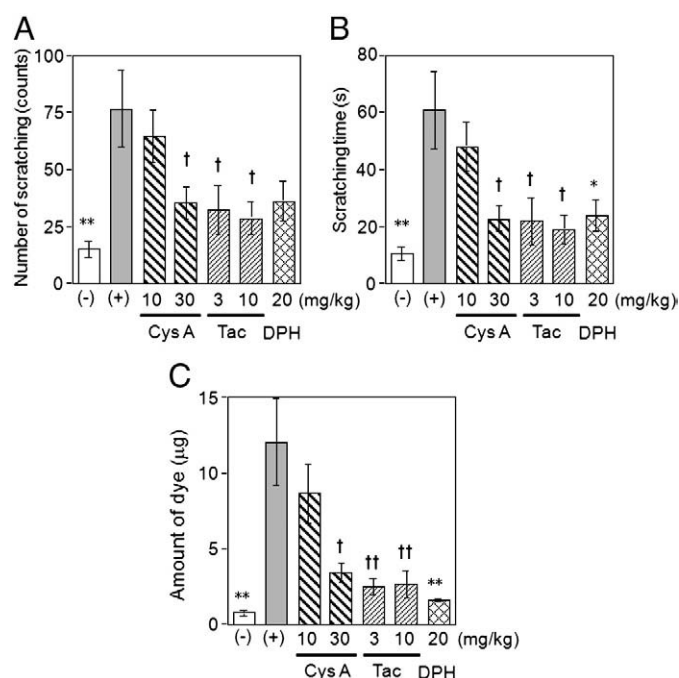


Fig. 5. Passive cutaneous anaphylaxis (PCA) of tacrolimus and cyclosporine A in ICR mice. The number of scratch attempts (A) and duration of scratching (B) were evaluated for 1 h after PCA challenge. The vascular permeability was assessed by measuring the amount of Evan's Blue dye induced by PCA (C). Each value represents the mean $\pm$ S.E.M. of 8 or 9 mice. (–), no challenge; (+), PCA; Cys A, cyclosporine A; Tac, tacrolimus; DPH, diphenhydramine. *, ** P <0.05, 0.01 vs. (+) (Student or Welch t -test). †, †† P <0.05, 0.01 vs. (+) (Dunnett test).

given 8 h before the final DNFB challenge (Fig. 1). Because topical treatment with Cys A at a concentration of 0.1% had no effect on scratching behavior (Supplementary Fig. 3), Cys A was administered orally. Cys A suppressed scratching behavior most effectively when given 4 h before challenge, and ear swelling when given 1 h before. The effect of Cys A on scratching behavior was prolonged, and peaked between 4 h and 8 h; similar results were observed with tacrolimus. However, although Cys A maximally inhibited ear swelling when given 1 h before challenge, tacrolimus did so when given 8 h before (Fig. 3).

Time to maximum drug concentration (T_{max}) after oral administration of Cys A in healthy adult humans is approximately 1.6 h (Avramoff et al., 2007; Piñero-López et al., 2007), whereas that of tacrolimus ointment is approximately 6 h in atopic dermatitis patients (Kawashima et al., 1997). Our results regarding ear swelling suggest the possibility of a negative correlation with the T_{max} of these drugs. Furthermore, the results at 24 h after challenge were similar to these at 6 h. This discrepancy with respect to the most effective administration timing of Cys A for suppression of scratching behavior and ear swelling might be due to a variety of factors; the reasons are unclear at present. The present findings suggest that Cys A and tacrolimus suppressed scratching by the same mechanism—the CaN pathway—but that the mechanisms responsible for their effects on inflammation differed.

The molecular weight of Cys A is approximately 1200, and that of tacrolimus is approximately 800. Generally, compounds with a molecular weight higher than 500 do not penetrate normal skin. However, compounds with higher molecular weights, such as tacrolimus, can penetrate inflammatory skin when the skin-barrier is broken. In ear lobes repeatedly exposed to DNFB the skin-barrier is disrupted, and tacrolimus, but not Cys A, can penetrate the skin.

Immunological stimulation induces production of IL-2 via activation of nuclear factor of activated T-cells (NFAT) transcription in T

cells (Jain et al., 1992). When antigen-presenting cells interact with T cell receptors on T cells, phospholipase $C\gamma$ (PLC γ) is activated to hydrolyze phosphatidylinositol 4,5-diphosphate (PIP $_2$) in plasma membranes to inositol triphosphate (IP $_3$). Then, Ca $^{2+}$ mobilized by IP $_3$ from endoplasmic reticulum (ER) activates calcineurin (CaN), a calcium-dependent serine/threonine phosphatase, by binding a regulatory subunit and activating calmodulin (CaM) binding. CaN induces NFAT activation, which is important in the transcription of inflammatory genes necessary to produce a variety of cytokines. The tacrolimus–FKBP and Cys A–cyclophilin complexes bind to and inhibit the activity of CaN and suppress the transcription by NFAT (Fruman et al., 1992; Ho et al., 1996). Tacrolimus also suppresses the inflammatory cytokine gene expression dependent on the AP-1 pathway (Okamoto et al., 1994). Tacrolimus and Cys A can suppress human neutrophil chemotaxis of peripheral blood mononuclear cells by inhibiting IL-8, a chemoattractant of neutrophils (Sasakawa et al., 2000).

In contrast, steroids bind to glucocorticoid receptor (GR) in cytoplasm, and the glucocorticoid–GR complex is transported to the nucleus for binding to the glucocorticoid responsive element (GRE). Inhibitor of κ B (I κ B) masks the nuclear translocation signal of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) proteins and keeps them in an inactive state in the cytoplasm (Jacobs and Harrison, 1998). In addition, glucocorticoids prevent c-Jun phosphorylation followed by AP-1 activation by blocking the induction of the Jun amino-terminal kinase (JNK) signaling cascade (Caelles et al., 1997). The fact that tacrolimus and Cys A, but not dexamethasone, suppressed scratching behavior in the present experimental model suggests that the pruritus depends on the CaM–CaN pathway.

Degranulation of mast cells dependent on IgE is one of the most important factors in inducing scratching behavior in a mouse model of atopic dermatitis (Yamashita et al., 2005, 2009). Therefore, we examined whether the effects of tacrolimus and Cys A on scratching behavior and the increase in vascular permeability were caused by passive cutaneous anaphylaxis (PCA). We found that, in mice, oral administration of tacrolimus and Cys A inhibited PCA, scratching behavior, and vascular permeability. Topical application of tacrolimus did not inhibit PCA (data not shown), probably because the skin of the mouse ear lobes was normal when PCA was induced: tacrolimus, with its molecular weight of 800, could not penetrate the skin. In addition, tacrolimus and Cys A inhibited mast cells degranulation in inflammatory skin (Supplementary Table 2). We hypothesize that inhibition of IgE-dependent degranulation of mast cells is one mechanism underlying the tacrolimus- and Cys A-induced suppression of scratching behavior.

Tacrolimus at a concentration of 0.1%, Cys A at a dose of 30 mg/kg and dexamethasone at a concentration of 0.05% were treated for 10 days before the last DNFB challenge (Supplementary Fig. 1). Repeated treatment of tacrolimus and Cys A was suppressed ear swelling and scratching behavior, but dexamethasone failed to suppress the up-regulation of scratching behavior as well as single treatment. These data supported the difference of action mechanism between tacrolimus, Cys A and dexamethasone. Additionally, IL-4, IL-13 and IFN- γ , which are inflammatory cytokines, were suppressed by tacrolimus, Cys A and dexamethasone, however, IL-31 related itch (Sonkoly et al., 2006) was not suppressed by dexamethasone (Supplementary Fig. 2). The cytokine profile corresponded with ear swelling and scratching.

In conclusion, tacrolimus and Cys A similarly suppressed scratching behavior induced by application of a 0.15% DNFB solution. Both drugs also suppressed scratching behavior associated with PCA. These results suggest that the suppression of scratching behavior by tacrolimus and Cys A depends on the CaM–CaN pathway. Studies of the target cells and the exact mechanisms involved are now underway.

Acknowledgements

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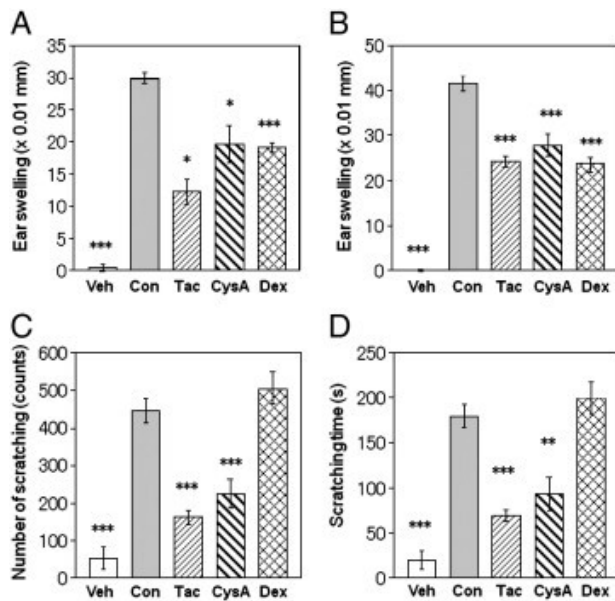
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [10.1016/j.ejphar.2010.07.031](https://doi.org/10.1016/j.ejphar.2010.07.031).

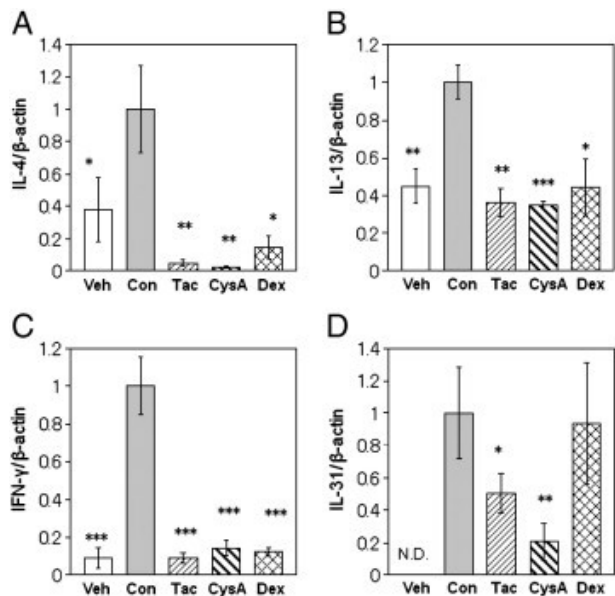
References

- Avramoff, A., Laor, A., Kitzes-Cohen, R., Farin, D., Domb, A.J., 2007. Comparative in vivo bioequivalence and in vitro dissolution of two cyclosporin A soft gelatin capsule formulations. *Int. J. Clin. Pharmacol. Ther.* 45, 126–132.
- Boguniewicz, M., Fiedler, V.C., Raimier, S., Lawrence, I.D., Leung, D.Y., Hanifin, J.M., 1998. A randomized, vehicle-controlled trial of tacrolimus ointment for treatment of atopic dermatitis in children. *Pediatric Tacrolimus Study Group. J. Allergy Clin. Immunol.* 102, 637–644.
- Caelles, C., Gonzalez-Sancho, J.M., Muñoz, A., 1997. Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes Dev.* 11, 3351–3364.
- Eisen, H.N., Carsten, M.E., Belman, S., 1954. Studies of hypersensitivity to low molecular weight substances. III. The 2, 4-dinitrophenyl group as a determinant in the precipitin reaction. *J. Immunol.* 73, 296–308.
- Frei, U., 1999. Overview of the clinical experience with neural in transplantation. *Transplant. Proc.* 31, 1669–1674.
- Fruman, D.A., Klee, C.B., Bierer, B.E., Burakoff, S.J., 1992. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. *Proc. Natl Acad. Sci. USA* 89, 3686–3690.
- Furue, M., Furukawa, F., Hide, M., Takehara, K., 2004. Guidelines for therapy for atopic dermatitis. *Jpn. J. Dermatol.* B114, 135–142.
- Ho, S., Cliststone, N., Timmermann, L., Northrop, J., Graef, I., Fiorentino, D., Nourse, J., Crabtree, G.R., 1996. The mechanism of action of cyclosporin A and FK506. *Clin. Immunol. Immunopathol.* 80, S40–S45.
- Inagaki, N., Goto, S., Nagai, H., Koda, A., 1984. Mouse ear PCA as a model for evaluating antianaphylactic agents. *Int. Arch. Allergy Appl. Immunol.* 74, 91–92.
- Inagaki, N., Igeta, K., Kim, J.F., Nagao, M., Shiraishi, N., Nakamura, N., Nagai, H., 2002. Involvement of unique mechanisms in the induction of scratching behavior in BALB/c mice by compound 48/80. *Eur. J. Pharmacol.* 448, 175–183.
- Inagaki, N., Igeta, K., Shiraishi, N., Kim, J.F., Nagao, M., Nakamura, N., Nagai, H., 2003. Evaluation and characterization of mouse scratching behavior by a new apparatus, MicroAct. *Skin Pharmacol. Appl. Skin Physiol.* 16, 165–175.
- Inagaki, N., Shiraishi, N., Igeta, K., Itoh, T., Chikumoto, T., Nagao, M., Kim, J.F., Nagai, H., 2006. Inhibition of scratching behavior associated with allergic dermatitis in mice by tacrolimus, but not by dexamethasone. *Eur. J. Pharmacol.* 546, 189–196.
- Inagaki, N., Shiraishi, N., Igeta, T., Nagao, M., Kim, J.F., Chikumoto, T., Itoh, T., Tanaka, H., Nagai, H., 2010. Depletion of substance P, a mechanism for inhibition of mouse scratching behavior by tacrolimus. *Eur. J. Pharmacol.* 626 (2–3), 283–289.

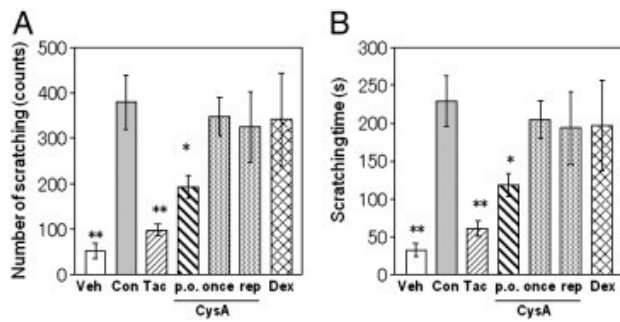
- Jacobs, M.D., Harrison, S.C., 1998. Structure of an IkappaBalpha/NF-kappaB complex. *Cell* 95, 749–758.
- Jain, J., Valge-Archer, V.E., Sinskey, A.J., Rao, A., 1992. The AP-1 site at –150 bp, but not the NF-kappa B site, is likely to represent the major target of protein kinase C in the interleukin 2 promoter. *J. Exp. Med.* 175, 853–862.
- Kawashima, M., Harada, S., Tamaki, K., Nakagawa, H., Shimada, S., Ishibashi, Y., 1997. A safety study of FK506 ointment in patients with atopic dermatitis. *J. Clin. Ther. Med.* 13, 1483–1492.
- Klein, P.A., Clark, R.A., 1999. An evidence-based review of the efficacy of antihistamines in relieving pruritus in atopic dermatitis. *Arch. Dermatol.* 135, 1522–1525.
- Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I., Schreiber, S.L., 1991. Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP–FK506 complexes. *Cell* 66, 807–815.
- Nagai, H., Matsuo, A., Hiyama, H., Inagaki, N., Kawada, K., 1997. Immunoglobulin E production in mice by means of contact sensitization with a simple chemical, hapten. *J. Allergy Clin. Immunol.* 100, S39–S44.
- Okamoto, S., Mukaida, N., Yasumoto, K., Rice, N., Ishikawa, Y., Horiguchi, H., Murakami, S., Matsushima, K., 1994. The interleukin-8 AP-1 and kappa B-like sites are genetic end targets of FK506-sensitive pathway accompanied by calcium mobilization. *J. Biol. Chem.* 269, 8582–8589.
- Piñeyro-López, A., Piñeyro-Garza, E., Torres-Alanís, O., Reyes-Araiza, R., Gómez-Silva, M., Waksman, N., E Salazar-Leal, M., Luján-Rangel, R., 2007. Evaluation of the bioequivalence of single 100-mg doses of two oral formulations of cyclosporin A microemulsion: a randomized, open-label, two-period crossover study in healthy adult male Mexican volunteers. *Clin. Ther.* 29, 2049–2054.
- Pritchard, D.I., 2005. Sourcing a chemical succession for cyclosporin from parasites and human pathogens. *Drug Discov. Today* 10, 688–691.
- Ruzicka, T., Bieber, T., Schöff, E., Rubins, A., Dobozy, A., Bos, J.D., Jablonska, S., Ahmed, I., Thestrup-Pedersen, K., Daniel, F., Finzi, A., Reitamo, S., 1997. A short-term trial of tacrolimus ointment for atopic dermatitis. *European Tacrolimus Multicenter Atopic Dermatitis Study Group. N Engl J. Med.* 337, 816–821.
- Sakurai, T., Inagaki, N., Nagai, H., 1994. The effect of anti-tumor necrosis factor (TNF)-alpha monoclonal antibody on allergic cutaneous late phase reaction in mice. *Life Sci.* 54, PL291–PL295.
- Sasakawa, Y., Sakuma, S., Higashi, Y., Sasakawa, T., Amaya, T., Goto, T., 2000. FK506 suppresses neutrophil chemoattractant production by peripheral blood mononuclear cells. *Eur. J. Pharmacol.* 403, 281–288.
- Sonkoly, E., Muller, A., Lauerma, A.I., Pivarsci, A., Soto, H., Kemeny, L., Alenius, H., Dieu-Nosjean, M.C., Meller, S., Rieker, J., Steinhoff, M., Hoffmann, T.K., Ruzicka, T., Zlotnik, A., Homey, B., 2006. IL-31: a new link between T cells and pruritus in atopic skin inflammation. *J. Allergy Clin. Immunol.* 117 (2), 411–417.
- Wahlgren, C.F., 1999. Itch and atopic dermatitis: an overview. *J. Dermatol.* 26, 770–779.
- Wahlgren, C.F., Scheynius, A., Häermark, O., 1990. Antipruritic effect of oral cyclosporin A in atopic dermatitis. *Acta Derm. Venerol. (Stockh.)* 70, 323–329.
- Yamashita, H., Michibata, Y., Mizukami, H., Ogihara, Y., Morita, A., Nose, M., 2005. Dermal mast cells play a central role in the incidence of scratching behavior in mice induced by multiple application of the hapten, 2,4,6-trinitrochlorobenzene. *Exp. Dermatol.* 14, 438–444.
- Yamashita, H., Tasaki, D., Makino, T., Matsuoka, K., Nose, M., Inagaki, N., Mizukami, H., 2009. The role of IgE and repeated challenge in the induction of persistent increases in scratching behavior in a mouse model of allergic dermatitis. *Eur. J. Pharmacol.* 605, 153–157.



Supplementary Fig. 2. Effects of repeatedly treatment of tacrolimus, Cys A and dexamethasone on the expression of cytokines. mRNA expressions interleukin-4 (IL-4), IL-13 and interferon- γ (IFN- γ) in the ear of BALB/c mice treated with dinitrofluorobenzene were detected by real time RT-PCR. Each value represents the mean $\pm$ S.E.M. of 4–8 mice. Veh, vehicle; Con, control; Tac, tacrolimus; Cys A, cyclosporine A; Dex, dexamethasone. *, **, *** P < 0.05, 0.01, 0.001 vs. Con (Student or Welch t -test).



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Supplementary Fig. 3. Effect of single or repeated treatment topically with Cys A on scratching behavior. Tacrolimus and dexamethasone were treated topically for 10 days (Tac and dex). Cys A was treated orally for 10 days (p.o.), or topically once and serial 10 days (once and rep; rep means repeated treatment). The number of scratch attempts (A) and duration of scratching (B) were measured from 0 to 2 h after the last DNFB challenge. Veh, vehicle; Con, control. *, ** $P < 0.05$, 0.01 vs. Con (Student or Welch t -test).