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INVESTIGATIVE REPORT

Prolonged Topical Application of Tacrolimus Inhibits Immediate Hypersensitivity Reactions by Reducing Degranulation of Mast Cells

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We studied the effects of prolonged topical application of tacrolimus on immediate hypersensitivity reactions. Tacrolimus, betamethasone or petrolatum was applied to the footpad of mice for up to 28 days after immunization, and the foot-swelling response to allergen was estimated. The reactions in tacrolimus-treated mice decreased significantly from day 14, while those in betamethasone-treated mice decreased significantly from day 7. Although the number of mast cells in the foot skin of each group did not differ significantly, the percentage of degranulated mast cells decreased in the tacrolimus-treated group on days 7 and 14. The TUNEL method indicated that there were no apoptotic mast cells in the foot skin of tacrolimus-treated mice. Continuous application of topical tacrolimus ointment may suppress immediate hypersensitivity reactions by reducing the degranulation of mast cells, rather than by decreasing their number. ***Key words: ovalbumin; betamethasone; IgE-mediated; mast cell number.***

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Topical tacrolimus has been widely used as an effective anti-inflammatory drug for atopic dermatitis (AD) (1–3). In patients with AD, pruritic oedematous erythema (most probably due to acute exacerbation) often coexists with chronic eczematous lesions, especially on the face. We often experience resolution of such swollen erythematous eruptions following continuous application of topical tacrolimus. Although it is reported that IgE-mediated hypersensitivity reaction in the early phase is suppressed after a single application of topical tacrolimus in ethanol, the mechanism of the inhibitory effect has not yet been clearly shown (4).

We studied the effects of continuous application of topical tacrolimus ointment on ovalbumin (OVA)-induced immediate hypersensitivity reactions. The results suggest that prolonged application of topical tacrolimus can suppress immediate hypersensitivity reactions by reducing degranulation of mast cells rather than by decreasing their number.

MATERIALS AND METHODS

Five-week-old male BALB/c mice (Shimizu Laboratory Supplies Co. Ltd, Kyoto, Japan) were immunized by i.p. injection of 1 μg OVA (Sigma Chemical Co., St Louis, MO, USA) and 1 mg aluminum hydroxide gel (alum) (5). We defined 14 days after immunization by OVA as day 0. From day 0, 0.1% tacrolimus hydrate (Protopic™; Fujisawa, Tokyo, Japan), 0.12% betamethasone valerate (Rinderon-V™; Shionogi, Tokyo, Japan) or petrolatum (Maruishi, Osaka, Japan) was applied to both soles on the hind limbs of each mouse once a day for up to 28 days. The mice (n =3 per group per each day) were provoked at day 0, day 3, day 7, day 14 and day 28 by intradermal injection of OVA to their soles 24 h after application. The sole thickness was measured immediately before and 20 min after injection using an engineer's micrometer. As a control, intradermal injection of saline was also administered to the contralateral sole.

At 20 min after each challenge, the sole skin was excised, fixed with formalin and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin (H&E) and toluidine blue. Immunohistochemical staining for c-kit (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) using Vectastain ABC-kit (Vector Laboratories, Inc. Burlingame, CA, USA) was also carried out as described previously (6). Apoptotic mast cells were detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) method.

For statistical evaluation, normality of distribution was confirmed using one factor ANOVA, then paired t test and Tukey-Kramer test were performed. A p value <0.05 was considered significant.

RESULTS

Effects of prolonged application of topical tacrolimus on the immediate hypersensitivity reaction

Mice were challenged with OVA by intradermal injection on day 0, day 3, day 7, day 14 or day 28. As seen in Fig. 1, the immediate hypersensitivity reactions began to be significantly suppressed from day 14 in the tacrolimus-treated group, and from day 7 in the betamethasone-treated group. The reaction in the petrolatum-treated group was not suppressed during the 28 days of observation.

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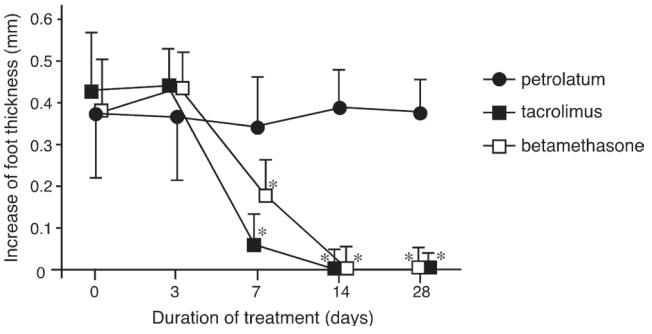

Fig. 1. Effects of prolonged application of topical tacrolimus and betamethasone on the immediate hypersensitivity reaction. Mice were challenged with ovalbumin by intradermal injection to the soles on days 0, 3, 7, 14 or 28. Increases in foot thickness were measured (n=3 mice/group/day). Data are expressed as mean±SD, independent experiments showing similar results. *p<0.05.

Number of mast cells in tacrolimus-treated skin

To examine the mechanisms underlying the suppressive effect of topical tacrolimus on the immediate hypersensitivity reaction, histological examination was carried out. The number of infiltrating mononuclear cells did not significantly differ among the three groups at each time point (data not shown). The numbers of mast cells were not significantly different in either the tacrolimusor betamethasone-treated group on toluidine blue staining (Fig. 2A) or c-kit immunostaining (Fig. 2B).

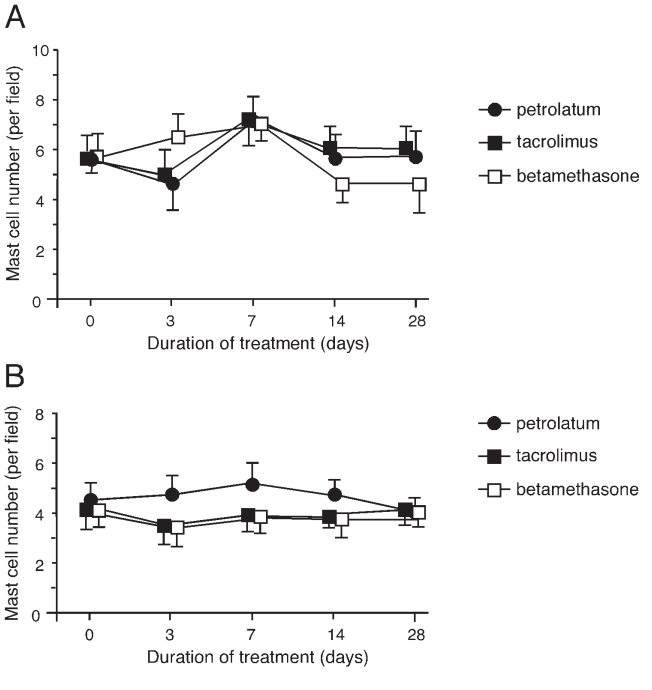

Fig. 2. The number of toluidine blue-positive mast cells. Dermal mast cells were counted to obtain the average number in three randomly selected fields of each section at a magnification of ×400 (A). The numbers of c-kit-positive mast cells did not significantly differ in either the tacrolimus- or betamethasone-treated group on c-kit staining (B).

In an *in vitro* study, it was reported that tacrolimus induces apoptosis of mast cells in a dose- and time-dependent manner (7), implying the possibility that continuous application of topical tacrolimus might induce apoptosis in mast cells *in vivo*. However, the TUNEL method did not demonstrate any apoptotic cells in tacrolimus- or betamethasone-treated foot skin. Therefore, it is suggested that apoptosis of mast cells does not contribute to the suppressive effect of tacrolimus on the immediate hypersensitivity reaction.

Inhibition of degranulation of mast cells in tacrolimus-treated skin

To examine whether topical tacrolimus suppresses the activity of mast cells, the percentage of degranulated mast cells was calculated. In the tacrolimus-treated group, but not in the betamethasone-treated group, the percentage of degranulated mast cells began to show a significant decrease on day 7 (Fig. 3). The average percentage of degranulation in the control group receiving saline injection to their sole was about 10%.

DISCUSSION

IgE-mediated hypersensitivity reaction is initiated when high affinity IgE receptors on mast cells are aggregated by multivalent allergen molecules (8). This provokes intracellular signals leading to degranulation and increased synthesis of cytokines and mediators of inflammation such as histamine, leukotrienes and chemotactic factors. Histamine causes vascular dilatation and increased vascular permeability, contributing to the early phase of hypersensitivity reaction, which occurs in the first 15–30 min following allergen exposures. The *de novo* synthesized cytokines and other mediators subsequently attract and activate inflammatory cells such as lymphocytes and basophils (9). The period occurring 6–12 h after allergen challenge is designated as the late phase reaction. In chronic lesions of AD, the total number of mast cells is increased, while in the acute phase of AD, mast cells are fragmented or degra-

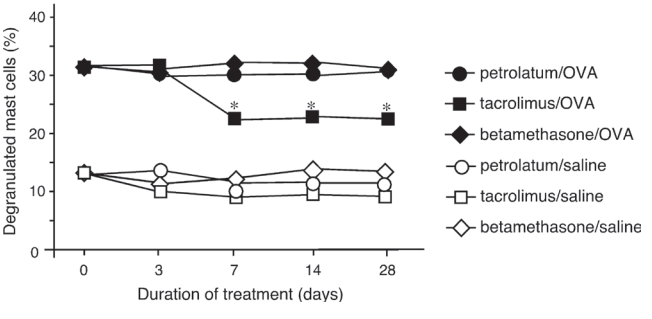

Fig. 3. Percentage of degranulated mast cells on toluidine blue-stained sections. OVA, ovalbumin-induced immediate hypersensitivity reaction. *p<0.05.

nulated (10). In patients with AD who have high serum IgE, inhalation of house dust mite sometimes induces pruritic erythematous lesions in addition to exacerbation of pre-existing eruption within a few hours (11). Patients with AD often notice such exacerbation and/or induction of swollen erythematous eruptions, mainly on the face, soon after exposure to relevant allergens such as house dust and pollen. It is thus suggested that IgE-dependent mast cell degranulation and mediator release may contribute to the inflammatory process of AD (12), although no direct evidence demonstrating their significance in AD is apparent. Clinical use of topical tacrolimus ointment is often effective for oedematous erythematous eruption. In this study, we found that continuous topical application of tacrolimus ointment suppresses the early phase of IgE-mediated hypersensitivity reaction.

Geba et al. (4) reported that a single topical application of tacrolimus diluted in ethanol inhibited the early phase of IgE-mediated hypersensitivity reaction in BALB/c mice (4). In contrast, we observed that the hypersensitivity reaction in mice continuously treated with tacrolimus began to decrease from day 14. This discrepancy may be explained by a difference in the vehicle diluting tacrolimus. Geba et al. used absolute ethanol for dilution, while we applied a petrolatum-based ointment as used in clinical practice. Our preliminary examination showed that 0.1% tacrolimus in ethanol is significantly more effective than tacrolimus ointment of the same concentration (data not shown).

It was previously shown that *in vitro* treatment of mast cells with tacrolimus inhibited activated release of histamine and cytokines (13, 14). In addition, mast cells treated *in vitro* with tacrolimus underwent apoptosis (7), suggesting that prolonged topical application of tacrolimus would reduce the number of mast cells in skin tissue. However, the present findings suggest that tacrolimus suppresses mast cell degranulation, but does not induce apoptosis in order to induce the inhibitory effect on IgE-mediated hypersensitivity shown by continuous topical application of tacrolimus. The previous data showed that tacrolimus (0.1–300 nM) concentration-dependently inhibited histamine release from mast cells (14), whereas tacrolimus induced mast cell apoptosis at a concentration of 1 μM (7). It is suggested that apoptosis of mast cells is not induced by the concentration of tacrolimus that inhibits histamine release from mast cells.

In this study, continuous topical application of corticosteroid over 7 days also significantly suppressed IgE-mediated footpad swelling, not by affecting mast cell numbers or by inhibiting its degranulation. Corticosteroid is known to be unable to inhibit IgE-dependent degranulation of mast cells (15). One of the putative mechanisms to explain this discrepancy is that steroids cause vasoconstriction by reducing the formation of vasodilator prostanoids via inhibition of phospholipase A2 (16) and cyclooxygenase (17). In addition, glucocorticoids induce a protein that nonspecifically inhibits the increase in vascular permeability (18, 19). It is, therefore, possible that repeated corticosteroid application over a continuous period inhibited the increase in vascular permeability, which suppressed IgE-mediated swelling.

Other studies have documented that topical application of steroids will reduce the number of mast cells (20, 21), which is contrary to our results. However, Finotto et al. (20) and Lawlor et al. (21) used the high-potency steroid fluocinonide and ultra-potent steroid clobetasol propionate, respectively,whereas we used the intermediate-potency steroid 0.12% betamethasone valerate.

Topical steroid is also reported to reduce the number of infiltrating cells in late phase reactions (6–8 h after provocation) by suppressing the production of cytokines or chemokines (20). In this study, however, the infiltrating cells did not reduce significantly in steroid-treated skin tissue obtained 20 min after OVA challenge. A likely explanation of this discrepancy is that we studied only the early phase of the immediate-type hypersensitivity reaction when no cell infiltration basically took place, and did not look at the infiltrate after 6–8 h (‘late phase’).

The mediator release and cytokine production by mast cells is closely linked to activation of Ca^{2+}-dependent phosphatase, calcineurin. Tacrolimus binds to its cytosolic receptor, FK-binding protein, and the drug-receptor complex then inactivates calcineurin (22). We suppose that continuous application of topical tacrolimus ointment *in vivo* suppresses calcineurin activity in local skin mast cells, resulting in the inhibition of mast cell degranulation, which may inhibit allergic inflammation and improve swollen erythematous eruptions in patients with AD. Histological examination of AD lesions before and after topical application is able to clarify the precise effect of this potent immunosuppressant on mast cell function, which could also demonstrate the role of mast cells in the pathogenesis of AD.

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