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Analysis of the Scratching Behavior in Mice with Imiquimod-induced Psoriasis-like Skin Inflammation

Kyosuke SATAKE ／ Daisuke HARADA ／ Yoshimi IKEDA
Yoshikazu MATSUMURA ／ Kensuke MYOJO ／ Hiroko YOSHITAKE
Toru AMANO ／ Katsuya KOBAYASHI

R&D Division, Kyowa Hakko Kirin Co., Ltd.

● **Abstract**

Pruritus is a common symptom in patients with psoriasis. However, the underlying mechanism of pruritus in psoriasis is not yet fully understood. As there have been few reports evaluating pruritus in animal models of psoriasis, we hypothesized that a mouse model of imiquimod-induced psoriasis would allow us to analyze the mechanisms of pruritus in psoriasis. In this study, we showed that the number of scratching was significantly increased in mice with psoriasis-like skin inflammation induced by repeated topical application of imiquimod cream compared with normal mice. ELISA and an immunohistochemical analysis revealed that the NGF production and intraepidermal nerve fiber density were significantly increased in the imiquimod-induced psoriatic skin lesions compared with normal skin, which is consistent with observations in psoriatic patients. The μ-opioid receptor antagonist naltrexone significantly decreased the number of scratching in the imiquimod-induced psoriatic model, suggesting that scratching in this model is an itch-associated behavior. In addition, the scratching behavior was dose-dependently inhibited by treatment with olopatadine hydrochloride, which is an approved antiallergic drug for the treatment of pruritus in patients with psoriasis vulgaris. These findings suggest that the pathogenic mechanism of scratching behavior observed in mice with imiquimod-induced psoriasis skin inflammation closely resembles that observed in psoriatic patients. Thus, the imiquimod-induced psoriatic model may be useful for clarifying the mechanism of pruritus and evaluating new therapies for pruritus in psoriatic patients.

Key Words: psoriatic, itch, NGF, innervation, anti-pruritics, olopatadine hydrochloride

INTRODUCTION

Psoriasis is one of the most common chronic inflammatory skin diseases, characterized by redness and scaly, raised plaques [1]. The prevalence of pruritus in patients with psoriasis vulgaris has been reported to vary between 70% and 90% [2-4]. Itch intensity has been shown to correlate with the severity of psoriasis [3]. Pruritus in psoriatic patients causes sleep disturbance and impairment of quality of life and can exacerbate dermatitis as a Koebner phenomenon [2-5]. Therefore, controlling itching in patients with psoriasis is important.

In Japan, only two antiallergic drugs with histamine H_1 receptor (H_1R) antagonistic activity are prescribed for the management of pruritus in patients with psoriasis vulgaris: olopatadine hydrochloride (olopatadine) and epinastine hydrochloride [6]. However, histamine does not seem to be involved in the development of pruritus in psoriasis, because there was no correlation between pruritus intensity and plasma histamine levels in psoriatic patients, and no marked difference was observed in the plasma histamine levels between psoriatic patients with and without pruritus [4]. Altered innervation is a major and widely discussed mechanism of itch in psoriasis. It has been reported that the number of C-fibers and the nerve growth factor (NGF) production are increased in the epidermal areas in psoriatic patients with itch [7,8]. However, although various mechanisms have been suggested, the pathogenesis of pruritus in psoriasis is not yet fully understood. Therefore, it is necessary to establish appropriate animal models to determine the mechanism of itching in psoriasis.

Kyowa Hakko Kirin Co., Ltd. address: 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka, 411-8731, Japan.
Corresponding author. Tel.: +81 55 989 3174; fax: +81 55 986 7430.
E-mail address: kyosuke.satake@kyowa-kirin.co.jp (K. Satake).

Several animal models of psoriasis have been developed and utilized over the years. However, there have been few studies focusing on pruritic behaviors. Recently, it has been reported that psoriasis-like skin inflammation is induced by topical treatment with the TLR7 agonist imiquimod in mice and humans [9,10]. Experimental data shows that imiquimod-induced dermatitis in mice closely resembles the skin lesions in psoriatic patients, not only in the phenotypic and histological characteristics, but also in the pathogenic mechanism, which depends on the IL-23/IL-17A axis [9].

In this study, we demonstrated that itch-associated scratching behavior was observed in imiquimod-induced psoriasis model in mice and that the pathogenic mechanism of scratching behavior observed in this model closely resembles that in psoriatic patients.

MATERIALS & METHODS

Experimental animals

Eight-week-old male BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). Mice were kept in a specific pathogen-free animal facility with a maintained temperature of 19-25 ℃, humidity of 30%-70% and 12-h day/night cycle and given access to food and water *ad libitum*. The experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, and the experimental protocol used in this study was approved by the Committee for Animal Experiments at Kyowa Hakko Kirin Co., Ltd. (Shizuoka, Japan).

Materials

Olopatadine was synthesized at Yokkaichi Plant, Kyowa Yuka Co., Ltd. (Mie, Japan). Naltrexone hydrochloride (naltrexone) was purchased from Sigma-Aldrich (Japan). Olopatadine and naltrexone were dissolved in distilled water and saline, respectively.

Imiquimod-induced psoriasis model

The mice received a daily topical dose of 25 mg of commercially available 5% imiquimod cream (Beselna Cream; Mochida Pharmaceutical, Tokyo, Japan) on their shaved back skin for 6 consecutive days. After the final application of imiquimod cream, the scratching behavior was measured. At 24 h after the final application of imiquimod cream, the mice were sacrificed, and skin samples were obtained. Transepidermal water loss (TEWL) was measured using a VapoMeter (Delfin, Kuopio, Finland) just before imiquimod treatment.

Measurement of NGF levels in imiquimod-treated skin

The obtained skin samples were minced and homogenized in phosphate-buffered saline containing a protease inhibitor (Complete™; Roche Diagnostics, Tokyo, Japan) with a Tissue Lyser. The precipitate was removed via centrifugation, and the supernatant was collected. The NGF (Promega, Tokyo, Japan) concentrations were analyzed with an assay kit in accordance with the manufacturer's protocol.

Immunohistochemical staining of skin sections for PGP9.5

The rostral back skin of each mouse was extracted and fixed in 10% formalin. The fixed samples were embedded in paraffin, and 30-μm sections were prepared. After deparaffinization, these sections were pre-incubated in 1% bovine serum albumin-phosphate-buffered saline (PBS) for 30 min at room temperature and then incubated overnight with 1 μg/mL anti-mouse PGP9.5 antibodies (Ultra Clone Limited, UK) at room temperature. After washing with PBS, these sections were incubated with biotinylated rabbit antirabbit IgG (Dako, Tokyo, Japan) at a dilution of 1:200 for 1 h at room temperature. The sections were washed with PBS and then incubated with streptavidine biotin complex. Peroxidase activity was visualized with a Liquid DAB Substrate chromogen system (Dako, Tokyo, Japan) and examined by microscope.

Evaluation of effects of drugs on scratching behavior in imiquimod-induced psoriasis model

Olopatadine at doses of 1 and 10 mg/kg was administered orally 1 day before the first imiquimod cream treatment and 1 h before each imiquimod cream treatment. The number of scratching was measured for 20 h after the final imiquimod cream treatment. Naltrexone at a dose of 3 mg/kg was subcutaneously administered after the final imiquimod cream treatment. The scratching behavior in mice was quantified using a SCLABA-Real System (Noveltec, Kobe, Japan) according to the manufacturer's instructions.

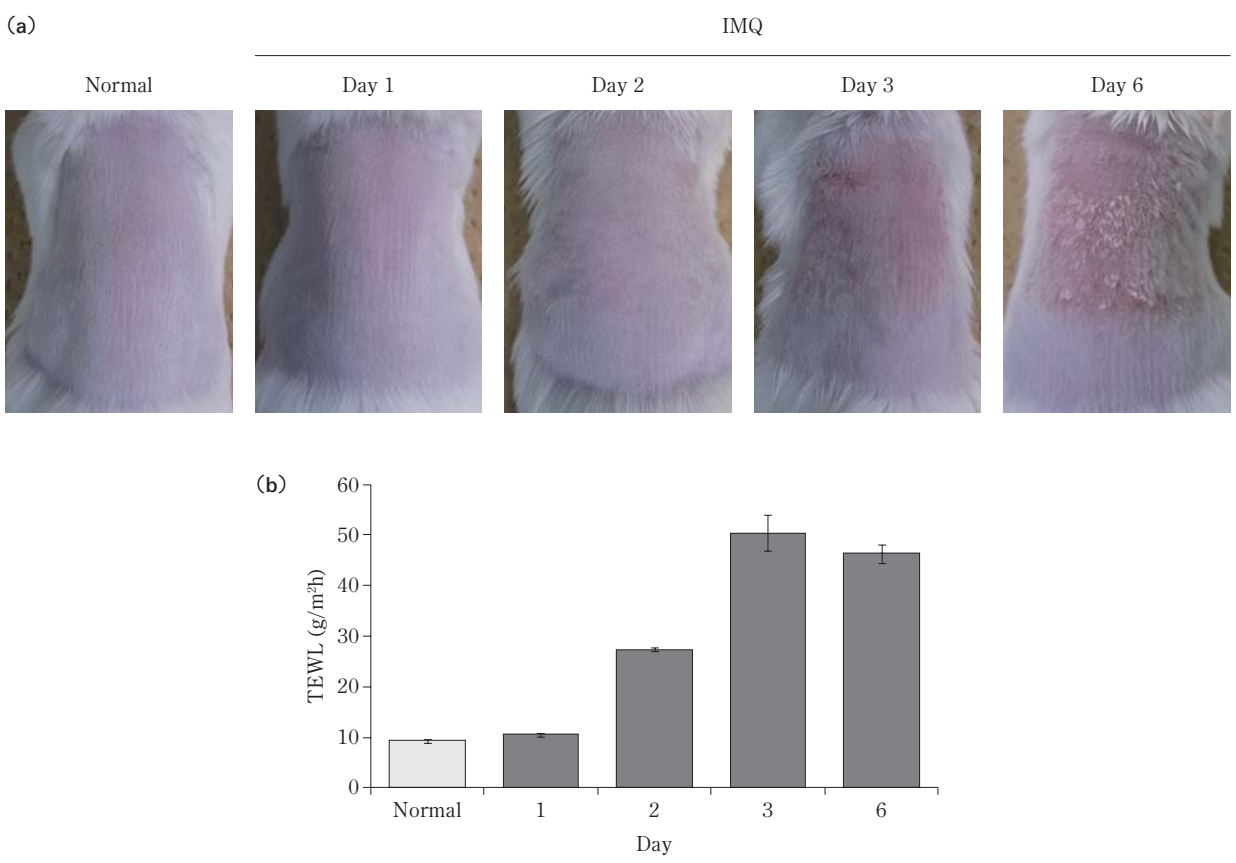
Statistical analyses

The data are presented as the mean + the standard error of the mean (S.E.M.). Student's t-test or the Aspin-Welch test following the F test was used for the analysis of the differences between two groups. Multiple comparisons among treatment groups were made using a one-way analysis of variance followed by Dunnett's test. In all tests, a p value of < 0.05 was considered to be statistically significant.

RESULT

Scratching behavior in mice with imiquimod-induced psoriasis-like skin inflammation

To assess whether or not scratching behavior could be observed in imiquimod-induced psoriasis model in mice, imiquimod cream was topically applied on the shaved back skin of mice once daily for 6 consecutive days. As reported previously [7], psoriatic phenotypes such as erythema and scales were observed from 3 days after the first imiquimod application and most prominently observed on Day 6. A typical example is shown in **Figure 1a**. A histopathological analysis revealed distinct acanthosis in the skin of imiquimod-treated mice (**Figure 3a**). TEWL, which is associated with the degree of skin barrier damage, increased with the frequency of



(a) Representative phenotypic presentation of mouse back skin after imiquimod cream treatment.
(b) TEWL was measured just before imiquimod treatment.
Each column and vertical bar represents the mean ± S.E.M. of 4 mice.

Figure 1　IMQ-induced skin inflammation in mice.

imiquimod application and plateaued on Day 3, consistent with the phenotypic changes of imiquimod-treated skin (**Figure 1b**).

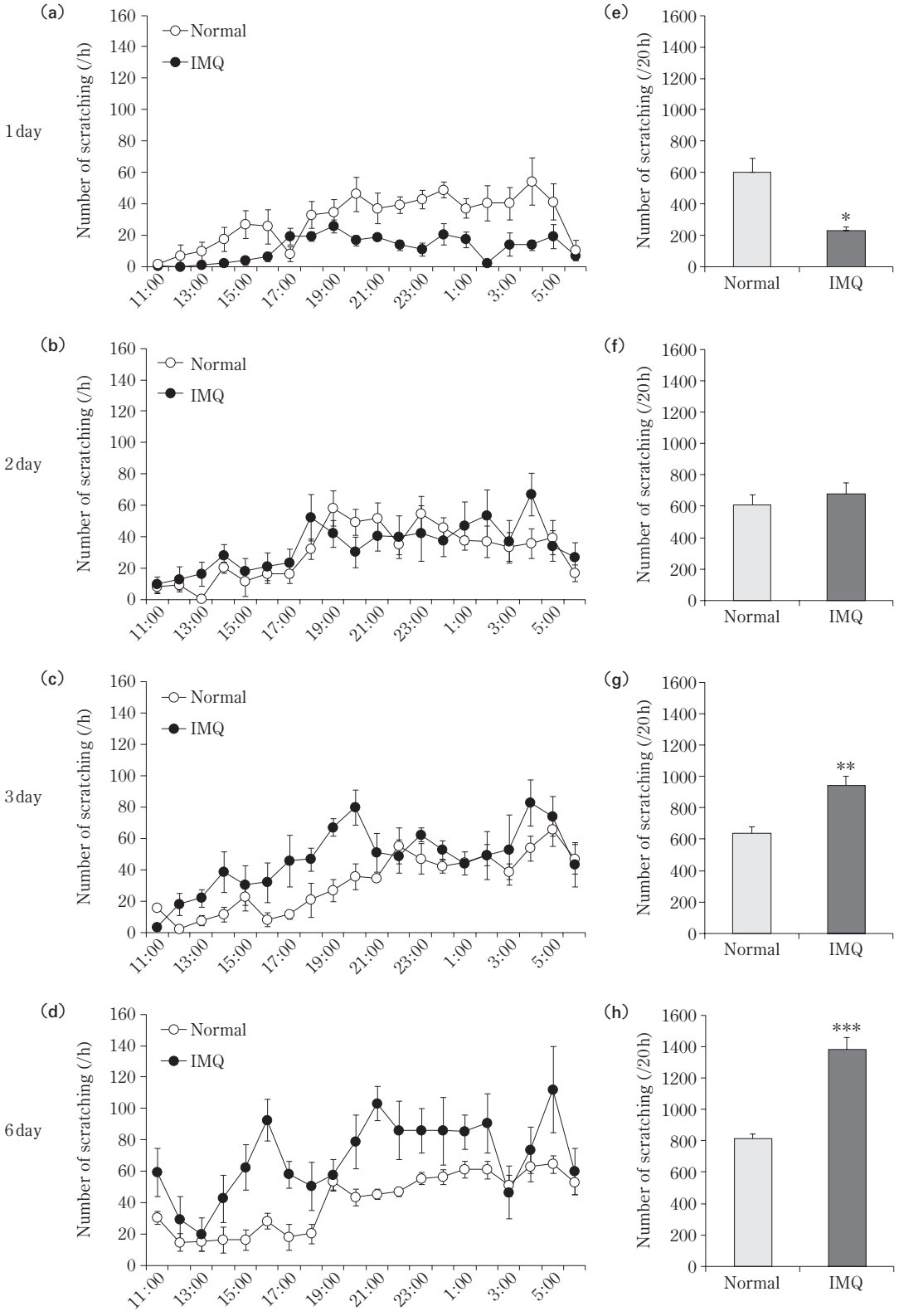
Scratching behavior was monitored in mice treated either once, twice, 3 times, or 6 times with imiquimod, and the number of scratching was measured for 20 h after the imiquimod treatment. The number of scratching in non-treated mice was measured as a control. As shown in **Figure 2a** and **2e**, the number of scratching was significantly decreased in mice with a single application of imiquimod cream compared with non-treated mice. In mice treated twice with imiquimod cream, which exhibited no phenotypic changes in the skin, the number of scratching was comparable to that in non-treated mice (**Figure 2b** and **2f**). In contrast, the number of scratching was significantly higher in mice with psoriasis-like skin inflammation induced by repeated treatment with imiquimod cream for 3 or 6 consecutive days than in non-treated mice (**Figure 2c**, **2d**, **2g**, and **2h**).

Nerve fiber distribution and NGF expression in imiquimod-induced psoriatic skin

To evaluate the neurite outgrowth in the epidermis of imiquimod-induced psoriatic skin, nerve fibers in the skin were detected by immunohistochemical staining for PGP9.5. As shown in **Figure 3a**, in the skin of non-treated mice, PGP9.5-positive nerve fibers were observed in the dermis, but were rarely observed in the epidermis. On the other hand, in the imiquimod-induced psoriatic skin, PGP9.5-positive nerve fibers were observed in both the dermis and epidermis and reached the uppermost layer of the epidermis (**Figure 3a**). The NGF levels in the skin of mice treated with imiquimod for 6 consecutive days was significantly increased compared with that in non-treated mice (**Figure 3b**).

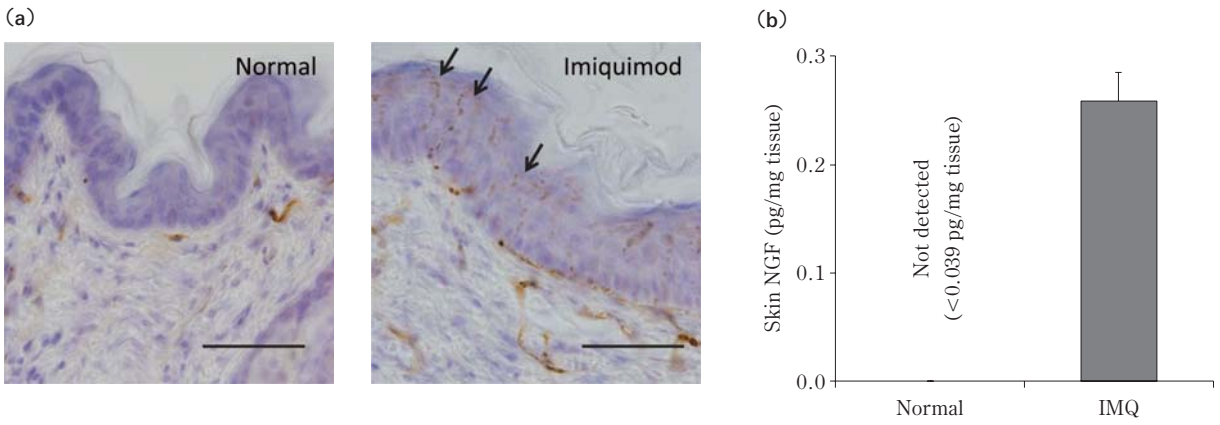
Effect of naltrexone on the scratching behavior in mice with imiquimod-induced psoriasis

In order to ascertain whether or not the scratching behavior observed in mice with imiquimod-induced psoriasis-like skin inflammation was caused by pruritus, the effect of naltrexone, a μ-opioid receptor antagonist,



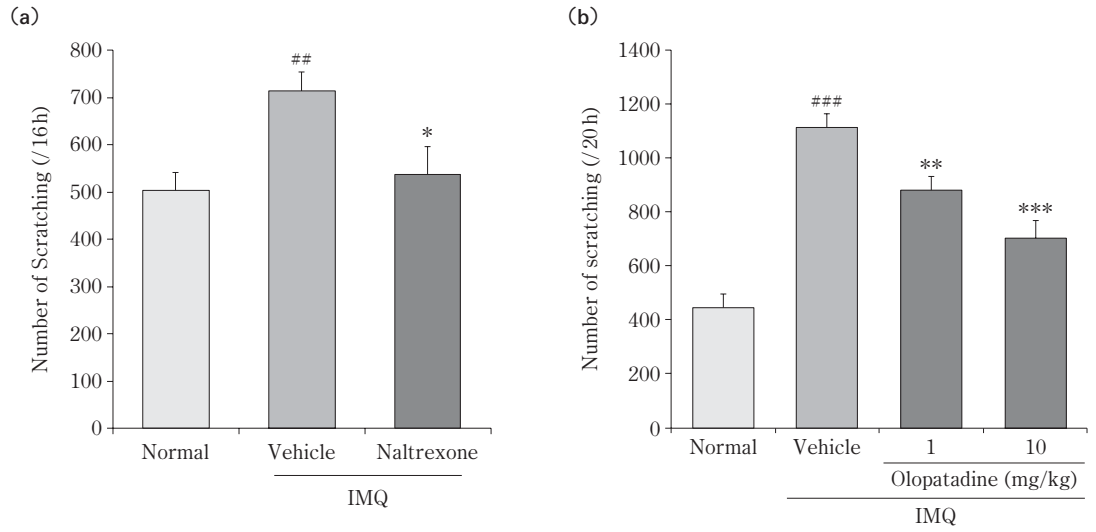
Scratching behavior was quantified using a SCLABA-Real system after the application of imiquimod cream to the back of BALB/c mice for 1 day (a, e), 2 days (b, f), 3 days (c, g) and 6 days (d, h).
(○) Normal group; (●) Imiquimod cream-treated group. Values are the mean ± S.E.M. of 4-8 mice.
Each column and vertical bar represents the mean + S.E.M. of 4-8 mice.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to normal group by Aspin-Welch test.

Figure 2 Changes in scratching behavior induced by repeated application of imiquimod in mice.



(a) Representative skin samples from normal and imiquimod-treated mice. PGP9.5 immunoreactivity was seen in the epidermis (arrows). Scale bars = 100 μm.
(b) The NGF levels in the homogenized skin tissue were measured by ELISA. IMQ: imiquimod cream.
Each column and vertical bar represents the mean + S.E.M. of 4 mice.

Figure 3　Altered innervation in the skin of imiquimod-induced psoriasis-like mice.



(a) Naltrexone was subcutaneously administered to the back skin 5 h after the final imiquimod cream treatment. The number of scratching was measured for 16 h after the naltrexone administration. $^{\#\#}P < 0.01$ compared to the normal group by Student's t-test. $^{*}P < 0.05$ compared to the vehicle group by Student's t-test.
(b) Olopatadine was administered orally 1 day before the first imiquimod cream treatment and 1 h before each imiquimod cream treatment. The number of scratching was measured for 20 h after the final imiquimod cream treatment. IMQ: imiquimod cream. Each column and vertical bar represents the mean + S.E.M. of 8 mice. $^{\#\#\#}P < 0.001$ compared to the normal group by Student's t-test. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the vehicle group by Dunnett's test.

Figure 4　Effects of naltrexone and olopatadine on the scratching behavior induced by repeated application of imiquimod cream.

on the scratching behavior in imiquimod-induced psoriasis model in mice was evaluated. Five hours after the final imiquimod application, mice were subcutaneously administered naltrexone once at 3 mg/kg, and the number of scratching was measured for 16 h, starting just after the naltrexone administration. The number of scratching was significantly lower in the naltrexone-treated group than in the vehicle-treated group (**Figure 4a**), suggesting that the scratching behavior observed in imiquimod-treated mice is indeed an itching-associated response.

Effect of olopatadine on the scratching behavior in mice with imiquimod-induced psoriasis

Next, we evaluated the effect of olopatadine, an antiallergic drug prescribed for the management of pruritus in patients with psoriasis vulgaris, on the scratching behavior in imiquimod-treated mice. Olopatadine at doses of 1 and 10 mg/kg was orally administered once daily from 1 day before the first imiquimod cream treatment to Day 6. As shown in **Figure 4b**, the increase in the number of scratching incidents was significantly suppressed by olopatadine in a dose-dependent manner.

DISCUSSION

In the present study, we demonstrated that repeated application of imiquimod cream resulted in a significant increase in scratching behavior in mice. TLR7 agonist is a known mediator of pruritus, and intradermal injection of imiquimod induces transient scratching behavior in mice[11]. However, we found that the number of scratching was significantly decreased by a single application of imiquimod cream compared with non-treated animals. However, this may be simply because of the difference in the route of administration.

It has been reported that imiquimod cream induces irritation as an adverse effect in clinical use[12], suggesting that irritation is also induced by a single application of imiquimod in mice. Itch is attenuated by allodynia induced by capsaicin[13]. Therefore, we speculate that the scratching behavior was attenuated by the irritation induced by the first application of imiquimod cream and that irritation maybe desensitized by repeated imiquimod application. No prominent changes in the imiquimod-induced skin inflammation and scratching behavior were observed until two days into treatment. At that point, however, a significant increase in persistent scratching behavior was observed, consistent with the manifestation of psoriatic phenotypes in imiquimod-treated skin. Therefore, it can be assumed that the scratching behavior induced by repeated application of imiquimod cream is associated with the development of psoriatic skin lesions rather than a direct effect of imiquimod.

The μ-opioid receptor antagonist naltrexone decreased pruritogen-evoked scratching, but not capsaicin-evoked wiping[14], so that itching-associated behavior can be distinguished from pain-associated behavior. In the present study, the scratching behavior in the imiquimod-induced psoriasis model was significantly inhibited by naltrexone treatment, suggesting that scratching behavior induced by repeated application of imiquimod cream is caused by itching rather than pain.

Pruritus is a common symptom for most psoriasis patients. Many possible mediators have been suggested to modulate itch sensation in psoriasis, but none has been clearly demonstrated to be a causative agent of itching[4]. The most commonly discussed theory is the importance of altered innervation in psoriatic skin. The itch sensation is mediated by afferent C-fibers[15]. The outgrowth of C-fibers is facilitated by NGF[16,17]. In healthy skin, most C-fibers terminate at the epidermal-dermal junction, and few invade the epidermis[7]. However, C-fibers abnormally innervate into the epidermis of psoriatic skin lesions, which can lead to hypersensitivity[7], and this increased innervation may be induced by increased expression of NGF in the epidermis[8]. Consistently, immunohistochemical analyses showed that the number of PGP9.5-positive nerve fibers was increased in the epidermis of imiquimod-induced psoriatic skin compared with non-treated skin. NGF expression was also greater in imiquimod-induced psoriatic lesions. Taken together, these findings suggest that increased innervation is one of the mechanisms of itch in the imiquimod-induced psoriasis model.

The present study clearly demonstrated that olopatadine treatment suppresses the increase in scratching behavior induced by repeated application of imiquimod cream in mice. Olopatadine is an antiallergic agent with H_1R antagonistic action that is applied to treat the signs and symptoms of allergic rhinitis, chronic urticaria, eczema dermatitis and also pruritus in psoriasis vulgaris in Japan[6]. However, histamine does not seem to be involved in the development of pruritus in psoriasis[4], suggesting that olopatadine shows anti-pruritic activity independent of its H_1R antagonistic activity. Olopatadine has been reported to inhibit skin inflammation and scratching behavior in oxazolone-induced contact dermatitis model mice by suppressing substance P and NGF production[19]. However, olopatadine did not suppress the increases in the epidermal thickness and NGF production in the imiquimod-treated back skin even at doses that significantly inhibited the scratching behavior (data not shown), suggesting that olopatadine reduces scratching behavior in this model through mechanisms other than inhibiting skin inflammation and NGF production. Very recently, Wong et al. reported that vascular endothelial growth factor (VEGF) is partially associated with pruritus in imiquimod-induced psoriasiform dermatitis in mice[20]. It has been reported that olopatadine, but not other antihistamines, inhibited the increase in VEGF in the nasal lavage fluid of toluene-2,4-diisocyanate-indeuced rhinitis model rats[21]. Accordingly, olopatadine may suppress the scratching behavior in the imiquimod-induced psoriasis model by inhibiting substance P and VEGF production. Further studies are needed to clarify the mechanisms of the inhibitory effects of olopatadine on the scratching behavior in imiquimod-induced psoriatic model mice and patients with psoriasis.

In conclusion, in the present study, we demonstrated that the imiquimod-induced psoriatic model is useful for evaluating the scratching behavior and clarifying the mechanisms of pruritus in psoriasis patients. This imiquimod-induced psoriatic model may provide a more

effective way of preventing pruritus in psoriatic patients.

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