研究成果の刊行に関する一覧表

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Immunopharmacology and Inflammation

Depletion of substance P, a mechanism for inhibition of mouse scratching behavior by tacrolimus

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ABSTRACT

Itching is the most important problem in atopic dermatitis and tacrolimus has been suggested to attenuate the itching by topical application. However, the anti-itch mechanism of tacrolimus has not been well elucidated. In the present study, an allergic dermatitis accompanied by frequent scratching behaviors was induced by repeated paintings with 2,4-dinitrofluorobenzene (DNFB) acetone solution onto the mouse ear and the effects of tacrolimus and dexamethasone on the dermatitis and associated scratching behavior were comparatively examined. Repeated DNFB paintings caused a typical dermatitis accompanied by elevated serum immunoglobulin E (IgE) and frequent scratching behaviors. Both tacrolimus and dexamethasone given topically for 10 days before the final challenge significantly inhibited the ear swelling and reduced the expression of interferon-γ mRNA. Dexamethasone inhibited the accumulation of eosinophils completely, although tacrolimus did not. Both drugs did not affect the elevation of serum IgE levels. Tacrolimus significantly inhibited the scratching behavior, whereas dexamethasone failed to affect it. Repeated DNFB challenge depleted substance P in the dermis. Treatment with tacrolimus before the final challenge completely inhibited the recovery of substance P content, whereas dexamethasone facilitated the recovery. DNFB-induced ear swelling and scratching behavior were significantly inhibited by FK888, a tachykinin NK_1 receptor antagonist. Therefore, substance P seems to participate in the induction of ear swelling and scratching behavior upon final challenge with DNFB, and depletion of substance P by tacrolimus in the dermis contributes to its inhibition of ear swelling and scratching behavior at least in part.

1. Introduction

Atopic dermatitis is a complex eczematous skin disease accompanied by severe itching and sufficient therapeutic strategies have not been established (Furue et al., 2004; Wahlgren, 1992). The onset of the disease is believed to be dependent on both genetic and environmental factors. The elevated serum immunoglobulin E (IgE) is also a characteristic feature in many patients (Hoffman et al., 1975; Sampson and Albergo, 1984). The severe itching is the most important problem because of its significant impairment of the patient's quality of life. Furthermore, induced scratching worsens the dermatitis resulting in more itching (Wahlgren, 1999). The typical skin lesion in atopic dermatitis is a resultant from repeated scratching. Therefore, regulation of itching and/or scratching is very important and beneficial for the treatment of atopic dermatitis.

Topical glucocorticoids are very important and very effective remedy for the treatment of atopic dermatitis. It is well known, however, that prolonged usages with high doses of glucocorticoids frequently cause a variety of adverse effects (Barnetson and White, 1992; Leung and Barber, 2003). Furthermore, inappropriate usage of topical glucocorticoids has been emphasized as one cause for the recent increase in the number of adult patients with severe symptoms in Japan (Furue et al., 2004). Recently, tacrolimus, a macrolide immunosuppressant, has been introduced to the treatment of atopic dermatitis as an ointment (Nakagawa et al., 1994; Bieber, 1998). Both short- and long-term monotherapy with tacrolimus ointment improves moderate to severe atopic dermatitis in adult and pediatric patients (Cheer and Plosker, 2001). Although the activity is less potent than glucocorticoids, it could be applied safely for lesions in the face and neck and its anti-itch property has been suggested (Ständer and Luger, 2003; Katoh et al., 2004; Ständer et al., 2006).

Previously, we examined the effects of tacrolimus and dexamethasone on the dermatitis and scratching behavior in mice caused by repeated 2,4-dinitrofluorobenzene (DNFB) paintings, and indicated that tacrolimus exhibits potent inhibition for scratching behavior caused by DNFB painting in spite of its relatively weak anti-inflammatory activity (Inagaki et al., 2006). In contrast, although dexamethasone exhibits potent anti-inflammatory activity, it does not affect the scratching behavior. Furthermore, we indicated that tacrolimus inhibits nerve fiber extension into the epidermis and

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suggested that it contributes to the inhibition of mouse scratching behavior by tacrolimus. Although some investigators also reported the inhibitory activity of tacrolimus for mouse scratching behavior (Takano et al., 2004; Maekawa et al., 2006; Tanaka et al., 2007; Nakano et al., 2008), the mechanism of tacrolimus for inhibiting mouse scratching behavior has not been elucidated.

In the present study, therefore, we induced an allergic dermatitis in the mouse ear with some features of atopic dermatitis accompanying frequent scratching behavior, and the inhibitory mechanism of itching by tacrolimus was further investigated. We report here that depletion of substance P by tacrolimus in the dermis contributes to its inhibition of ear swelling and scratching behavior at least in part.

2. Materials and methods

2.1. Animals

Male BALB/c mice, 8–10 weeks of age, were obtained from Japan SLC, Inc. (Hamamatsu, Japan). They were housed in an air-conditioned animal room with a temperature of 22 ± 1 °C and a humidity of 60 ± 5%. Experiments were undertaken following the guideline for the care and use of experimental animals of Gifu Pharmaceutical University with permission by the Animal Experiment Committee of our university.

2.2. Antigens

2,4-Dinitrofluorobenzene (DNFB, Sigma Chemical Co., St. Louis, MO, USA) and dinitrophenylated *Ascaris suum* extract (DNP-Asc) were used as antigens. DNFB was dissolved in acetone at a concentration of 0.15% and applied topically to cause cutaneous reaction. DNP-Asc was mixed with aluminum hydroxide gel and injected intraperitoneally to induce IgE production.

2.3. Drugs

Tacrolimus (FK506, Astellas Pharma Inc., Tokyo, Japan), dexamethasone (Sigma), and FK888 (N^2-[(4*R*)-4-hydroxy-1-(1-methyl-1H-indol-3-yl) carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-3-(2-naphthyl)-L-alaninamide, Astellas) (Fujii et al., 1992; Miyayasu et al., 1993) were used. Tacrolimus and dexamethasone were dissolved in ethanol at concentrations of 0.1 and 0.05%, respectively, and given topically onto the ear of mice. FK888 was dissolved in dimethyl sulfoxide and administered intravenously at doses of 0.1 and 1 mg/kg. The dose of each drug employed was determined according to our preliminary experiments.

2.4. Induction of mouse ear dermatitis

Mice were immunized by injecting 10 μg DNP-Asc and 1 mg aluminum hydroxide gel intraperitoneally to stimulate IgE production. Dermatitis was evoked in the mouse ear by repeated paintings with 25 μl of 0.15% DNFB acetone solution onto each surface of both ears, 100 μl in total. The DNFB challenge was repeated 5 times every other day, from 2 weeks after the immunization. Two weeks after the fifth challenge, mice received a final DNFB challenge similarly. Control mice were treated with vehicle without DNP-Asc immunization.

Ear dermatitis was evaluated by measuring the ear thickness using a dial thickness gauge (R12-1A, Ozaki MFG Co., Ltd., Tokyo, Japan). Blood samples for the measurement of serum IgE, and tissue specimens for the histopathological observation and examination of mRNA expression were obtained from mice occasionally. Mouse scratching behavior was observed after the final DNFB challenge. Tacrolimus and dexamethasone were administered topically for 10 days before the final challenge. FK888 was administered intravenously 2 min before the final challenge. In one experiment, 50 nmol/10 μl of substance P (Sigma) was injected into the ear lobe instead of the final challenge, and the effect of FK888 was observed similarly.

2.5. Histopathological observation

Excised ear specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections with 5 μm thick were prepared and observed after hematoxylin and eosin, toluidine blue or Luna staining. Localization of substance P was observed immunohistochemically by staining with rabbit anti-substance P polyclonal antibodies (AB1566, Nihon Millipore, Tokyo, Japan), biotin-conjugated goat anti-rabbit immunoglobulin polyclonal antibodies (E0432, Dako, Glostrup, Denmark) and peroxidase-conjugated streptavidin (K1016, Dako). Localized substance P visualized by an enzyme reaction in the dermis was quantitatively evaluated by LEICAQwin (Leica Microsystems Heiderberg GmbH, Mannheim, Germany) and indicated as the percentage stained area in the dermis.

2.6. Measurement of serum IgE

Dinitrophenyl (DNP)-specific IgE was measured by means of a captured ELISA (Nagai et al., 1997a; Hirano et al., 1989). In brief, IgE in samples was trapped with anti-mouse IgE antibody (monoclonal, ε-heavy chain-specific, rat IgG1, Serotec Ltd., Oxford, UK) and detected by enzyme reaction after incubating with biotinylated antigen (dinitrophenylated bovine serum albumin) and then with peroxidase-conjugated streptavidin (Dako). After the enzyme reaction, absorbance at 492 nm was measured. Biotinylated antigen was prepared using a biotinylation kit (Pierce Biotechnology Inc., Rockford, IL, USA). Mouse monoclonal IgE against DNP residue (SPE-7, Sigma) was used as a standard.

2.7. Detection of cytokine mRNA expression

Characteristics of the DNFB-induced cytokine mRNA expression were evaluated by means of reverse transcriptase-polymerase chain reaction (RT-PCR) (Nagai et al., 1997a,b). In brief, total RNA was extracted from excised ears and cervical lymph nodes of mice using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan). cDNA was prepared from the RNA by reverse transcription (Superscript II, Gibco BRL, Grand Island, NY, USA) and then amplified by PCR (denaturation: at 94 °C for 1.5 min, annealing: at 62 °C for 1.5 min, extension: at 72 °C for 1.5 min, 35 cycles) using ampliTaq DNA polymerase (Takara Taq, Takara Holdings Inc., Kyoto, Japan) on a thermalcycler (Trio-Thermoblock, Biometra GmbH, Goettingen, Germany). Resultant products were electrophoresed on 2% agarose gel containing ethidium bromide. The bands were recorded by Polaroid camera (Polaroid 665 film, Nippon Polaroid, Tokyo, Japan).

Effects of drugs on the cytokine mRNA expression were evaluated by means of real-time RT-PCR. In brief, total RNA was extracted from excised ears of mice using Isogen. cDNA was prepared from the RNA by reverse transcription (Superscript II, Gibco BRL) and then amplified by PCR (denaturation: at 95 °C for 3 min, annealing and extension: at 95 °C for 15 s and at 63.1 °C for 1 min, 60 cycles) in the presence of syber green (Takara) on iCycler (Bio-Rad, Hercules, CA, USA). PCR products were analyzed by iCycler iQ Real-Time Detection System (Bio-Rad).

Primers employed are listed in Table 1.

2.8. Evaluation of scratching behavior

Mouse scratching behavior was automatically detected and objectively evaluated with an apparatus, MicroAct (Neuroscience, Inc., Tokyo, Japan) (Inagaki et al., 2002, 2003). A small magnet (1 mm in diameter, 3 mm long, coated with teflon) was inserted subcutaneously into both hind paws under ether anesthesia before the start of

Table 1
Primers employed for detecting mRNA.

RT-PCR		
β-actin	Sense	5′ GTG GGC CGC TAG GCA CCA 3′
	Anti-sense	5′ CGG TTG GCC TTA GGG TTC AGG GGG G 3′
IFN-γ	Sense	5′ TAC TGC CAC GGC ACA GTC ATT GAA 3′
	Anti-sense	5′ GCA GCG ACT CCT TTT CCG CTT CCT 3′
IL-4	Sense	5′ ACG GAG ATG GAT GTG CCA AAC GTC 3′
	Anti-sense	5′ CGA GTA ATC CAT TTG CAT GAT GC 3′
Real-time RT-PCR		
β-actin	Sense	5′ GAT CTG GCA CCA CAC CTT CT 3′
	Anti-sense	5′ GGG GTG TTG AAG GTC TCA AA 3′
IFN-γ	Sense	5′ GAG GAA CTG GCA AAA GGA TG 3′
	Anti-sense	5′ GCT GAT GGC CTG ATT GTC TT 3′
IL-4	Sense	5′ CAG CAA CGA AGA ACA CCA CAG 3′
	Anti-sense	5′ ATC GAA AAG CCC GAA AGA GTC 3′
IL-13	Sense	5′ CAC GGC CCC TTC TAA TGA GG 3′
	Anti-sense	5′ CTG CCT CTC CCC AGC AAA GT 3′

experiment. It was confirmed that the operation and magnet did not affect the mouse behavior. The mouse with magnets was placed in an observation chamber (11 cm in diameter, 18 cm high), which 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 a computer. Under our experimental conditions, the apparatus detected consecutive scratching behavior consisting of 3 or more beats. Results of scratching behavior are given as both incidence of consecutive scratching behavior and total scratching time in an indicated period after DNFB painting.

2.9. Statistics

The data are expressed as the mean values with standard error. Statistical significance of the difference between two experimental groups was evaluated by Student's or Welch's *t*-test, and among three experimental groups by Dunnett's multiple comparison test. When the *P* value was smaller than 0.05, the difference was considered to be significant.

3. Results

3.1. Characterization of the mouse ear dermatitis

Immunized mice were treated with DNFB 5 times repeatedly, and finally challenged 2 weeks later. Changes in histopathological features and ear swelling are shown in Fig. 1. After the first DNFB challenge, apparent ear swelling was not induced and only a small number of inflammatory cells were detected in the dermis at 6 h after the challenge. Repeated challenge given every other day caused a potent inflammation with a significant scab formation. Thickening of epidermis and dermis, and potent accumulation of inflammatory cells were observed. Although ear thickness increased significantly, the increase in thickness 6 or 24 h after the fifth challenge was relatively small. During a period of 2 weeks between the fifth and final challenge, the scab was dropped off and the inflammation waned. Upon final challenge, however, potent inflammation recurred. The ear swelling was very potent and an apparent swelling was detected from 1 h after the challenge.

Serum samples were collected from mice 1 day before the first challenge and 6 days after the fifth challenge and specific IgE was measured. As shown in Fig. 2A, DNP-specific IgE was induced by the

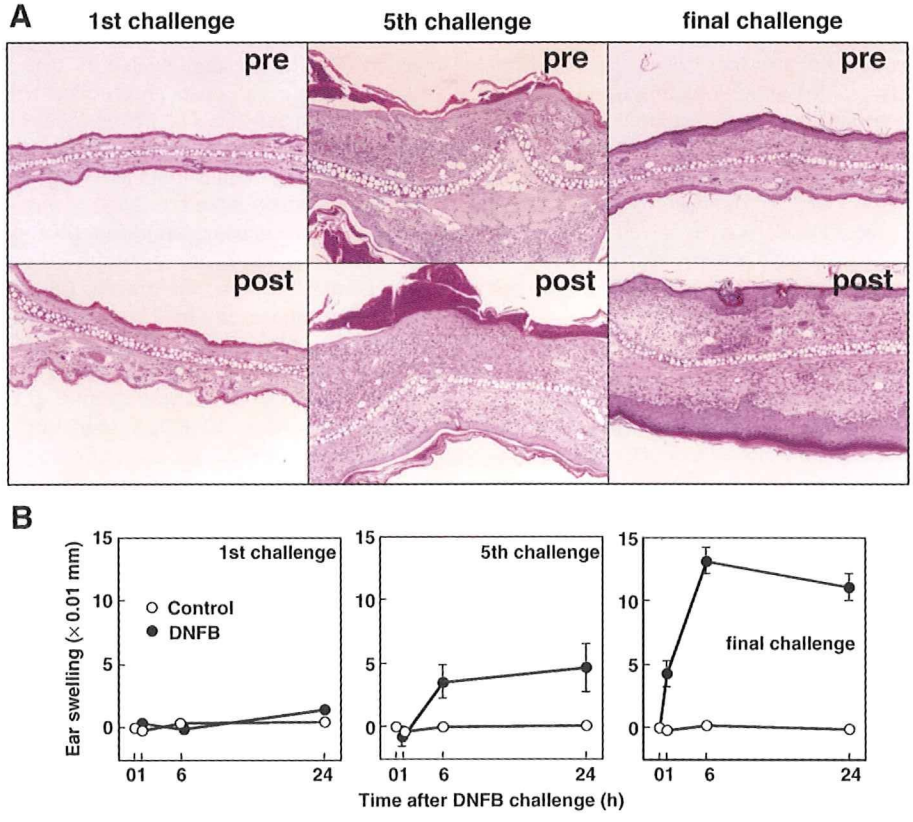

Fig. 1. Changes in histopathological features and ear swelling of the mouse ear treated with DNFB repeatedly. A: Histopathological images of skin lesions. Hematoxylin–eosin staining, original magnification: ×90, pre: before challenge, post: 6 h after challenge. B: Changes in ear swelling after the first, fifth and final challenges. Mean ± S.E.M. for 6 mice.

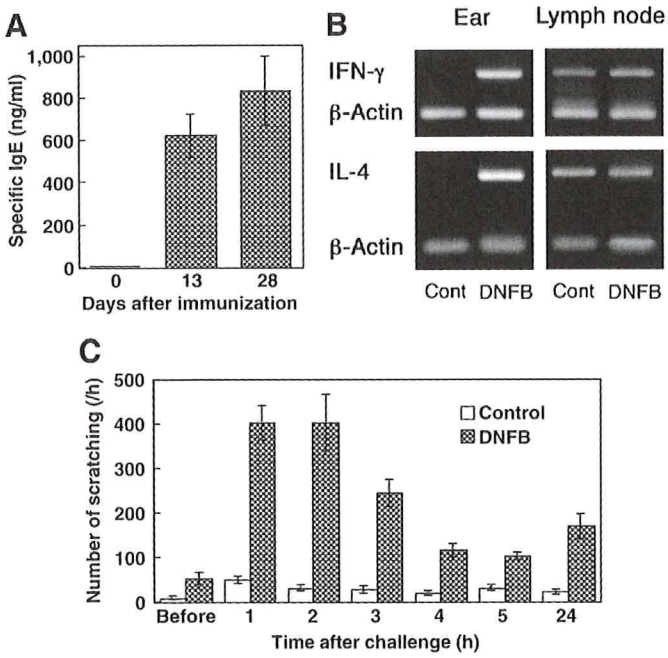

Fig. 2. Serum IgE levels, cytokine mRNA expression and scratching behavior in mice treated with DNFB repeatedly. A: DNP-specific IgE levels in the sera. Serum samples were obtained before immunization, 1 day before the first challenge and 6 days after the fifth challenge. Mean ± S.E.M. for 5 or 6 mice. B: Expression of IFN-γ and IL-4 mRNA in the ear and cervical lymph node. Ears and lymph nodes were excised 4 h after the final challenge. C: Changes of incidence of scratching behavior before and after the final challenge. Values indicate the incidence in each hour. Mean ± S.E.M. for 6 mice.

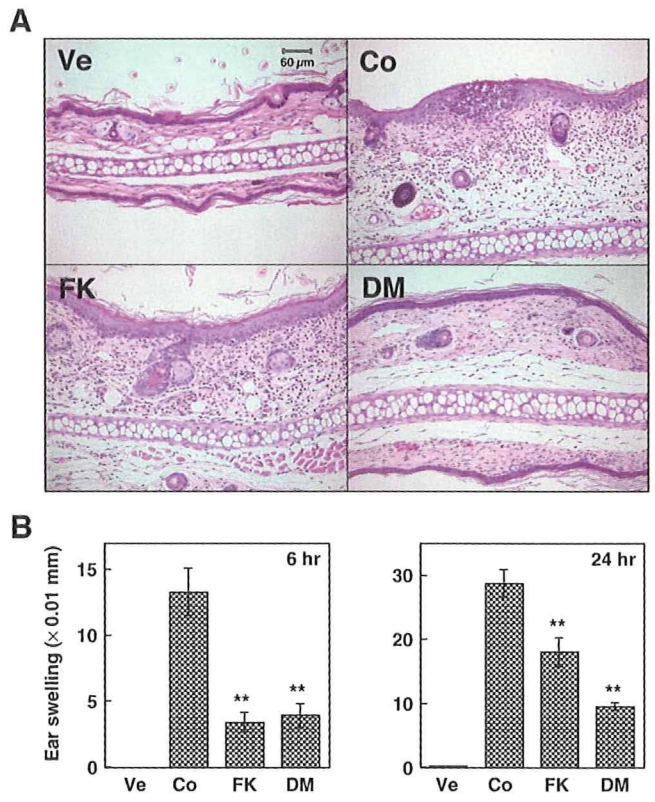

Fig. 3. Effects of tacrolimus and dexamethasone on the histopathological features and ear swelling caused by the final challenge with DNFB. A: Histopathological images of skin lesions stained by hematoxylin and eosin. Skin lesions were excised 24 h after the final challenge. B: Ear swelling. Ear thickness was measured 6 and 24 h after the final challenge. Mean ± S.E.M. for 6 or 8 mice. $^{**}P<0.01$, Ve: vehicle, Co: control, FK: tacrolimus, DM: dexamethasone.

immunization and repeated DNFB challenge potentiated the production. Ear lobes and cervical lymph nodes were excised 4 h after the final challenge, and cytokine mRNA expression was evaluated. As shown in Fig. 2B, although apparent alteration of IFN-γ and IL-4 mRNA expression was not observed in the lymph node, both cytokine mRNA expression was potently induced in the ear lesion. The change in incidence of scratching behavior after the final challenge is indicated in Fig. 2C. Upon the final challenge, frequent scratching behaviors were induced. The incidence was very high in the first 2 h and then declined gradually.

3.2. Effects of tacrolimus and dexamethasone on the dermatitis

Effects of tacrolimus and dexamethasone were examined by topical application. The drug application was performed daily for 10 days from the fifth day after the fifth DNFB challenge through the day of the final challenge.

Histpathological images at 24 h after the final challenge are shown in Fig. 3A. In control mice, significant thickening of epidermis and dermis, and potent accumulation of inflammatory cells including mast cells (confirmed by toluidine blue staining, Supplemental Fig. 1) and eosinophils (confirmed by Luna staining, Supplemental Fig. 2) were observed. Dexamethasone potently inhibited the inflammation histopathologically when comparing to tacrolimus, and completely prevented the eosinophil accumulation. However, the drugs did not inhibit mast cell accumulation apparently. In contrast, as shown in Fig. 3B, both drugs significantly depressed the ear swelling at 6 and 24 h after the final challenge. The inhibition by tacrolimus at 6 h was comparable to that of dexamethasone, but that at 24 h was reduced.

Results of scratching behavior observed for 2 h after the final challenge are shown in Fig. 4A. Upon challenge with DNFB, frequent scratching behavior was induced. Treatment with tacrolimus significantly inhibited the scratching behavior both in incidence and time, although dexamethasone did not affect the scratching behavior. Results of specific IgE levels at 24 h after the final challenge are indicated in Fig. 4B. Immunization and repeated DNFB challenge elevated the serum IgE levels, and treatment with the drugs potentiated the elevation slightly. IFN-γ, IL-4 and IL-13 mRNA expressions in the ear lesion at 4 h after the final challenge were examined. As shown in Fig. 4C, IFN-γ mRNA expression was apparently potentiated by the DNFB challenge. IL-4 and IL-13 mRNA expressions were slightly potentiated, although the level was very low. The increased expression of the cytokine mRNA was apparently inhibited by the drugs.

Ear lesions were excised 24 h after the final challenge, and localization of substance P was observed immunohistochemically. As shown in Fig. 5A, although immunoreactive substance P was localized in the dermis of vehicle-treated mice, in DNFB challenged control mice, substance P disappeared completely. In tacrolimus-treated mice, substance P could not be detected, whereas substance P was observed in dexamethasone-treated mice. Results of changes in substance P content are indicated in Fig. 5B. At 5 days after the repeated DNFB challenge, substance P could not be detected. During the following 10 days, substance P content gradually increased to the normal level in control mice. However, substance P disappeared completely 24 h after the final challenge. Tacrolimus treatment for 10 days before the final challenge completely inhibited the recovery of substance P content. In contrast, the recovery of substance P content was facilitated by dexamethasone, and the release of recovered substance P was inhibited only partially.

3.3. Role of substance P in the ear dermatitis

The role of substance P in the ear swelling and scratching behavior was examined using tachykinin NK_1 receptor antagonist, FK888. The

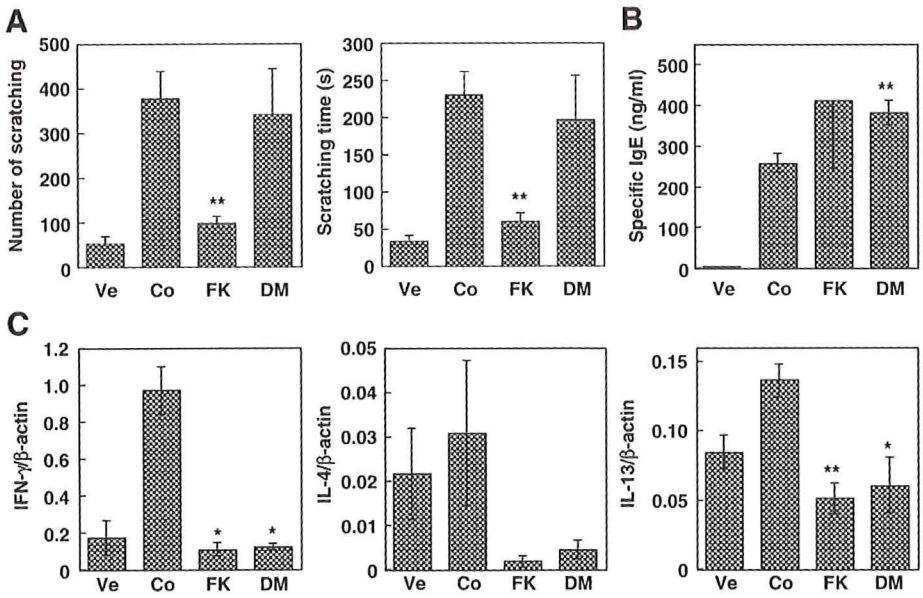

Fig. 4. Effects of tacrolimus and dexamethasone on the scratching behavior, serum IgE level and cytokine mRNA expression. A: Scratching behavior induced after the final challenge was evaluated for 2 h and indicated as incidence and total scratching time in 2 h. Mean ± S.E.M. for 6 or 7 mice. B: DNP-specific IgE levels in the sera. Serum samples were obtained 24 h after the final challenge. Mean ± S.E.M. for 7 or 8 mice. C: Expression of IFN-γ, IL-4 and IL-13 mRNA in the ear. Ears were excised 4 h after the final challenge. Mean ± S.E.M. for 4 mice. $^{*}P<0.05$, $^{**}P<0.01$, Ve: vehicle, Co: control, FK: tacrolimus, DM: dexamethasone.

drug was given to mice intravenously 2 min before the final challenge. As shown in Fig. 6A and B, FK888 at a dose of 1 mg/kg significantly inhibited the ear swelling at 6 and 24 h and the scratching behavior.

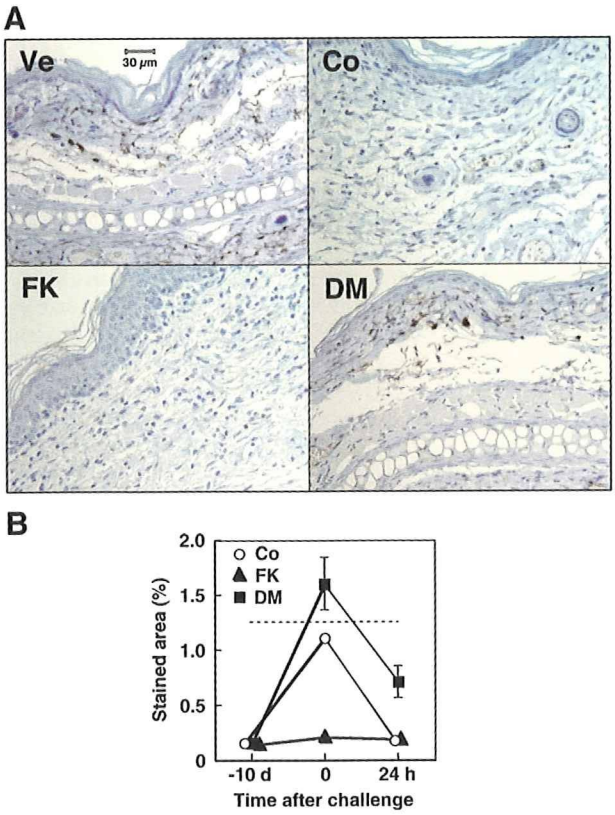

Fig. 5. Effects of tacrolimus and dexamethasone on the localization of substance P. A: Immunohistochemical images of the skin lesion. Skin specimens were excised 24 h after the final challenge. B: Changes of substance P content in the dermis. Skin samples were excised 10 days and just before, and 24 h after the final challenge, and substance P was detected with anti-substance P antibodies. Mean ± S.E.M. for 3 mice. Dotted line: normal level, Ve: vehicle, Co: control, FK: tacrolimus, DM: dexamethasone.

Furthermore, whether substance P induces scratching behavior in DNFB-treated mice was examined. Substance P was injected into the ear lobe instead of the final DNFB challenge. As shown in Fig. 6C, substance P induced scratching behavior although the incidence was small, and FK888 inhibited the induced scratching behavior completely.

4. Discussion

In the present study, we induced an allergic dermatitis in the mouse ear by repeated paintings with DNFB and examined the anti-scratch activity and mechanism by tacrolimus. As a result, we demonstrated that tacrolimus depletes substance P content in the dermis and that the depletion of substance P contributes to the inhibition of scratching behavior by tacrolimus.

Previously, we employed an allergic dermatitis to observe the effects of tacrolimus and dexamethasone on the dermatitis and associated scratching behavior (Inagaki et al., 2006). The dermatitis was induced in the clipped mouse back by repeated paintings with a hapten, DNFB. Repeated hapten application to the mouse skin induces a dermatitis bearing some features of atopic dermatitis (Nagai et al., 1997b; Kitagaki et al., 1997). The dermatitis caused by repeated DNFB paintings on the mouse back exhibits typical skin lesions accompanied by elevated serum IgE, Th2 cytokine expression, inflammatory cell accumulation and frequent scratching behavior (Inagaki et al., 2006). Tacrolimus applied topically exhibits potent inhibition for scratching behavior caused by DNFB painting in spite of its relatively weak anti-inflammatory activity, although dexamethasone exhibits potent anti-inflammatory activity without affecting the scratching behavior. Furthermore, we indicated that tacrolimus inhibits nerve fiber extension into the epidermis and suggested that the inhibition contributes to the reduced expression of scratching behavior by tacrolimus.

In the present study, we induced a dermatitis in the mouse ear by repeated paintings with DNFB under a different experimental protocol. DNFB acetone solution was epicutaneously challenged 5 times every other day in the ear of mice immunized with DNP-Asc 2 weeks before. The immunization was aimed to potentiate the IgE production. Two weeks after the repeated challenge, mice were challenged again and the dermatitis and associated scratching behavior were observed. Repeated challenge and final challenge

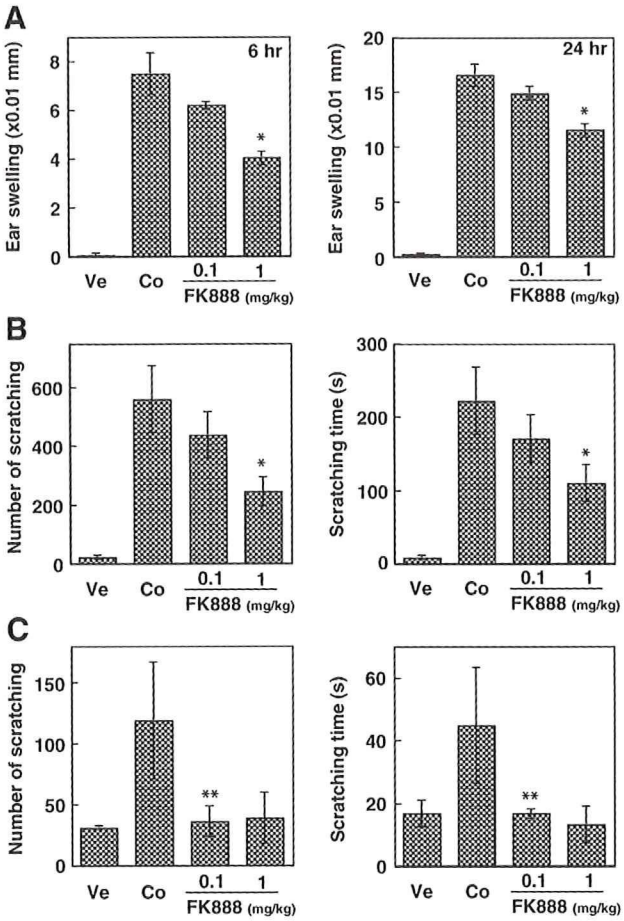

Fig. 6. Effects of FK888 on the ear swelling and scratching behavior. A: Ear swelling was evaluated 6 and 24 h after the final challenge. Mean ± S.E.M. for 3–6 mice. B: Scratching behavior was observed for 2 h after the final challenge and indicated as incidence and total scratching time in 2 h. Mean ± S.E.M. for 3–6 mice. C: Substance P was injected into the ear instead of the final challenge and scratching behavior was observed for 1 h. Mean ± S.E.M. for 3 mice. $^{*}P<0.05$, $^{**}P<0.01$, Ve: vehicle, Co: control.

were separated by 2 weeks to observe the secondary response and tacrolimus and dexamethasone were administered topically for 10 days before the final challenge. In the ear dermatitis model, repeated DNFB challenge caused a potent skin lesion accompanied by elevated serum IgE, Th2 cytokine expression, inflammatory cell accumulation and frequent scratching behavior similar to our previous back skin dermatitis model (Inagaki et al., 2006). Similar to the case in the back skin dermatitis, topical application of tacrolimus for 10 days before the final challenge significantly inhibited the scratching behavior after the final challenge. Furthermore, in contrast to the back skin dermatitis, we indicated that tacrolimus also significantly inhibited the ear swelling at 6 and 24 h after the final challenge. Inhibitory activity for ear swelling by tacrolimus seems to be more potent in 6 h than in 24 h after the challenge suggesting that tacrolimus may inhibit an allergic response that appeared immediately more effectively than that in later phase (de Paulis et al., 1991; Sperr et al., 1996). In contrast, although dexamethasone exhibited a potent inhibition of ear swelling and eosinophil accumulation, it did not affect the scratching behavior. These results confirmed our previous conclusions that tacrolimus inhibits scratching behavior caused by DNFB through mechanisms that could not be inhibited by dexamethasone (Inagaki et al., 2006).

Substance P is a neuropeptide that is released from sensory nerve fibers to cause or expand inflammation (Foreman, 1988; Scholzen et al., 1998). Released substance P stimulates mast cells to cause mediator release. The mast cell derived vasoactive mediator, histamine, potentiates inflammation and induces itching. Thus, substance P potentiates inflammation and itching through mast cell activation. Furthermore, substance P is reported to be a putative mediator for itching and induces scratching behavior in mice directly as well as indirectly through mast cell activation (Andoh et al., 1998; Thomsen et al., 2002). On the other hand, it is well known that topical tacrolimus exhibits burning or pruritus as a frequent adverse effect in the application area, which is resolved within the first week of treatment (Ruzicka et al., 1997; Singalavanija et al., 2006). In 2007, Ständer et al. demonstrated that topical tacrolimus stimulates the release of substance P and calcitonin gene-related peptide from primary afferent nerve fibers in murine skin and that the released neuropeptides stimulate skin mast cells to release inflammatory mediators. These reports strongly suggest the importance of substance P in itching.

In the present study, we observed the localization of substance P in the skin immunohistochemically and indicated that immunoreactive substance P in the dermis completely disappeared after 5 times repeated challenge with DNFB. Therefore, DNFB stimulation seems to stimulate substance P release from storage sites. During 10 days to the final challenge, substance P content recovered to near normal levels. Upon final challenge with DNFB, however, restored substance P in the dermis again disappeared in 24 h. Topical treatment with tacrolimus for the 10-day period before the final challenge completely prevented the recovery of substance P content in the dermis, and substance P could not be detected at the final challenge. Therefore, stimulation with DNFB could not induce substance P release in tacrolimus-treated mice. In contrast, dexamethasone facilitated the restoration of substance P during the treatment period for 10 days. However, dexamethasone failed to inhibit substance P release completely, and substantial amount of substance P released from the storage sites. These results and Ständer's report (Ständer et al., 2007) suggest that substance P to be restored in the dermis is continuously released by repeated topical application with tacrolimus, and that depletion of substance P seems to be maintained. The lack of substance P release seems to be a mechanism for the inhibition of ear swelling and scratching behavior caused by DNFB by tacrolimus. Actually, we demonstrated that substance P plays a role in inducing ear swelling and scratching behavior in this dermatitis model using a tachykinin NK_1 receptor antagonist. We further confirmed that substance P induces small numbers of scratching behavior in DNFB-treated mice, although intact mice do not exhibit scratching behavior (data not shown). Repeated inflammatory stimulation may increase the skin reactivity against substance P, although the underlying mechanism is not unclear at present.

In conclusion, we indicated that tacrolimus treatment depletes substance P content in the dermis and that the depletion of substance P contributes to the inhibition of scratching behavior by tacrolimus at least in part. Although stimulation of substance P release by tacrolimus is suggested to be involved in the depletion, effect of tacrolimus on the substance P production and degradation should also be examined. Furthermore, substance P content and its localization should also be determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2009.09.043.

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REVIEW

Topical tacrolimus as treatment of atopic dermatitis

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Abstract: Atopic dermatitis (AD) is a common, chronic, relapsing, severely pruritic, eczematous skin disease. The mainstays of treatment for AD are topical tacrolimus and topical steroids. Tacrolimus, a calcineurin inhibitor, not only complements existing treatment options but also overcomes some of the drawbacks of topical steroid therapy when given topically and thus meets the long-term needs of patients in preventing disease progression. Topical tacrolimus has been widely recognized in terms of its short- and long-term efficacies and safety, and it is also accepted as a first-line treatment for inflammation in AD. The recent proactive use of topical tacrolimus may emphasize a long-term benefit of this calcineurin inhibitor for AD treatment. To reduce possible long-term adverse effects, it is important to monitor its topical doses in daily clinics.

Keywords: atopic dermatitis, topical tacrolimus, topical steroids, dose, proactive use, adverse effects

Introduction

Atopic dermatitis (AD) is a common, chronic or chronically relapsing, severely pruritic, eczematous skin disease. In our previous Kyushu University Ishigaki Atopic Dermatitis Study cohort, 71.6% of AD patients regressed spontaneously, whereas 5.5% of non-AD individuals developed AD among nursery school children during the 3-year follow-up.[1] The incidence rate of AD in Japanese elementary school students was approximately 3% from 1981 to 1983, but increased to about 6% to 7% in the 1990s.[2] In recent years, the percentage of adolescent- and adult-type AD has also been increasing.[3] As the first-line therapy for AD in Japan, topical steroids, topical tacrolimus and emollients are the mainstays in addition to oral antihistamines, but not topical pimecrolimus which is unavailable in Japan. The purpose of this article is to review recent research on topical tacrolimus as a treatment for AD.

Impact of topical tacrolimus on topical steroid therapy

The clinical usefulness of topically active corticosteroids must be judged in the light of their anti-inflammatory activity and propensity to cause undesirable local side effects, as well as their systemic side effects from absorption through the skin. Topical corticosteroids are generally used for patients with AD of variable clinical severity. When the disease is severely exacerbated, it is likely that patients use more creams and ointments than when it is under reasonable control. Although their frequency is low, topical corticosteroids induce various cutaneous adverse effects in locally applied

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sites, such as telangiectasia, skin atrophy, hypertrichosis, perioral dermatitis, fungal/bacterial/viral infection and atrophic cutaneous striae;[4,5] these side effects have long been known. On the other hand, the efficacy and safety of topical corticosteroids when used appropriately are also well-recognized.[6,7] However, it is not uncommon for patients to express irrational fear and anxiety regarding the use of topical corticosteroids.[7,8] This 'steroid phobia' may be accentuated by media publicity as well as by the common misconception that topical corticosteroids are analogous to anabolic steroids or oral steroids. Moreover, physician's and/or pharmacist's advice to apply topical corticosteroids 'sparingly' or 'thinly' with accompanying messages to exercise caution against steroid adverse effects usually influences patients to interpret such warnings negatively, giving rise to steroid phobia which causes poor adherence to treatment.[9] Experience in dermatology outpatient clinics has suggested that many patients are reluctant to use even mild topical corticosteroids, which may have important implications in terms of patient compliance and subsequent response to treatment.[7]

Before topical tacrolimus was commercially available in Japan, we collected clinical data from 1271 AD patients (210 infantile, 546 childhood, and 515 adolescent and adult AD) who had been followed for at least 6 months in outpatient clinics.[5] All of the patients were treated with topical steroids and moisturizing emollients. The clinical severity of AD in the majority of the patients improved or remained unchanged after 6 months of conventional therapy. However, 19% of the adolescent and adult AD patients remained in a very severe or severe state or experienced exacerbation ('uncontrolled' group).[5] After topical tacrolimus became commercially available in Japan in 1999, we collected clinical data from 215 patients with adolescent- and adult-type AD who had been followed by topical tacrolimus and steroid combination for at least 6 months in outpatient clinics.[10] Very interestingly, the clinical severity of AD improved remarkably in the majority of the patients after 6 months of combination topical therapy. Only 6% of the patients with adolescent- and adult-type AD showed uncontrolled disease.[10] In addition, a reduction in the dose of the topical steroid by add-on tacrolimus application clearly attenuated the incidence and intensity of steroid-induced side effects.[10,11]

Clinical effects of topical tacrolimus on AD

Recent meta-analysis studies have shown the clinical efficacy of topical tacrolimus against AD.[12,13] El-Batawy et al reported the results of meta-analysis from 10 papers on randomized control trials involving 2771 topical tacrolimus-treated patients and 2824 controls.[13] It was demonstrated that topical tacrolimus was significantly more effective than the vehicle. Compared with topical steroids, both 0.1% and 0.03% tacrolimus ointments were as effective as moderate potency steroids, and more effective than a combined steroid regimen. Also, tacrolimus was more effective than mild steroids. Chronic AD lesions of the face and flexures are the most justified indications for topical tacrolimus.[13]

In 2 randomized, double-blind studies of 632 adults with AD, Hanifin et al reported that patients treated with 0.1% and 0.03% tacrolimus ointment showed significantly greater improvement than those treated with the vehicle for all efficacy parameters evaluated, including the percentage body surface area affected, the total score and individual scores for signs of AD, the patient's assessment of pruritus, and Eczema Area and Severity Index (EASI) score.[14] Specifically, the 0.1% concentration was more effective than the 0.03% concentration.[14] Although tacrolimus is a very potent immunosuppressant, its clinical potency remains limited because of its low transepidermal absorption. When applied for 1 week, 0.1% tacrolimus ointment was shown to be more potent than 0.1% alclomethasone dipropionate ointment.[15] Both 0.03% and 0.1% tacrolimus ointments were significantly more effective than 1% hydrocortisone acetate when applied for 3 weeks in children.[16] The efficacy of 0.1% tacrolimus ointment was similar to that of 0.1% hydrocortisone butyrate ointment and 0.12% betamethasone valerate ointment when applied for 3 weeks in adults.[17,18] The continuous efficacy of topical tacrolimus has been confirmed in a previous long-term study, in which marked or excellent improvement or clearance of disease was reported in 54%, 81%, and 86% of patients at week 1, month 6, and month 12, respectively.[19] Adverse events such as burning sensation (47% of patients) were common but tended to occur only when initiating treatment.[19] In a 2-year long-term application trial, marked or excellent improvement or clearance of disease was reported in 90% and 93.1% of patients at weeks 10 and 104, respectively.[20] Adverse events such as burning sensation occurred in 79.2% (450/568) of patients within 1 year; however, the incidence of irritation side effects decreased to only 5.5% (23/418) of patients who applied the ointment for more than 1 year.[20] Similar short-term and long-term efficacies of topical tacrolimus were previously reported in children with AD.[21–25] Taken together, the important finding is that the efficacy of topical tacrolimus is not attenuated under long-term continuous application for at least 2 years. The blood concentration of absorbed tacrolimus is very low

and usually does not exceed 3 ng/mL (most patients are below the detection limit of 0.5 ng/mL).

Adverse effects of topical tacrolimus

The most common adverse events induced by topical tacrolimus are sensation of skin burning or irritation, especially among patients with severe or extensive disease. However, these events are usually brief and resolve during the first few days of treatment.[26] Unlike topical corticosteroids, topical tacrolimus does not cause skin atrophy.[27]

Bacterial and viral infections are commonly associated with AD.[28,29] As tacrolimus is an immunosuppressive drug, topical tacrolimus therapy may potentially increase the risk of cutaneous infections. However, Fleischer et al reported that treatment of AD with tacrolimus ointment was not associated with an increase in cutaneous infections.[30] A systematic review of 1554 AD patients treated with tacrolimus ointment in 5 clinical trials revealed that the 12-week adjusted incidence rates of all cutaneous infections in patients treated with the vehicle, 0.03% tacrolimus ointment, and 0.1% tacrolimus ointment were 18.0%, 24.8%, and 17.7% for adult patients, and 20.9%, 19.6%, and 23.6% for pediatric patients, respectively.[30] The incidence of any individual cutaneous infection in the tacrolimus group was not significantly higher than that in the vehicle group, with the exception of folliculitis in adults.[30] Several other reports also supported these findings.[5,10,31,32] One of the major concerns in topical tacrolimus therapy in AD patients is the possible increase in herpes infection and subsequent Kaposi's varicelliform eruption; however, recent long-term studies have clearly demonstrated that topical tacrolimus does not increase herpes infection.[31,32]

In March 2005, the Food and Drug Administration (FDA) issued a public health advisory informing healthcare professionals and patients about the potential risk of developing cancer from the use of topical calcineurin inhibitors (http://www.fda.gov/bbs/topics/ANSWERS/2005/ANS01343.html). This concern is based on information from animal studies, case reports in a small number of patients, and knowledge of how drugs in this class work. Specifically, in an issued statement, the FDA concluded that it may take 10 years or longer in human studies to determine if the use of topical calcineurin inhibitors is linked to cancer development. Very recently, Arellano et al have investigated the association between topical immunosuppressant therapy and development of lymphoma in 293,253 patients and found no increased risk of lymphoma in those treated with topical calcineurin inhibitors.[33] Moreover, Margolis et al found no association between exposure to topical calcineurin inhibitors and an increased risk of non-melanoma skin cancers in 5000 adults with dermatitis.[34]

Irrational fear of drugs always hampers patients' adherence and compliance to therapy. McNeill and Koo stated that animal and human studies suggest that topical tacrolimus is a safe alternative to topical steroids, with only transient burning sensation as the major known adverse effect compared with those of topical steroids, including long-term side effects.[35] Therefore, currently available data do not suggest that 'unknown risks' of topical tacrolimus need be any more concerning than the known side-effects of the topical steroids.[35]

Recent topics on topical tacrolimus

Fleischer et al compared the efficacy and safety of 0.1% tacrolimus ointment and 1% pimecrolimus cream in adult patients with moderate to very severe AD. In their study, 281 patients (141 treated with tacrolimus and 140 treated with pimecrolimus) were randomized to a multicenter, investigator-blinded, 6-week study. Tacrolimus-treated patients had significantly greater improvements in EASI score than pimecrolimus-treated patients (mean % reduction from baseline to study end: 57% vs 39%, respectively; $P = 0.0002$). Treatment success rate was also significantly higher among the tacrolimus-treated patients than among the pimecrolimus-treated patients (40% vs 22% at study end; $P = 0.001$), as well as improvement in the percentage of total body surface area affected (a reduction of 49% vs 34% at study end; $P = 0.01$).[36]

With regard to itch sensation, Hon et al evaluated the clinical efficacy of topical tacrolimus for itch reduction in children with AD. Seven children (3 and 4 girls) with AD were treated with topical tacrolimus for a consecutive 2-week period after a 1-week run-in. The clinical severity of AD was assessed using the SCORing Atopic Dermatitis scale. Sleep disturbance, as reported by the patients, and nocturnal scratching documented by a wrist movement monitor (DigiTrac), were evaluated at baseline and throughout treatment. Itch and sleep disturbance scores were reduced. Moreover, scratching activity, as documented by the DigiTrac movement recorder, was reduced from 115.0 g/min (64.8–215.5) to 71.5 g/min (51.0–118.0) ($P = 0.028$) after 2 weeks of treatment, showing that tacrolimus is effective in relieving itch in children with AD.[37] In another study, the efficacy and safety of tacrolimus ointment for pediatric patients with AD was evaluated by meta-analysis.[38] In this previous study, topical tacrolimus

induced remission of pediatric AD, and the effects of 0.03% and 0.1% tacrolimus ointment were better than those of 1% hydrocortisone acetate and 1% pimecrolimus. However, no significant difference was found between the 0.03% tacrolimus group and the 0.1% tacrolimus group.

The long-term safety of topical tacrolimus was previously assessed by Remitz et al[39] using an open-label study, with the incidence of adverse events being the primary end-point. In the study, 466 children with AD aged 2 to 15 years applied 0.03% or 0.1% tacrolimus ointment twice daily for up to 29.5 months. The most common application site events were skin burning and pruritus, but their prevalence decreased over time. There was no increase in viral infections or other adverse events over time, and laboratory profiles were consistent with those reported in atopic populations. Substantial improvement in all efficacy end-points was observed by week 2 and was maintained throughout the study.[39] Reitamo et al conducted a 4-year follow-up study of AD therapy with 0.1% tacrolimus ointment where both children and adults applied the ointment continuously or intermittently twice daily during episodes of active disease plus an additional week after remission over a follow-up period of up to 4 years.[40] The intent-to-treat population comprised 782 patients (median age, 22 years; range 2–72) who remained in the study for up to 4 years. Approximately half of the patients discontinued the study prematurely; the median follow-up period was 1422 days. The median tacrolimus ointment use during the first week was 31.2 g, and ointment use decreased during the first year and then remained stable for the remainder period of the study. The median cumulative tacrolimus use was 271.5 g at month 6, 462.5 g at month 12, 739.9 g at month 24, 1029.3 g at month 36 and 1320.8 g at month 48. Altogether 51.8% of the patients discontinued prematurely and the main reasons were withdrawal of consent (13.3%), loss to follow-up (11.3%) and lack of efficacy (9.4%). Adverse events led to discontinuation in 3.7% of the patients. The most frequent application site events were skin burning and pruritus most commonly occurring in adult patients during the initial treatment period; prevalence decreased after the first week and remained at a low level throughout the study. In general, calculated daily hazard rates showed no indication of increased risk of adverse events with prolonged treatment. The total affected body surface area decreased substantially upon treatment onset, and treatment efficacy was maintained until the end of the study with smaller but continuous improvements throughout the follow-up period. Overall, 75% of the patients and 76% of the investigators rated their satisfaction with the treatment as excellent, very good or good at the end of the study or at the time of premature discontinuation. The safety profile of the intermittent or continuous long-term application of 0.1% tacrolimus ointment for up to 4 years was consistent with that established from shorter studies and implied no cause for concern. In addition, 0.1% tacrolimus ointment demonstrated sustained efficacy as reflected by the expression of high satisfaction with the treatment by both patients and investigators.[40] In another study, the long-term safety of tacrolimus was evaluated by Krueger et al who reviewed its pharmacokinetics after topical application in adult and pediatric patients with moderate to severe AD from all clinical trials in which tacrolimus blood levels were obtained.[41] In their study, 0.03% or 0.1% tacrolimus ointment was applied twice daily. During the 12 clinical efficacy trials of tacrolimus ointment, single blood samples were obtained at various times relative to tacrolimus ointment application. In the pharmacokinetic studies, the concentration of 89% to 95% of tacrolimus whole blood samples was <1 ng/mL; the mean maximum concentrations ranged from 0.2 to 1.6 ng/mL and the mean area under the blood concentration-time curves (0–12 hours) ranged from 1.4 to 13.1 ng $\times$ h/mL. Likewise, in the clinical efficacy trials, the concentration of the majority (85%–99%) of tacrolimus samples was <1 ng/mL. Furthermore, Krueger et al showed that tacrolimus ointment is associated with minimal systemic absorption and no evidence of systemic accumulation in patients with moderate to severe AD.[41]

One of the recent topics on topical tacrolimus is that proactive intermittent low-dose application can control acute disease and prevent exacerbations. For example, a 12-month European multicenter randomized study previously demonstrated that the proactive, twice-weekly application of 0.03% tacrolimus ointment can maintain AD in remission and reduce the incidence of disease exacerbation (DE) in children with AD.[42] During the initial open-label period, 267 children with AD applied 0.03% tacrolimus ointment twice daily for up to 6 weeks to all affected areas. When an Investigator Global Assessment (IGA) score of ≤ 2 was achieved, the patient entered the disease control period (DCP) and was randomized to receive tacrolimus (n = 125) or vehicle ointment (n = 125) twice weekly for 12 months. Exacerbations were treated with 0.03% tacrolimus ointment twice daily until an IGA ≤ 2 was regained, then randomized treatment was restarted. The outcome measure was the number of DEs during the DCP that required substantial therapeutic intervention. Proactive application of 0.03% tacrolimus ointment significantly reduced the number of DEs during the DCP that required substantial

therapeutic intervention and the percentage of DE treatment days, and increased the time to first DE requiring intervention.[42] Furthermore, similar beneficial clinical effects by this type of proactive treatment with 0.1% tacrolimus ointment have been reported in patients with adult AD.[43]

Breneman et al also evaluated the long-term efficacy and safety of 3-times-weekly use of tacrolimus ointment for preventing AD relapse.[44] Adult and pediatric patients with moderate to severe AD who were clear of the disease after up to 16 weeks of treatment with tacrolimus ointment were randomized in a double-blind fashion to 3-times-weekly treatment with either tacrolimus ointment (0.03% or 0.1%) or vehicle for 40 weeks. The number of flare-free treatment days was considered as the primary end-point. A total of 125 patients were randomized to tacrolimus and 72 patients to vehicle. The mean number of flare-free treatment days was 177 for tacrolimus and 134 for vehicle ($P = 0.003$). The median time to first relapse was 169 days for tacrolimus and 43 for vehicle ($P = 0.037$). Maintenance therapy with tacrolimus ointment was associated with significantly more flare-free days than vehicle treatment, as well as a significantly longer time until first relapse.[44]

Conclusions

The major side effect of topical tacrolimus is a transient burning sensation which usually subsides within several days of continuous application. Topical tacrolimus has already been commercially available for 10 years in Japan, and so far there have been no case reports on the development of internal and cutaneous malignancies due to topical tacrolimus. Topical tacrolimus is presently considered a safe and beneficial drug, and in conjunction with topical steroids, plays a major role in the management of AD. Careful follow-up and dose monitoring are however essential in order to foster this life-changing drug in dermatology-related medical fields.

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Disclosures

The authors disclose no conflicts of interest.

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ORIGINAL ARTICLE

Severity scores, itch scores and plasma substance P levels in atopic dermatitis treated with standard topical therapy with oral olopatadine hydrochloride

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ABSTRACT

Atopic dermatitis (AD) is a common chronic or chronically relapsing, severely pruritic, eczematous skin disease. Recently, substance P (SP) has been demonstrated to be one of the important neuropeptides for mediating itch–scratch and stress–scratch cycles. In this study, we examined the severity scores, itch scores and plasma SP levels in 19 patients with AD treated with standard topical therapy with or without an oral antihistamine, olopatadine hydrochloride, for 4 weeks. The standard therapy decreased SCORAD scores, itch behavioral rating scores and plasma SP levels at post-treatment in the mass, but the topical therapy with olopatadine was more effective than the topical therapy alone, suggesting a potential additive effect.

Key words: atopic dermatitis, guideline, olopatadine hydrochloride, topical therapy.

INTRODUCTION

Atopic dermatitis (AD) is a chronic relapsing skin disease, often associated with elevated levels of serum immunoglobulin E and a personal and/or familial history of allergy.[1] AD is a major skin disease of children that is increasing in both developed and developing countries.[2–5] Acute phases of the disease are primarily characterized by extreme pruritus (itching), which in turn leads to excoriation. Excoriation further exacerbates the underlying inflammation, setting up an "itch–scratch" cycle, resulting in chronic lesions.[6] There are a variety of known itch-associated mediators, including histamine, neuropeptides, opioids, growth factors and cytokines.[7] Among various neuropeptides, substance P (SP) has been postulated to play an important role in AD. SP promotes the production of nerve growth factor from keratinocytes and the release of histamine, leukotrienes or tumor necrosis factor from mast cells, leading to sensory nerve fiber sprouting and augmentation of skin inflammation.[8,9] Interestingly, it has also been pointed out that stress worsens dermatitis via SP-dependent neurogenic inflammation in mice.[10] Thus, SP is currently considered to be one of the key pruritogenic factors.[6,7] In keeping with this notion, abnormal expression of SP was reported in the skin lesions of AD.[11] Furthermore, it was reported that the increased plasma levels of SP significantly correlated with the disease severity in patients with AD.[12]

Standard therapy for AD is constituted of topical steroids, topical calcineurin inhibitors, emollients and oral antihistamines.[1] The standard therapy has been documented to significantly improve the severity scores and pruritus as well as quality of life in patients with AD.[13] Randomized controlled clinical trials revealed the itch-relieving effects of antihistamines in AD.[14] Olopatadine hydrochloride is a second-generation, non-sedative antihistamine that is widely used in Japan.[15,16] The antihistamine has been shown to potentially reduce the serum SP levels in AD when compared to other antihistamines.[17] In this study,

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Score	Itching During the Day
0	None
1	Mild itching, not annoying and not troublesome
2	Moderate itching, annoying and troublesome, may interfere with daily activities
3	Severe itching, very annoying, substantially interfering with daily activities
4	Very severe itching, interfering with daily activities
Score	**Itching During the Night**
0	None
1	Mild itching, not annoying or interfering with sleep
2	Moderate itching, annoying and troublesome, may interfere with sleep
3	Severe itching, very annoying, substantially interfering with sleep
4	Very severe itching, interfering with sleep

Table 1. Behavioral rating scores (BRS)

we examined the severity scores, itch scores and plasma SP levels in patients with AD treated with standard topical therapy with or without oral olopatadine hydrochloride.

METHODS

Patients and scoring systems

Nineteen patients with AD (14 men and five women; mean age, 27.3 years) were enrolled in this study. Nine patients (six men and three women; mean age, 24.1 years) were treated with topical therapy alone, and 10 patients (eight men and two women; mean age, 30.1 years) were treated with topical therapy with oral olopatadine hydrochloride daily (10 mg/day). The disease severity was scored by SCORAD.[18] The intensity of itch was measured by behavioral rating scores (behavioral rating scores [BRS]; 0–8 points in total) which was used in the previous clinical trial (Table 1).[14] The SCORAD scores ranged 30.3–100.4 (mean ± SD, 63.71 ± 23.20) in the group treated with topical therapy with olopatadine and from 19.0–68.5 (mean ± SD, 36.26 ± 17.48) in the group treated with topical therapy alone. The patients with higher SCORAD scores tended to be allocated to the group treated with topical therapy with oral olopatadine. All patients were treated for 4 weeks, and the clinical scoring as well as the blood sampling was performed at pre- and post-treatment. This study was approved by the Ethics Committee of Kyushu University.

Measurement of plasma SP level

Considering the instability of SP, peripheral blood was sampled in the presence of anti-proteinase solution (Kyowa Medex, Tokyo, Japan), and plasma SP levels were measured using an enzyme-linked immunosorbent assay method.[19] The plasma SP levels were examined in 37 healthy adult volunteers (normal controls) as well as in the 19 patients described above.

Statistical analysis

The data were analyzed using a Wilcoxon signed-rank test and multiple regression analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Efficacy of standard therapy on clinical scores of AD

The 4-week standard therapy for AD clearly and significantly reduced the disease severity in the mass as assessed by SCORAD (Fig. 1a). As this study was not randomized, patients with severe symptoms tended to be allocated to the topical therapy plus olopatadine group (Fig. 1b,c). The significant reduction of SCORAD scores was clearly observed in the group treated with topical therapy plus olopatadine (Fig. 1b). The decrease of SCORAD was very little in the group treated with topical therapy alone, however, all of the cases except one were kept in good control (Fig. 1c). The subjective itch intensity measured by BRS had a tendency to decrease after the 4-week standard therapy in total (Fig. 2a, $P = 0.075$). In the group treated with topical therapy plus olopatadine, the BRS significantly reduced at post-treatment (Fig. 2a), whereas it was not the case in the group treated with topical therapy alone (Fig. 2c).

Severe cases with higher SCORAD scores in the group treated with topical therapy with olopatadine

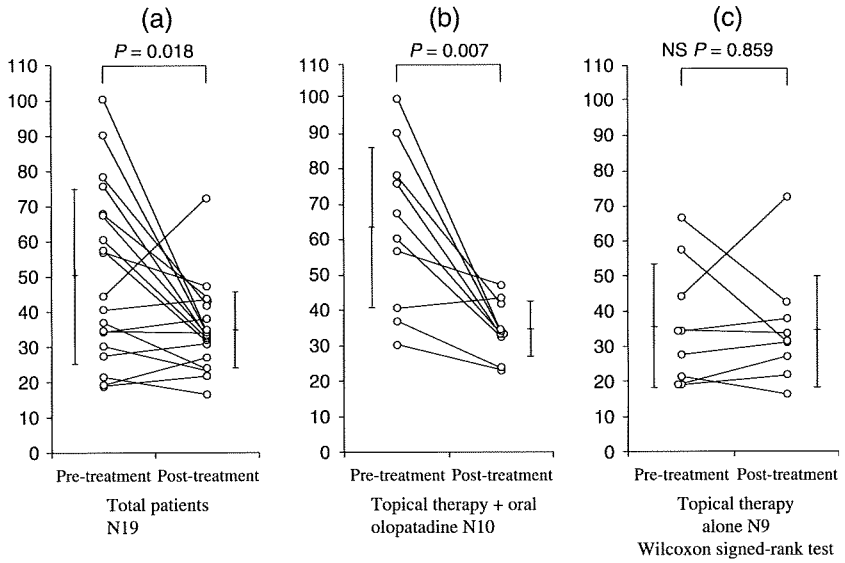

Figure 1. Pre- and post-treatment SCORAD in the patients treated with topical therapy with or without olopatadine. (a) Total patients; (b) group treated with topical therapy + olopatadine; (c) group treated with topical therapy alone.

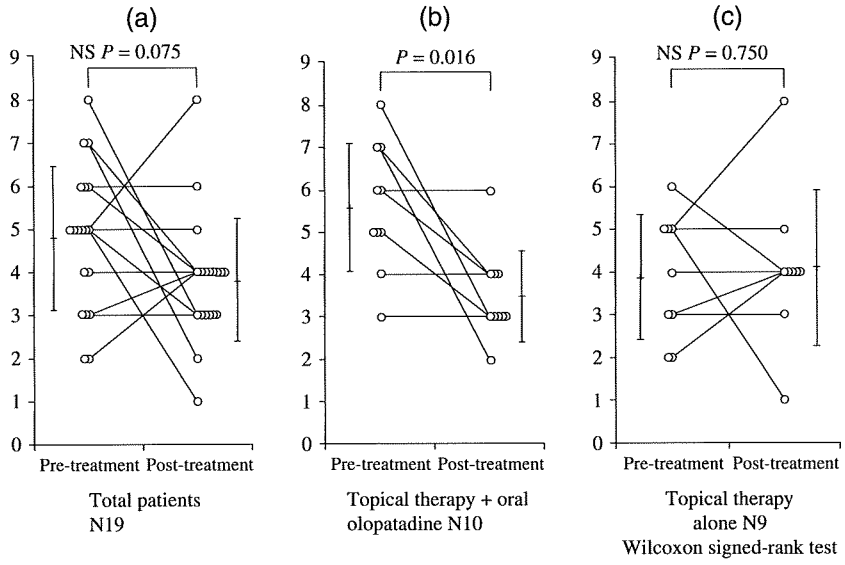

Figure 2. Pre- and post-treatment itch behavioral rating scores in the patients treated with topical therapy with or without olopatadine. (a) Total patients; (b) group treated with topical therapy + olopatadine; (c) group treated with topical therapy alone.

tended to use larger amounts of topical steroids, and the average amount of topical steroids used during the 4-week treatment tended to be larger in the topical therapy plus olopatadine group (topical steroids, 88.5 ± 88.9 g; topical tacrolimus, 15.0 ± 30.1 g) than in the topical therapy alone group (topical steroids, 42.2 ± 45.5 g; topical tacrolimus, 5.78 ± 11.2 g), although the difference was not statistically significant. However, multiple regression analysis clearly demonstrated that the dose of topical steroids contributed more to SCORAD reduction than did the administration of oral olopatadine (Table 2). In contrast, the administration of oral olopatadine contributed more to BRS-reduction than did the dose of topical steroids.

Plasma SP levels in AD

The plasma SP levels in patients with AD at pretreatment were 76.77 ± 109.89 pg/mL and were significantly higher than those of normal controls (16.46 ± 5.18 pg/mL). The plasma SP levels tended to normalize after the 4-week standard therapy in total (Fig. 3a, $P = 0.053$). The tendency for SP-normalization was observed in the group treated with topical therapy plus olopatadine (Fig. 3b), but not in the group treated with topical therapy alone (Fig. 3c).

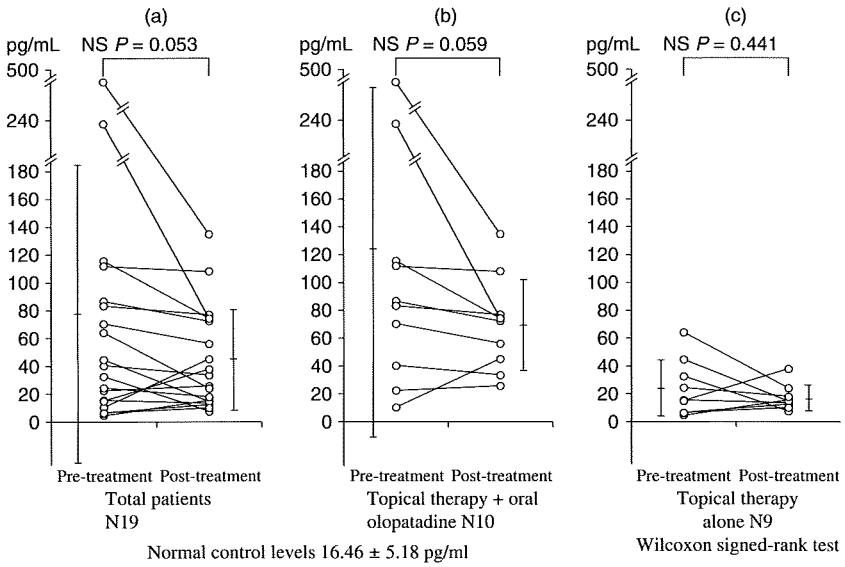

Figure 3. Pre- and post-treatment plasma substance P levels in the patients treated with topical therapy with or without olopatadine. (a) Total patients; (b) group treated with topical therapy + olopatadine; (c) group treated with topical therapy alone.

Table 2. Contribution of oral olopatadine and the dose of topical steroids to SCORAD reduction and behavioral rating score (BRS) reduction

		Oral olopatadine	Dose of topical steroids
***P*-values**	**SCORAD reduction**	0.020833	**0.002409**
	BRS reduction	**0.064572**	0.14763

DISCUSSION

In the present study, we examined the disease severity (SCORAD) and pruritus (BRS) in patients with AD at pre- and post-treatment, aiming to clarify the efficacy of standard therapy on the objective and subjective symptoms of AD.[20] Both SCORAD and BRS apparently were reduced after the standard treatment of AD. Severely affected patients with higher SCORAD scores tended to be treated with topical therapy plus oral olopatadine. Thus, it might not be surprising that the beneficial clinical effects were more evident in the group treated with topical therapy plus olopatadine than in the group treated with topical therapy alone. This may be partly due to the fact that severe cases with higher SCORAD scores in the group treated with topical therapy plus olopatadine tended to use larger amounts of topical steroids. Another possibility is that the more disrupted skin barriers in the severely affected patients may have enhanced percutaneous absorption of topical steroids, resulting in more rapid improvement than expected.[21,22] In addition, the anti-pruritic effects of olopatadine may ameliorate the itch–scratch cycle.[23] However, it is of note that the topical therapy alone could keep all of the patients except one in good control throughout the study. When the contribution of the dose of topical steroids and administration of oral olopatadine were compared, our results demonstrated that the dose of topical steroids contributed more to SCORAD reduction than to BRS reduction, and vice versa, the administration of oral olopatadine contributed more to BRS reduction than to SCORAD reduction. These data may explain the fact that the SCORAD did not correlate well with the BRS in this study (data not shown). The additive anti-pruritic effects by antihistamines have already been published in a randomized controlled study,[14] and such anti-pruritic effects would surely be beneficial to prevent excoriation in AD.

In accordance with the previous reports,[12] the circulating SP levels were elevated in patients with AD in the present study. The plasma SP levels apparently showed a tendency to decrease at post-treatment. However, the plasma SP level did not seem to be a sensitive marker for disease severity of AD ($r = 0.326$, $P = 0.173$) compared to other sensitive laboratory markers such as TARC which can range 10–40 000 pg/mL,[24,25] because the distribution window of actual values of plasma SP levels was relatively small (10 to <500 pg/mL). The declining tendency of plasma SP levels was again more evident in the group treated with topical