Therapeutic approach to mite-induced intractable dermatitis using novel immunomodulator FTY720 ointment ( fingolimod) in NC/Nga mice

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Abstract
Background: The increasing incidence and prevalence of atopic dermatitis (AD) demands new therapeutic approaches for treating the disease. We investigated the therapeutic efficacy of immunomodulator FTY720 ointment ( fingolimod) for mite-induced intractable AD using an NC/Nga mouse model.

Methods: Female NC/Nga mice that developed severe AD were divided into four groups: (1) FTY720 (0.001% FTY720 ointment), (2) tacrolimus ( tacrolimus hydrate ointment) (3) betamethasone ( betamethasone ointment), and (4) ointment base ( hydrophilic petrolatum), all of which received treatment six times per week. Therapeutic efficacy after two weeks was evaluated in terms of AD severity, histochemical observations ( epidermal hypertrophy, mast cell accumulation, and CD3 T cell infiltration), transepidermal water loss ( TEWL), and epidermal barrier function ( filaggrin expression).

Results: Betamethasone treatment showed little effect, confirming that the AD was intractable. In the FTY720 group, AD improved significantly compared with the ointment base group, as did epidermal hypertrophy, mast cell accumulation, and CD3 T cell infiltration. In contrast, AD in the tacrolimus and betamethasone groups did not improve significantly, nor did epidermal hypertrophy or mast cell accumulation. Furthermore, in the FTY720 group, TEWL decreased significantly compared with the ointment base group, and filaggrin expression significantly increased compared with the betamethasone and ointment base groups.

Conclusions: FTY720 ointment is a promising candidate for treatment of intractable AD. These findings also provide the first evidence that FTY720 ointment ameliorates epidermal barrier function.

Introduction
Atopic dermatitis (AD) is the most common skin disease and can significantly compromise quality of life due to sleep disruption, social awkwardness, and emotional distress. Most infants who present with mild AD will outgrow their skin disease in later childhood. Recent studies have indicated that defects in epidermal barrier function (tight junction [ TJ] and stratum corneum [ SC] barriers) contribute greatly to triggering and perpetuating skin inflammation associated with AD. With AD, the skin is characterized by increased transepidermal water loss and reduced levels of ceramides and filaggrin. Claudin-1 and filaggrin play a critical role in TJ and SC barrier formation, respectively. In AD, therapeutic targets include not only the inflammatory response but also dry skin associated with epidermal barrier dysfunction.

The novel immunomodulator FTY720 ( fingolimod) was synthesized by structural modification of myriocin ( ISP-I ), a compound from Isaria sinclairii. FTY720 was discovered by Tetsuro Fujita ( F) in collaboration with Taito Co., Ltd. ( T; Mitsui Sugar, Tokyo, Japan) and Yoshitomi Pharmaceutical Industries, Ltd. ( Y; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) in Japan. As a result of structural modification studies of ISP-I, the reduction of toxicity and the enhancement of immunosuppressive activity were acquired. FTY720 has been reported to be effective not only in preclinical transplantation models, but also in preventing development of various immunologic diseases in animal models, including rheumatoid arthritis, myasthenia gravis, multiple sclerosis, type 1 diabetes.
diabetes mellitus, and AD. FTY720 was approved for treatment of human multiple sclerosis in the Russia in 2010. Thereafter, it has been approved for use in at least 50 countries (United States, European Union, Japan, etc.).

The mechanism of action of FTY720 differs from that of established immunosuppressants, such as tacrolimus hydrate and cyclosporine. In vivo, FTY720 is rapidly phosphorylated by sphingosine kinase 2 to phospho-FTY720 — the active form of the drug. Phospho-FTY720 is an agonist of sphingosine 1-phosphate receptor [S1PR]. Interestingly, phospho-FTY720 is able to bind to four S1PRs (S1P1, S1P3, S1P4, and S1P5), rendering it a potentially useful agonist of S1PR. This signaling induces internalization and intracellular partial degradation of the receptor. As a result, FTY720 suppresses the immune response by sequestering circulating mature lymphocytes from the blood and peripheral tissue to the secondary lymphoid tissue and thymus. In contrast, S1P2 binds S1P but not phospho-FTY720. At oral therapeutic doses, FTY720 does not affect T cell and B cell responses in vitro or in vivo. Because FTY720 treatment allows for preservation of many aspects of immune function, including the total number of lymphocytes, capacity for lymphocyte activation in the lymph nodes and tissue, capacity for generating antibodies, and innate immune response, there is only a limited increase in susceptibility to infectious disease, such as herpes virus infection and urinary tract infection. Furthermore, immune memory function is not impaired.

NC/Nga mice have been used as a murine model of human AD. In conventional circumstances, the human AD-like skin lesions spontaneously appear with hyper-immunoglobulin E (IgE) production, while in a specific pathogen-free environment, mice show neither AD nor hyper-IgE production. We previously reported that oral FTY720 in combination with betamethasone ointment significantly improved spontaneous and mite-induced AD in an NC/Nga mouse model.

In the present study, we examined the local efficacy of FTY720 ointment for treating established steroid-resistant (intractable) AD using an NC/Nga mouse model.

**Methods**

**Animals and ethics**

Nine-week-old female NC/Nga mice bred under specific pathogen-free (SPF) conditions were purchased from Japan SLC Inc., Shizuoka, Japan. The mice were bred and maintained under SPF conditions (23 ± 1 °C and 47–67% humidity, under a 12 h light/dark cycle), and given γ-ray-irradiated food (RCF-1; Orientalbio Co., Ltd., Kyoto, Japan) and distilled water ad libitum. This study was performed according to a protocol approved by the Institutional Animal Care Committee of Setsunan University (Nos. 12-11-16-02-S-239 and 12-12-16-02-S-278). Throughout the experimental procedures, every effort was made to minimize animal suffering and the number of animals used.

**Drugs**

2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride (FTY720, fingolimod) was kindly provided by Yoshitomi Pharmaceutical Industries, Ltd., Japan. 0.001% FTY720 ointment was prepared by mixing 0.5 mL of 0.1 mg/mL FTY720 with 4.5 g of hydrophilic petrolatum. Betamethasone valerate ointment (0.12%

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**Fig. 1.** Therapeutic effects of each treatment on atopic dermatitis in NC/Nga mice. Female NC/Nga mice with severe skin lesions were treated for two weeks with (1) FTY720 (0.001% FTY720 ointment, 100 mg/affected area, n = 8, ●), (2) tacrolimus (tacrolimus hydrate ointment, 100 mg/affected area, n = 8, □), (3) betamethasone (betamethasone ointment, 100 mg/affected area, n = 8, △), or (4) ointment base (hydrophilic petrolatum, 100 mg/affected area, n = 8, ▲) six times per week. (a) Each value is the mean, while the vertical bar with small horizontal bars indicates the standard deviation. The significance of the difference in atopic dermatitis (AD) score was examined using the Mann–Whitney U test. *(the FTY720 group vs. betamethasone group), **(the FTY720 group vs. the ointment base group), *+(the tacrolimus group vs. the ointment base group), and *(the tacrolimus group vs. the ointment base group) denotes P < 0.05. (b) Representative pictures, illustrating different skin symptoms at the end of the observation period in each group.
Rinderon-V ointment) was purchased from Shionogi Pharmaceutical Co., Ltd., Osaka, Japan. Tacrolimus hydrate ointment (0.1% Protopic ointment) was purchased from Astellas Pharma Inc., Tokyo, Japan.

**Induction of AD**

Ten mg of freeze-dried mite *Dermatophagoides farinae* crude extract (Df) (Mite Extract-Df; Cosmo Bio Co., Ltd., Tokyo, Japan) was mixed with 4 g hydrophilic petrolatum (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) at a concentration of 2.5 mg Df/1 g hydrophilic petrolatum (hereafter referred to as Df ointment). The mice were anesthetized with chloral hydrate, and the hair on their back was shaved with an electric shaver. AD was induced by topical application of 100 mg Df ointment on the shaved dorsal skin. Barrier disruption was achieved using 150 μL 4% sodium dodecyl sulfate treatment on the shaved dorsal skin three hours prior to Df ointment application. These procedures were repeated twice per week for 4 weeks.22

**AD severity**

AD severity was evaluated daily. Development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The AD score was defined as the sum of the individual scores.22

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**Fig. 2.** Serum immunoglobulin E concentration. Female NC/Nga mice with severe skin lesions were treated for two weeks with (1) FTY720 (0.001% FTY720 ointment, 100 mg/affected area, n = 8, ●), (2) tacrolimus (tacrolimus hydrate ointment, 100 mg/affected area, n = 8, ○), (3) betamethasone (betamethasone ointment, 100 mg/affected area, n = 8, □), or (4) ointment base (hydrophilic petrolatum, 100 mg/affected area, n = 8, △) six times per week. Serum immunoglobulin E (IgE) concentration was measured by two-site enzyme immunoassay. Each value is the mean, while the vertical bar with small horizontal bars indicates the standard deviation.

**Fig. 3.** Effect of each treatment on epidermal hypertrophy in NC/Nga mice. Female NC/Nga mice with severe skin lesions were treated for two weeks with (1) FTY720 (0.001% FTY720 ointment, 100 mg/affected area, n = 8), (2) tacrolimus (tacrolimus hydrate ointment, 100 mg/affected area, n = 8), (3) betamethasone (betamethasone ointment, 100 mg/affected area, n = 8), or (4) ointment base (hydrophilic petrolatum, 100 mg/affected area, n = 8) six times per week. (a) Skin sections from each mouse and age-matched NC/Nga mice with no symptoms (n = 4) were stained with hematoxylin and eosin. Epidermal thickness was measured under a microscope at a magnification of ×200. Each value is the mean, while the vertical bar with small horizontal bars indicates the standard deviation. The significance of the difference in epidermal hypertrophy was examined using the Mann–Whitney U test. NS, not significant. (b) Representative pictures, illustrating different extents of epidermal hypertrophy at the end of the observation period in each group.
Study protocol

This study was composed of two separate experiments. Female NC/Nga mice with skin lesions were divided into four groups: (1) FTY720 (0.001% FTY720 ointment, 100 mg/affected area, n = 8), (2) tacrolimus (tacrolimus hydrate ointment, 100 mg/affected area, n = 8), (3) betamethasone (betamethasone ointment, 100 mg/affected area, n = 8), and (4) ointment base (hydrophilic petrolatum, 100 mg/affected area, n = 8), all of which received treatment six times per week. Meanwhile, age-matched NC/Nga mice (n = 4), which did not undergo Df ointment application, were used as controls. To ensure that the amount of FTY720 in the ointment did not cause any decrease in the number of CD3\(^+\)T cells in peripheral blood, the number of CD3\(^+\)T cells was measured on days 0, 7, and 14 by flow cytometry. Treatment was continued for two weeks. These treatment regimens were similar to those reported by Yamamoto et al.\textsuperscript{22} Clinical symptoms of AD were checked every day and scored as described above.

Measurement of serum IgE level

Measurement of serum IgE level was based on our previous study.\textsuperscript{10} On days -7, 0, 7, and 14 after elicitation, peripheral blood samples were collected, and the levels of serum IgE antibody were measured by means of a two-site enzyme immunoassay kit (Mouse IgE ELISA MAX Standard Set; BioLegend, Inc., San Diego, CA, USA).

Histochemical staining

AD-associated epidermal hypertrophy and the number of infiltrated mast cells were assessed according to methods used in our previous study.\textsuperscript{10} After the treatment period, lesional or nonlesional dorsal skin was removed, fixed with 10% buffered formalin solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan), embedded in paraffin according to the conventional method, and cut into 3- to 4-\(\mu\)m sections. The sections were stained with hematoxylin and eosin (Mayer's Hematoxylin Solution; Wako Pure Chemical Industries, Ltd.) and sodium tetrabromo-fluorescein (Wako Pure Chemical Industries, Ltd.), and with toluidine blue (0.05% Toluidine Blue Solution; Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The number of mast cells in the dermis was counted under the microscope and quantified using imaging software (Image J).

Immunostaining for CD3\(^+\) T cells

The number of infiltrated CD3\(^+\) T cells was assessed according to methods used in our previous study.\textsuperscript{10} Paraffin embedded tissue was cut into 3- to 4-\(\mu\)m sections. The sections were stained for CD3 using polyclonal goat anti-mouse CD3 antibody (Santa Cruz Biotechnology, Inc., CA, USA) and peroxidase-conjugated polyclonal anti-goat IgG antibody (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan); visualization was carried out with diaminobenzidine. The number of CD3\(^+\) T cells in the dermis was quantified under a microscope at a magnification of ×200. Each value is the mean, while the vertical bar with small horizontal bars indicates the standard deviation. The significance of the difference in mast cell infiltration was examined using the Mann–Whitney U test. NS, not significant.
dermis was counted under the microscope and quantified using Image J.

Measurement of transepidermal water loss

On days -7, 0, 7, and 14 after elicitation, transepidermal water loss (TEWL) of the shaved dorsal skin was measured by means of a TEWA meter (TM 120; Courage and Khazaka, Köln, Germany). Resulting data were analyzed by a microprocessor, and were expressed in g/m²/h.

Immunostaining for filaggrin

After the treatment period, lesional or nonlesional dorsal skin was removed, fixed with 4% buffered paraformaldehyde solution (Wako Pure Chemical Industries, Ltd.), embedded in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and cut into 10-µm frozen sections with a cryostat. The sections were stained for filaggrin using a polyclonal rabbit anti-filaggrin IgG antibody (Covance Inc., Princeton, NJ, USA). Then, the sections were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) antibody (Life Technologies, Carlsbad, CA, USA) for 1 h at 20 °C. Finally, the cell nuclei were stained with Hoechst 33342 (Lonza Group Ltd., Walkersville, MD, USA) for 1 min at room temperature and examined under a fluorescence microscope; the fluorescence intensity of filaggrin was then measured using imaging software (Image J).

Statistical analysis

The significance of differences in AD score, epidermal hypertrophy, serum IgE level, and TEWL were evaluated using the Mann–Whitney U test or the paired Student’s t test, where appropriate. P < 0.05 was considered statistically significant.

**Results**

**Therapeutic effect**

In the betamethasone group, no marked improvement in AD was observed; the mean AD scores before and after treatment were 6.3 ± 1.2 and 4.1 ± 1.2, respectively (Fig. 1). These results indicate that the severe skin lesions in this mouse model were indeed steroid resistant. In the tacrolimus group, the therapeutic efficacy was limited, and improvement was not significant compared with the ointment base group; the mean AD scores before and after treatment were 6.0 ± 1.3 and 2.8 ± 1.0, respectively (Fig. 1). In the FTY720 group, improvement was significant compared with the betamethasone and ointment base groups (P < 0.05); the mean AD scores before and after treatment were 5.9 ± 1.0 and 1.8 ± 1.3, respectively (Fig. 1). The results of this study were similar to those of our previous study investigating oral therapy.11

**Serum IgE level**

As shown in Figure 2, IgE concentration was elevated after topical application of Df ointment on the shaved dorsal skin. No decrease in IgE concentration was observed in any of the four groups, and there was no significant difference in IgE concentration among the groups (Fig. 2).

**Histochemical study**

AD-associated epidermal hypertrophy improved significantly in the FTY720 group but not in the betamethasone or tacrolimus...
groups (Fig. 3). As for mast cell infiltration into the dermis, the number of mast cells was significantly lower in the FTY720 group than in the ointment base group. In addition, the number of mast cells was comparable in the FTY720 group and in mice without symptoms. The tacrolimus and betamethasone groups, however, showed no difference compared with the ointment base group (Fig. 4). There was no significant difference in mast cell degranulation among groups (data not shown). With regard to inflammatory cell infiltration into the dermis, the numbers of CD3⁺ T cells in the FTY720, tacrolimus, and betamethasone groups were significantly decreased compared with those in the ointment base group (Fig. 5). This result in the betamethasone group is similar to that of our previous study. However, the number of infiltrating CD3⁺ T cells with oral FTY720 therapy was not decreased, but it was significantly decreased in the FTY720 ointment group compared with that in the ointment base group.

Recuperative effect on skin barrier function

TEWL in the FTY720 and tacrolimus groups decreased significantly from before treatment to after treatment (FTY720 group: 60 ± 12 g/m²/h → 26 ± 14 g/m²/h; tacrolimus group: 63 ± 10 g/m²/h → 45 ± 14 g/m²/h) (P < 0.05). On day 14 after the first treatment, TEWL in the FTY720 group was significantly decreased compared with that in the betamethasone (59 ± 30 g/m²/h) and ointment base groups (48 ± 14 g/m²/h). TEWL after treatment was similar to that observed in age-matched NC/Nga mice with no symptoms (28 ± 23 g/m²/h). In the other groups, no significant decrease in TEWL was observed (Fig. 6). Furthermore, the intensity of filaggrin expression in the FTY720 group was significantly greater than that in the betamethasone and ointment base groups, and was similar to age-matched NC/Nga mice with no symptoms (Fig. 7).

Discussion

The incidence of AD has been increasing in recent years throughout the world, especially in children. As for steroid-resistant AD, Leung et al. reported that Staphylococcus aureus infection induces resistance to corticosteroids, while Li et al. reported that glucocorticoid receptor-β controls expression of histone deacetylase 2 by inhibiting glucocorticoid response; this inhibition is the cause of steroid insensitivity. With regard to steroid-resistant AD, Sears et al. reported that 69% of patients with AD treated with hydrocortisone buteprate showed excellent or good results. In other words, the remaining 31% of patients were considered to have steroid-resistant AD. In another respect, tacrolimus hydrate ointment is particularly useful for treating skin lesions on the face or neck, but 1.9% of patients develop opportunistic infection (e.g., eczema herpeticum). Furthermore, in another study, NC/Nga mice treated with tacrolimus hydrate ointment did not achieve...
complete remission. Thus, it seems necessary to develop a more effective regimen to treat steroid-resistant AD.

We previously reported that oral administration of FTY720 completely prevented spontaneous development of AD in NC/Nga mice,
and FTY720 in combination with betamethasone ointment provided dramatic relief of spontaneous and mite-induced AD.
In this study, we examined the local therapeutic efficacy of FTY720 ointment for established steroid-resistant AD using an NC/Nga mouse model.

In this study, 0.001% FTY720 ointment had no effect on the number of CD3 T cells in peripheral blood; on days 0, 7, and 14, the numbers of CD3 T cells in the FTY720 group were 517 ± 97 cells/μL, 503 ± 137 cells/μL, and 574 ± 200 cells/μL, respectively. The number of mast cells infiltrating into the dermis was significantly lower in the FTY720 group compared with the ointment base group. Additionally, application of FTY720 ointment was not related to IgE production in mite-induced AD. These results are well matched with those of our previous study.

As described in our previous paper, FTY720 binds to S1PRs, except S1P2. Mast cells express two of the five S1PRs (S1P1 and S1P3). In mast cells, S1P1 regulates migration toward antigens and sites of inflammation. S1P2 is important for proper mast cell degranulation induced by antigen-mediated cross-linking of FcRRI. In addition, Kleinjan et al. indicated the possibility that FTY720 might induce apoptosis of mast cells, potentially acting preferentially on newly recruited mast cells, whereas resident mast cells are unaffected. Furthermore, the number of CD3 T cells infiltrating into the dermis was significantly lower in the FTY720 group than in the ointment base group, and was similar to the number in age-matched NC/Nga mice with no symptoms. Kleinjan et al. also indicated that topical treatment with FTY720 impaired T cell proliferation and Th2 cell differentiation. In future studies, the mechanism underlying the decrease in CD3 T cells should be further investigated. In either case, these results suggest that treatment with FTY720 ointment is associated with a low risk of infection.

In the present study, we demonstrated that TEWL improved significantly after FTY720 treatment, and was a similar level as that in age-matched NC/Nga mice with no symptoms. Furthermore, the intensity of filaggrin expression in the FTY720 group was significantly increased compared with that in the betamethasone and ointment base groups. The Th2 cytokines (IL-4, IL-13, and IL-31) and the Th22 cytokine IL-22 reduce inflammation.29 S1P2 is a receptor for S1P, which prolongs skin allograft survival by decreasing T cell infiltration into grafts but not cytokine production in vivo.29 The immunosuppressive activity found in Isaria sinclairii metabolite, J Antibiot 1994;47:208–15.

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11. Acknowledgments

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Conflict of interest

The authors have no conflict of interest to declare.

Authors’ contributions

The study was designed and conducted, and wrote the manuscript. YY, RB, TJ, and TK participated in the study design and assisted with writing the manuscript. SO, AK, JTC, and SS conducted the study.

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