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Immediate-type allergic and protease-mediated reactions are involved in scratching behavior induced by topical application of *Dermatophagoides farinae* extract in NC/Nga mice

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Abstract

Atopic dermatitis (AD)-like dermatitis can be induced by repeated topical application of an ointment containing *Dermatophagoides farinae* body (Dfb) extract in NC/Nga mice.

This AD-like murine model also exhibits a biphasic increase in the number of scratching behavior after topical application of Dfb ointment. In this study, we investigated the possible mechanisms underlying the scratching behavior in each phase. An increase in the content of mast cell derived mediators such as histamine and 5-hydroxytryptamine in the lesional skin and increased vascular permeability were observed in the early phase after the Dfb ointment application. Chlorpheniramine (H₁ receptor antagonist) and cromoglycate (mast cell stabilizer) reduced the scratching behavior in the early phase but not that in the later phase. Furthermore, the content of various endogenous pruritogens such as interleukin-31 and thymic stromal lymphopoietin in the lesional skin was increased 1 or 24 hours after the Dfb ointment application. Elevated expression of

proteinase-activated receptor-2 (PAR-2) was also observed in the epidermis. Finally, gabexate (serine protease inhibitor) reduced the scratching behavior in both phases, and anti-PAR2 antibody also showed a tendency to reduce both scratching behaviors. These findings suggest that immediate-type allergic reactions caused by mast cell degranulation and PAR-2 activation by proteases are involved in the scratching behavior in this AD-like model.

Abbreviations

5-HT, 5-hydroxytryptamine

AD, atopic dermatitis

Dfb, *Dermatophagoides farina* body

ET, endothelin

FcεRI, high-affinity IgE receptors

GRP, gastrin releasing peptide

IgE, immunoglobulin E

IL, interleukin

PAR-2, proteinase-activated receptor-2

PBS, phosphate-buffered saline

PG, prostaglandin

SDS, Sodium dodecyl sulfate

SP, substance P

Th, T helper

TSLP, thymic stromal lymphopoietin

Key words

atopic dermatitis, house dust mites, protease, pruritogen

Introduction

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases characterized by a disturbed barrier function and abnormal immune response to various exogenous antigens (1). Most patients with AD are suffering from a strong pruritus, an unpleasant sensation provoking the desire to scratch, which thereby aggravates eczema

and causes an itch-scratch cycle. Immunohistochemical studies have demonstrated that the density of peripheral nerve fibers is increased in the skin of patients with AD than in healthy controls (2, 3). Moreover, the skin of AD patients reveals a higher tendency of provoking itch due to reduced itch threshold and prolonged itch duration to pruritic stimuli (4). Oral antihistamines have been widely used to relief pruritus for decades, but their efficacies are limited in patients with AD (5). Therefore, it is suggested that other endogenous pruritogens such as neuropeptides, neurotransmitters, cytokines, proteases and arachidonic derivatives are involved in AD-associated pruritus (4).

Proteinase-activated receptor-2 (PAR-2) is a G protein-coupled receptor that is activated by proteolytic cleavage of the N terminus of the receptor (6). In the skin, PAR-2 is known to be expressed by many cell types, especially by keratinocytes (7, 8). House dust mites of *Dermatophagoides* species such as *Dermatophagoides farinae* are the major source of allergens associated with pathogenesis of AD (9, 10), which have been shown to contain several allergens with proteolytic activity (11). It is suggested that mite-derived serine proteases activate PAR-2 on keratinocytes to produce a release of cytokines by cleaving of the peptide corresponding to the N-terminus of PAR-2 at the activation site (12). Furthermore, PAR-2 has been suggested to play an important role in pruritus, based on the following observations that markedly enhanced expression of PAR-2 on primary afferent nerve fibers was observed in the lesional skin of patients with AD and PAR-2

agonists provoked enhanced and prolonged itch by intradermal injection to the lesional skin of AD patients (13). These findings suggest that proteases derived from house dust mites may contribute to the pathogenesis of AD by aggravating the skin inflammation and inducing pruritus due to the activation of PAR-2.

In NC/Nga mice, dermatitis can be induced by repeated topical application of an ointment containing *Dermatophagoides farinae* body (Dfb) extract, accompanied by spontaneous scratching behavior, elevation of serum immunoglobulin E (IgE) levels, infiltration of various inflammatory cells into the lesional skin and increased expression of T helper (Th) 1 and Th2 cytokines in lymph node cells (14, 15). These clinical, histopathological and immunological characteristics are similar to those observed in patients with AD. We previously demonstrated that the number of scratching behavior was increased in a biphasic fashion after topical application of Dfb ointment in this AD-like model (16). However, possible mechanisms underlying the scratching behavior in each phase remain unclear.

Therefore, to clarify which pruritogenic mediators play an important role for the induction of scratching behavior in this model, we first examined the content of endogenous pruritogens in the lesional skin 1 or 24 hours after topical application of Dfb

ointment, and then found an increased content of various pruritogens related to mast cell degranulation and PAR-2 activation in this model. Judging from these data, we next focused on clarifying the involvement of immediate-type allergic reactions and PAR-2 activation in the induction of scratching behavior in this model.

Materials and methods

Animals

Female 7-week-old NC/Nga mice were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan). One-hundred and sixty-five mice were used in this study and five mice were maintained in one cage. The mice were housed in the specific pathogen free animal facility under conditions of controlled temperature ($23 \pm 3^{\circ}\text{C}$) and relative humidity ($50 \pm 20\%$), and fed a laboratory diet and ultraviolet sterile water. All of the animal experimental procedures were approved by the Ethics Committee for Animal Experiments of Maruho Co., Ltd. (Kyoto, Japan, approved number: 13-184, 14-018), and conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals at Maruho Co., Ltd.

Reagents

Sodium dodecyl sulfate (SDS) and chlorpheniramine maleate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were dissolved in distilled water. Evans blue dye (Nacalai tesque, Inc., Kyoto, Japan), cromoglycate (Sigma-Aldrich, St. Louis, MO), gabexate mesylate (Sigma-Aldrich) and anti-PAR-2 antibody (SAM-11; Santa Cruz Biotechnology, Inc., Dallas, TX) were dissolved in saline.

Induction of dermatitis

Under isoflurane anesthesia, the back of mouse was shaved with an electric clipper, and residual hair was removed using a depilatory cream on day 0. The dorsal skin was then challenged with topical application of an ointment containing Dfb extract (Biostir® AD; Biostir Inc., Kobe, Japan) or the base (hydrophilic petrolatum; Nikko Pharmaceutical Co., Ltd., Gifu, Japan). The application of Dfb ointment was repeated twice a week for 2 weeks (day 3, 7, 10 and 14). The skin barrier was disrupted with 4% SDS (150 μ L) 2 hours before each application of Dfb ointment. Only the mice with dermatitis score of 5 or more on day 14 were used for experiments, and subjected to the final application of Dfb ointment on day 17 for measurement of scratching behavior.

Evaluation of dermatitis

The severity of dermatitis was evaluated prior to every barrier disruption. The developments of 1) hemorrhage, 2) dryness and 3) hyperplasia were scored as 0 (none), 1 (mild), 2 (moderate) and 3 (severe). The sum of the individual scores was taken as the dermatitis score (minimum: 0, maximum: 9) in each mouse.

Histological analysis

The dorsal skins were excised 24 hours after final application of Dfb ointment (day 18).

The skin samples were fixed with 4% paraformaldehyde, embedded in paraffin and sectioned into 4 μ m slices. The sections were stained with hematoxylin-eosin and toluidine blue.

Measurement of scratching behavior

The number of scratching behavior was automatically measured under an unattended environment using MicroAct (Neuroscience, Inc., Tokyo, Japan). Under isoflurane anesthesia, a small magnet was implanted subcutaneously into both hind paws of mouse on day 8 or 9. The mice were acclimated for approximately 1 hour in an observation

chamber with food and water, and treated with the skin barrier disruption followed by

Dfb ointment on day 14 and 17. Then, the measurement of the number of scratching

behavior (events) was started and kept for 24 hours.

Drug administration

Chlorpheniramine (10 mg/kg, p.o.), cromoglycate (100 mg/kg, s.c.), gabexate (10 mg/kg,

s.c.) and anti-PAR-2 antibody (0.2 mg/kg, i.v.) were administrated 60, 10, 30 and 60

minutes before application of Dfb ointment followed by the scratch measurement on day

17, respectively. Dose and time of the administration were determined based on the

previous reports (17-20). Control animals received each vehicle in a volume of 10 mL/kg.

Evaluation of vascular permeability

Intravenous injection of 0.5% Evans blue dye (250 μ L) was performed immediately before

final application of Dfb ointment or hydrophilic petrolatum. One hour later, the mice

were sacrificed and the dorsal skins were excised. Each skin sample was cut into small

pieces and digested in 0.7 mL of 1N KOH at 37°C overnight. Thereafter, the dye was

extracted by adding 9.3 mL of a mixture of 0.6N H₃PO₄ solution and acetone (5:13) and

vigorously shaking. After removal of the precipitates by filtration, the amount of dye in

supernatant was measured colorimetrically at 620 nm by using a microplate reader

(Molecular Devices, LLC., Sunnyvale, CA).

Measurements of endogenous pruritogens

The skin samples of 5 mm in diameter were collected 1 and 24 hours after topical application of Dfb ointment. The samples were homogenized in 150 μ L of ice-cold phosphate-buffered saline (PBS) containing a protease inhibitor cocktail set III (Calbiochem, San Diego, CA) using a ShakeMaster (Biomedical Sciences, Inc., Tokyo, Japan) and centrifuged at $10,000 \times g$ for 10 minutes at 4 °C. The concentrations of histamine, 5-hydroxytryptamine (5-HT) and gastrin releasing peptide (GRP) (Cusabio Biotech, Co. Ltd., Wuhan, China); substance P (SP) (Enzo Life Sciences, Inc., Farmingdale, NY); interleukin (IL) -31 (USCN Life Science Inc., Wuhan, China); thymic stromal lymphopoietin (TSLP), prostaglandin (PG) E₂, endothelin (ET) -1 and periostin (R&D Systems, Inc., Minneapolis, MN) in the supernatants were measured by enzyme-linked immunoassay (ELISA) according to the manufacturer's protocol.

Immunohistochemical analysis

The skin samples excised on day 18 were fixed in 4% paraformaldehyde and immersed in PBS containing 30% sucrose. These samples were embedded in OCT compound (Sakura Finetek USA, Inc., Torrance, CA) and sectioned using a CM1950 Cryostat (Leica Biosystems Nussloch GmbH, Wetzlar, Germany) at 20 μ m thickness. After blocking in

PBS containing 1% bovine serum albumin (Sigma-Aldrich), the sections were incubated with a goat anti-PAR-2 antibody (1:50 dilution; Santa Cruz Biotechnology, Inc.) for 2 hours at room temperature. Subsequently, the sections were washed with PBS and incubated with a rhodamine-conjugated anti-goat antibody (1:100 dilution; Santa Cruz Biotechnology, Inc.) for 1 hour at room temperature. Images of immunolabeled sections were captured with a confocal laser scanning microscopy (LSM510; Carl Zeiss MicroImaging GmbH, Jena, Germany).

Statistical analysis

Data are expressed as the mean $\pm$ standard error of mean (SEM). A significant difference between two groups in all measurements except dermatitis score was evaluated by F-test followed by Student's or Aspin-Welch's *t*-test. Wilcoxon test was used for dermatitis scores. A *P* value of less than 0.05 was considered statistically significant.

Results

Repeated topical application of Dfb ointment induces AD-like skin lesions and biphasic increase in the number of scratching behavior in NC/Nga mice

The dermatitis score was gradually and significantly increased from day 3 to day 17 in Dfb ointment-applied mice, whereas it was hardly changed in control mice treated with hydrophilic petrolatum (Fig. 1a, b). Histopathological observation revealed that

epidermal hyperplasia and dermal infiltration of inflammatory cells such as lymphocytes and mast cells in the lesional skin of Dfb ointment-applied mice (Fig. 1c, d). Furthermore, the number of scratching behavior was increased in Dfb ointment-applied mice in a biphasic fashion; a transient increase in the first one hour (early phase) followed by a persistent increase lasted from about 4 to 24 hours (later phase) (Fig. 1e).

Content of endogenous pruritogens is increased 1 or 24 hours after topical application of Dfb ointment

To clarify which pruritogenic mediators are involved in the induction of scratching behavior in each phase, the content of endogenous pruritogens was measured in the lesional skin 1 or 24 hours after topical application of Dfb ointment. The content of histamine and 5-HT, which are secreted by mast cell degranulation, was significantly increased 1 hour after topical application of Dfb ointment compared with control mice. A significant increase in 5-HT content was also observed at 24 hours post-application of Dfb ointment (Fig. 2a, b). A significant increase in the content of SP and GRP, which are neuropeptide contained in the primary sensory neurons, was observed in the lesional skin 1 hour after topical application of Dfb ointment (Fig. 2c, d). In contrast, IL-31 content was significantly increased 24 hours after the Dfb ointment application (Fig. 2e). Moreover,

the content of TSLP and PGE₂, which are mainly released from keratinocytes by PAR-2 activation (21, 22), was remarkably increased 1 and 24 hours after topical application of Dfb ointment (Fig. 2f, g). A significant increase in ET-1 and periostin content was also observed in both time points (Fig. 2h, i). These observations suggest that immediate-type allergic reaction caused by mast cell degranulation and PAR-2 activation in the lesional skin may occur in the early phase and later phase of scratching behavior in this model.

Immediate allergic reactions in the lesional skin is induced in the early phase after topical application of Dfb ointment

Repeated topical application of Dfb ointment was reported to induce an elevation of total IgE levels in plasma on day 13 in this model (16). In sensitized individuals, mast cell degranulation induced by environmental antigens is accompanied by secretion of chemical mediators such as histamine, results in a dramatic increase in vascular permeability which occurs within minutes of allergen exposure (23). Then, to investigate involvement of these immediate-type allergic reactions in this model, we first measured extravasated Evans blue dye in the lesional skin 1 hour after topical application of Dfb ointment or hydrophilic petrolatum. The amount of dye was significantly increased in the lesional skin of Dfb ointment-applied mice compared with that in normal skin of control

mice after topical application of hydrophilic petrolatum (Fig. 3a), indicating a basal increase in vascular permeability in Dfb ointment-applied mice. When compared within Dfb ointment-applied mice, the amount of dye in the Dfb ointment-treated skin was significantly greater than that in the hydrophilic petrolatum-treated skin (Fig. 3a). Next, we investigated the effects of chlorpheniramine (H₁ receptor antagonist) and cromoglycate (mast cell stabilizer) on scratching behavior after topical application of Dfb ointment (Fig. 3b-g). Chlorpheniramine and cromoglycate reduced the number of scratching behavior in the early phase (Fig. 3c, f). The two drugs, however, hardly changed the subsequent number of scratching behavior until 24 hours; there was no difference in the number of scratching behavior in the later phase (Fig. 3d, g).

PAR-2 activation is involved in the induction of scratching behavior in Dfb ointment-applied mice

Allergens with protease activity have been identified in house dust mites such as *Dermatophagoides farinae* which are well known as major allergens in human AD (11). PAR-2 is activated by proteolytic unmasking of the N-terminal extracellular tethered ligand (6). These observations suggest that PAR-2 activation induced by allergens with protease activity contained in Dfb ointment may be involved in the induction of

scratching behavior in this model. Therefore, in order to further clarify the involvement of PAR-2 activation in this model, we first assessed the change of PAR-2 expression in the lesional skin by immunostaining, and then we found out an increased expression of PAR-2 in the epidermis of Dfb ointment-applied mice, compared with very little PAR-2 expression in the epidermis of control mice (Fig. 4a). Next, we examined the effects of gabexate (serine protease inhibitor) and anti-PAR-2 antibody on scratching behavior in this model (Fig. 4b-g). The protease inhibitor reduced the scratching behavior in the early and later phases (Fig. 4b-d), and anti-PAR-2 antibody exhibited a tendency to reduce the scratching behavior in both phases (Fig. 4e-g).

Discussion

In the present study, we investigated the mechanisms underlying the biphasic increase in scratching behavior after topical application of Dfb ointment in AD-like model. In sensitized individuals, mast cells have allergen-specific IgE bound to their surface high-affinity IgE receptors (FcεRI). When antigens cross-link the adjacent IgE antibodies, aggregation of FcεRI induces mast cell degranulation that results in the secretion of chemical mediators such as histamine and 5-HT. These immediate-type allergic reactions occur within minutes of allergen exposure, accompanied by a vasodilation and markedly

increased vascular permeability at the affected sites (23). It has been shown that repeated topical application of Dfb ointment elevates the levels of total IgE in plasma and increases the number of mast cells in the dermis in this model (16). In addition, this study demonstrated that the content of histamine and 5-HT was increased in the lesional skin 1 hour after topical application of Dfb ointment (Fig. 2a, b). An increase in vascular permeability was also observed in the lesional skin by measuring the amount of extravasated dye (Fig. 3a), which was consistent with timing of scratching behavior in the early phase. Furthermore, chlorpheniramine (H₁ receptor antagonist), and cromoglycate (mast cell stabilizer), which inhibits degranulation of mast cells (24), reduced the scratching behavior in the early phase (Fig. 3c, f). These results suggest that immediate-type allergic reactions are involved in the induction of scratching behavior in the early phase in this model.

It is suggested that PAR-2 plays an important role in mediating pruritus in AD. PAR-2 activation on keratinocytes has been reported to induce the production of TSLP and PGE₂ (21, 22), suggesting that allergens with protease activity may induce the scratching behavior via PAR-2-dependent mechanisms. Similar to the previous reports indicating that PAR-2 is highly expressed by keratinocytes throughout the epidermis in patients with AD (8), the expression of PAR-2 was increased in the epidermis of the lesional skin in this model (Fig. 4a). The present study also demonstrated an increased content of

TSLP and PGE₂ in the lesional skin (Fig. 2f, g). Furthermore, gabexate (serine protease inhibitor), which is capable of inhibiting several proteases including trypsin, tryptase and kallikreins (25), reduced the scratching behavior in both early and later phases, and anti-PAR-2 antibody showed a tendency to reduce the scratching behavior in both phases (Fig. 4b-g). PAR-2 agonists are shown to increase the release of pruritogens from mast cells (26). Moreover, PAR-2 activation on skin nerve fibers is suggested to mediate the pruritus in patients with AD (13, 27). These findings imply the possibility that gabexate and anti-PAR-2 antibody may inhibited the PAR-2-dependent mechanisms underlying scratching behavior in the early phase in this model. Recently, it has been reported that the synthetic peptide (SLIGRL) known as PAR-2 agonist is capable of activating not only PAR-2 but also the Mas-related G-protein-coupled receptor MrgprC11 (28). Moreover, SLIGRL-induced scratching is suggested to be mediated by MrgprC11, rather than PAR-2 (28). However, we used an ointment containing Dfb extract shown to contain several allergens with proteolytic activity. Additionally, mite-derived serine proteases have been reported to cleave the peptide corresponding to the N-terminus of PAR-2 at the activation site (29). Although endogenous proteases are produced by various cells in skin, it is probable that PAR-2 activation by proteases derived from house dust mites which contained in Dfb ointment is involved in the scratching behavior in this AD-like model.

Several mediators, such as neuropeptides, neurotransmitters, cytokines, proteases and arachidonic derivatives have been shown to play a crucial role in the pathophysiology of pruritus in patients with AD (4). The present study demonstrated an increased content of endogenous pruritogens in the lesional skin of this AD-like model, which is also observed in human AD (30-36). These results suggest that endogenous pruritogens in the pathogenesis of pruritus in this model may play a similar role in the patients with AD. Indeed, SP and GRP content were increased in the lesional skin 1 hour after the Dfb ointment application in this model (Fig. 2c, d). Neuropeptides such as SP released from sensory afferent nerve fibers are partly involved in the hypersensitivity of itch sensation and neurogenic inflammation (4, 37). Therefore, it is assumed that topical application of Dfb ointment may activate sensory afferent nerve fibers sprouted into the epidermis directly or indirectly via other mediators, resulting in release of neuropeptides from nerve fiber endings and increase in the number of scratching behavior in the early phase. Previous studies have demonstrated that intradermal injection of GRP was found to elicit scratching behavior in mice, at least partly, through activation of BB₂ bombesin receptors present in the mast cells, triggering their degradation (38). Recently, GRP-expressing nerve fibers were found in human skin and the majority of the nerve fibers coexpressed SP (39). These findings suggest that GRP released from the peripheral terminals of primary sensory neurons also increases scratching behavior in the early phase. In this

study, we found that the levels of some mediators such as histamine and SP in the skin were higher at 24 hours after topical application of hydrophilic petrolatum than 1 hour in control mice (Fig. 2a, c) and were similar to those at 24 hours in Dfb ointment-applied mice. Although the precise reason why the increase in histamine and SP content occurred in the skin of petrolatum-applied mice is not clear, it also was observed that the number of scratching behavior was slightly increased just by topical application of petrolatum on the back skin in control mice (16), and then it is assumed that the increase in the scratching behavior may affect the content of histamine and SP in the skin of petrolatum-applied mice.

IL-31 is reported to induce delayed itch sensations by skin prick testing in humans (40). It has been also reported that transgenic mice overexpressing IL-31 develop severe pruritus and skin lesions (41). In this study, IL-31 content was increased 24 hours after the Dfb ointment application (Fig. 2e), suggesting that scratching behavior may be attributed to the increased content of IL-31 in the later phase.

TSLP is highly expressed by epithelial cells, especially keratinocytes from patients with AD (36). Overexpression of TSLP in keratinocytes leads to the development of an AD-like skin phenotype with scratching behavior in mice (42, 43). Recently, TSLP has been reported to act directly on sensory neurons to trigger robust scratching behavior in mice (21). PGE₂ also provokes pruritus in normal skin and in patients with AD (44, 45).

Intradermal injection of a mixture of PGE₂ and histamine elicits itch of longer duration than could be accounted for by simple additive effects of PGE₂ and histamine in humans, suggesting that PGE₂ augments histamine-induced itch (46). It has been reported that intradermal injection of ET-1 causes pruritus via a histamine-independent mechanism in humans (47). Moreover, ET-1 induces dose-dependent scratching behavior in mice via an ET_A receptor in skin (48). We observed a significant increase in the content of TSLP, PGE₂ and ET-1 in the lesional skin 1 and 24 hours after the Dfb ointment application (Fig. 2f, g, and h). These findings suggest that the increased content of TSLP, PGE₂ and ET-1 is one possible molecular mechanism by which scratching behavior is increased in each phase.

Periostin is an extracellular matrix protein which sets up a vicious circle that links Th2 immune response and keratinocyte activation. Indeed, Th2 cytokines IL-4 and IL-13 stimulate fibroblasts to produce periostin, inducing production of TSLP from keratinocytes (33). The content of periostin as well as TSLP was increased 1 hour after the Dfb ointment application, and the level of periostin was increased continuously while that of TSLP was decreased 24 hours after the Dfb ointment application (Fig. 2f, i). It is therefore probable that the increase in the content of periostin may be one of the factors to induce the production of TSLP, leading to scratching behavior in this model.

In the present study, we used an ointment containing Dfb extract as an environmental allergen to induce scratching behavior in AD-like model in NC/Nga mice. Previous studies have reported that intradermal injections of histamine, 5-HT and compound 48/80, a potent mast cell-degranulating agent, elicit scratching behavior in mice (49, 50). Trypsin or tryptase-induced scratching behavior is shown to be triggered by PAR-2 activation (51, 52). Although scratching behavior is reported to be increased within 1 hour after the intradermal injections of pruritogens, a subsequent reaction after a rapid increase in scratching behavior has not been fully elucidated. Moreover, there is little report about AD-like murine model indicating that an increase in the number of scratching behavior is attributed to both mast cell degranulation and PAR-2 activation. To our knowledge, this is the first report showing a biphasic increase in the scratching behavior involving mast cell degranulation and PAR-2 activation.

In conclusion, we demonstrated the possible mechanisms underlying the biphasic increase in scratching behavior in AD-like murine model. Immediate-type allergic reactions caused by mast cell degranulation and PAR-2 activation by proteases might be involved in the scratching behavior in this AD-like model.

Acknowledgments

None.

Author contributions

Yoshihito Yamada performed the research, designed the research study, analysed the data and wrote the paper; Yuhki Ueda, Aki Nakamura, Shoji Kanayama, Rie Tamura and Kei Hashimoto performed the research; Tatsumi Matsumoto and Ritsuko Ishii wrote the paper.

Conflict of interests

The authors have declared no conflicting interests.

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Figure legends

Figure 1. Dermatitis score and scratching behaviour are increased after repeated topical application of Dfb ointment in NC/Nga mice. Dfb ointment (100 mg) was applied topically on the dorsal skin after disruption of the skin barrier with a depilatory cream on day 0 and with 4% SDS (150 μ L) on day 3, 7, 10, 14 and 17. Control animals (petrolatum) were treated with hydrophilic petrolatum instead of Dfb ointment. (a) Dermatitis score was evaluated prior to every barrier disruption. **: $P < 0.01$, compared with control mice (petrolatum). (b) Representative clinical features are shown in the lesional skin on day 17. The dorsal skin excised on day 18 was stained with (c) hematoxylin-eosin and (d) toluidine blue. Arrow indicates mast cells. Bar = 50 μ m. (d) The number of scratching behavior (events) was measured for 24 hours after topical application of Dfb ointment on day 14. Each data represent the mean $\pm$ SEM (n=5 for control mice, n=6 for Dfb ointment-applied mice). * $P < 0.05$, ** $P < 0.01$, compared with control mice (petrolatum).

Figure 2. Content of endogenous pruritogens is increased in the lesional skin after

topical application of Dfb ointment. The dorsal skins were excised 1 or 24 hours after

topical application of Dfb ointment. (a) histamine, (b) 5-HT, (c) SP, (d) GRP, (e) IL-31, (f)

TSLP, (g) PGE₂, (h) ET-1 and (i) periostin content in the skin was measured by ELISA.

Each column represents the mean $\pm$ SEM (n=5 for control mice, n=6 for Dfb

ointment-applied mice). * $P < 0.05$, ** $P < 0.01$, compared with control mice (petrolatum) at

each point. Petro, petrolatum; 5-HT, 5-hydroxytryptamine; SP, substance P; GRP, gastrin

releasing peptide; IL-31, interleukin-31; TSLP, thymic stromal lymphopoietin; PGE₂,

prostaglandin E₂; ET-1, endothelin-1.

Figure 3. Topical application of Dfb ointment induces increase in vascular permeability

in the lesional skin of AD-like mice, and chlorpheniramine and cromoglycate reduce the

scratching behavior in the early phase but not that in the later phase. (a) Evans blue

solution was intravenously injected immediately before topical application of Dfb

ointment (+) or hydrophilic petrolatum (-) on day 17. Increase in vascular permeability

was evaluated by measuring the amount of extravasated Evans blue dye in the dorsal

skin excised 1 hour after topical application of Dfb ointment. Each column represents the

mean $\pm$ SEM (n=6 for petrolatum-applied mice (-), n=5 for Dfb ointment-applied mice (+)).

* $P < 0.05$, compared with control mice treated with hydrophilic petrolatum. ## $P < 0.01$,

compared with Dfb ointment-applied mice treated with hydrophilic petrolatum. (b-d)

Chlorpheniramine (10 mg/kg, p.o., -1 hour) and (e-g) cromoglycate (100 mg/kg, s.c., -10 minutes) were administered before the last topical application of Dfb ointment on day 17.

The number of scratching behavior (events) was measured for 24 hours after topical application of Dfb ointment on day 17 (b, e). The total number of scratching behavior was counted in 0-1 hour (c, f) and 4-24 hours (d, g) after topical application of Dfb ointment.

Each data represent the mean $\pm$ SEM (n=5 for petrolatum + vehicle, Dfb + chlorpheniramine and Dfb + cromoglycate groups, n=6 for Dfb + vehicle groups). * P <

0.05, ** P < 0.01, compared with petrolatum + vehicle groups. # P < 0.05, ## P < 0.01,

compared with Dfb + vehicle groups. Petro, petrolatum; CPA, chlorpheniramine; CG, cromoglycate.

Figure 4. PAR-2 expression is increased in the lesional skin of AD-like mice, and protease inhibitor and anti-PAR2 antibody reduce the scratching in both early and later phases.

(a) The dorsal skin excised on day 18 was stained with PAR-2 antibodies (original magnification, $\times 200$). White broken line indicates the border between the epidermis and dermis. Bar = 50 μ m. (b-d) Gabexate (10 mg/kg, s.c., -30 minutes) and (e-g) anti-PAR-2 antibody (0.2 mg/kg, i.v., -1 hour) were administered before the last topical application of Dfb ointment on day 17. The number of scratching behavior (events) was measured for

24 hours after topical application of Dfb ointment on day 17 (b, e). The total number of scratching behavior was counted in 0-1 hour (c, f) and 4-24 hours (d, g) after topical application of Dfb ointment. Each data represent the mean $\pm$ SEM (n=5 for each group). * P < 0.05, ** P < 0.01, compared with petrolatum + vehicle groups. # P < 0.05, compared with Dfb + vehicle groups. Petro, petrolatum; GX, gabexate.

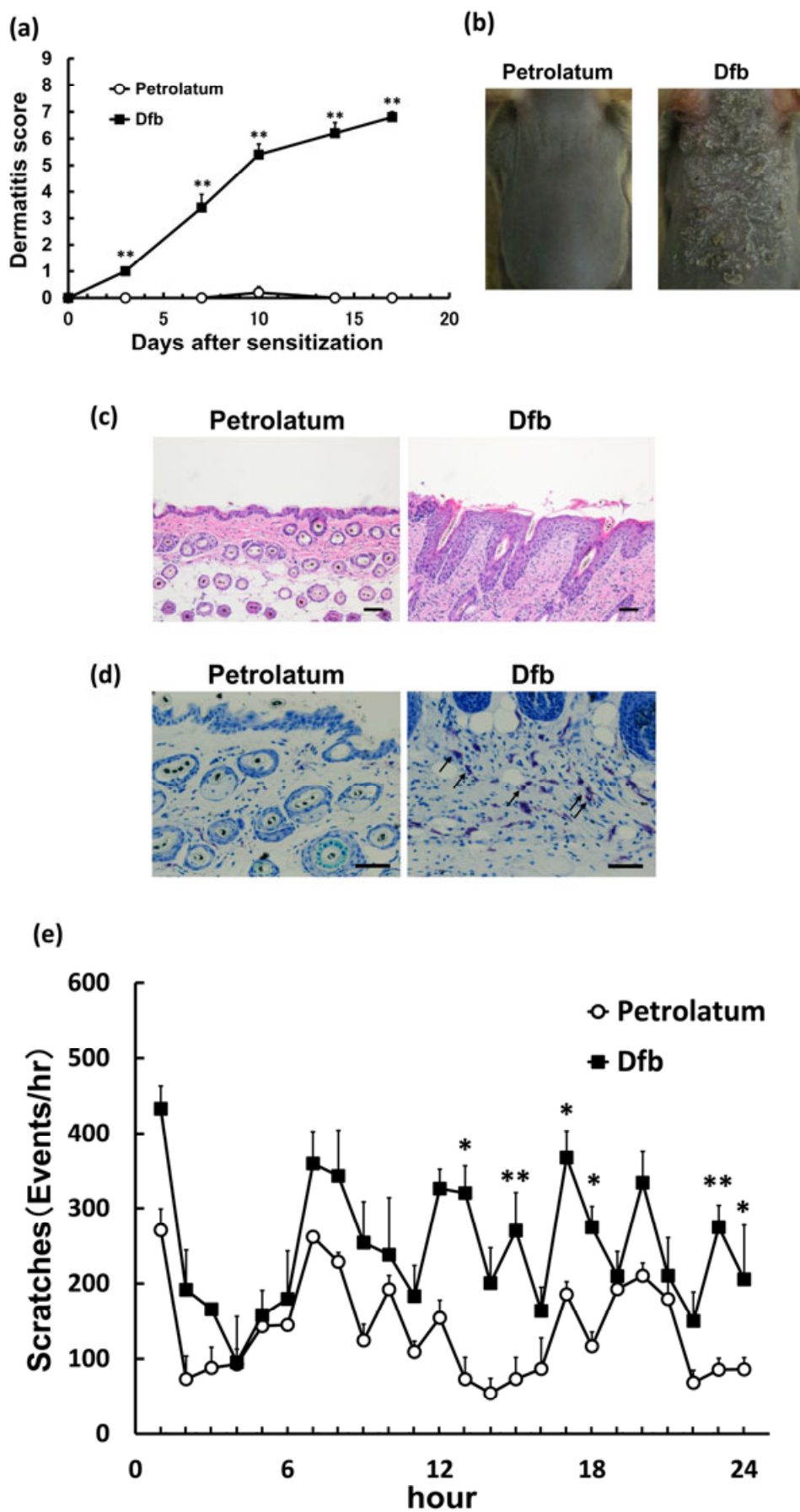


Figure 1

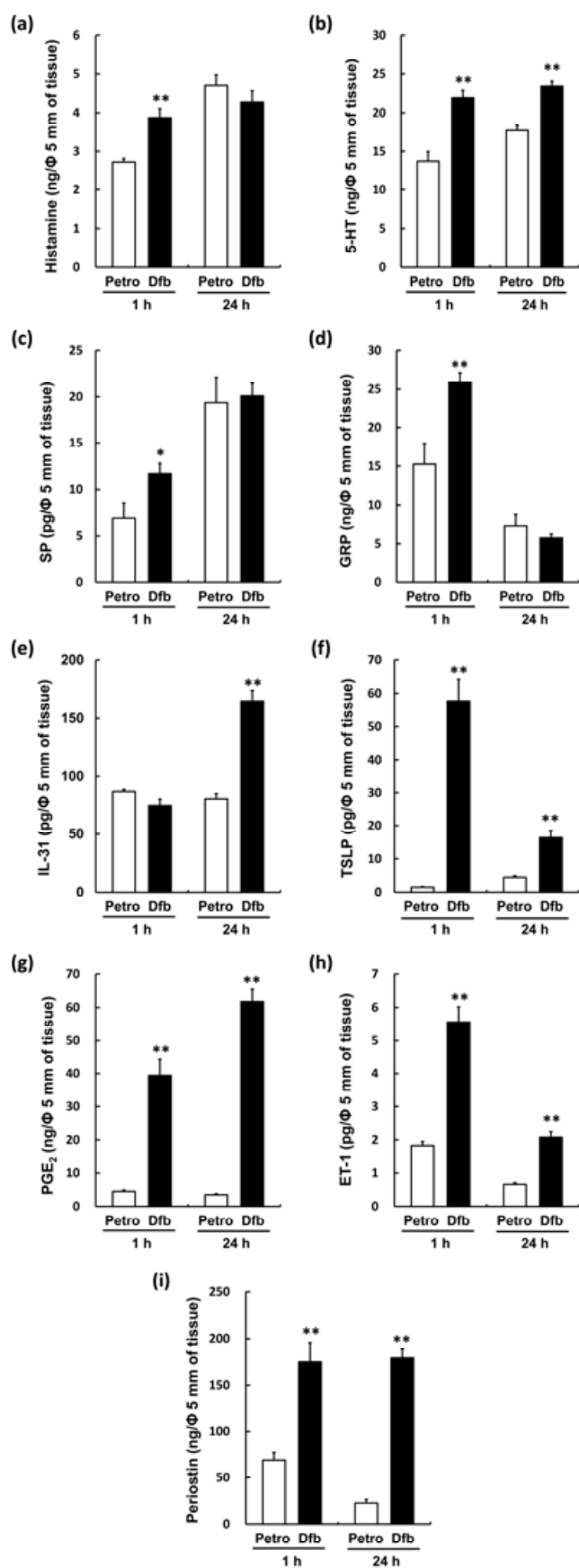


Figure 2

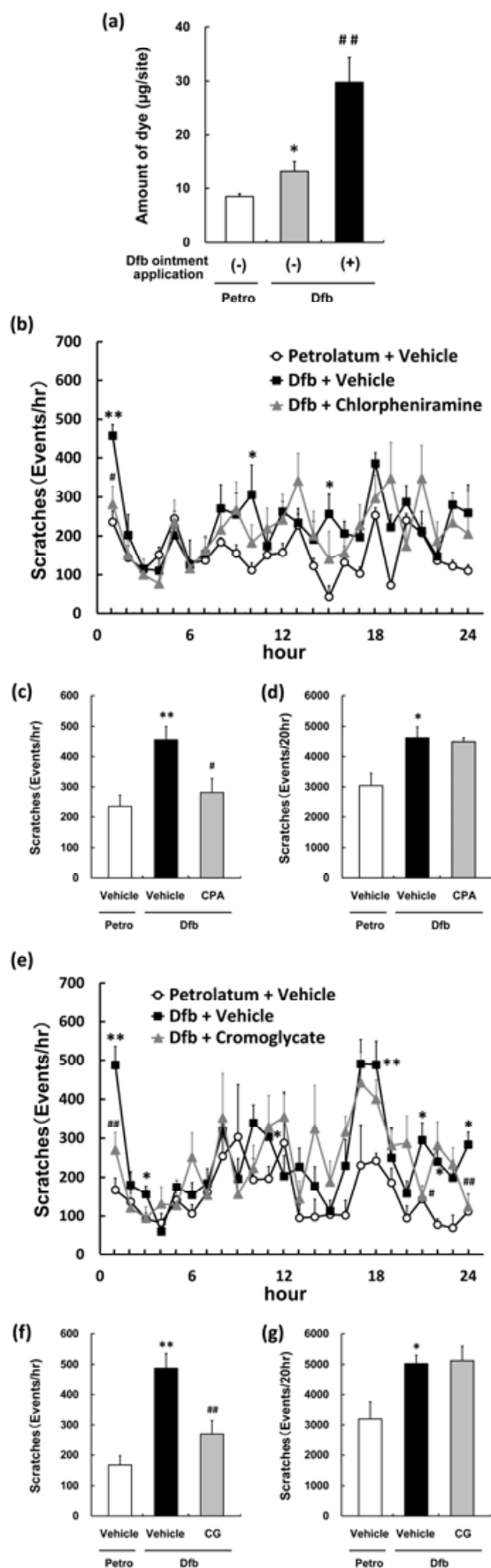


Figure 3

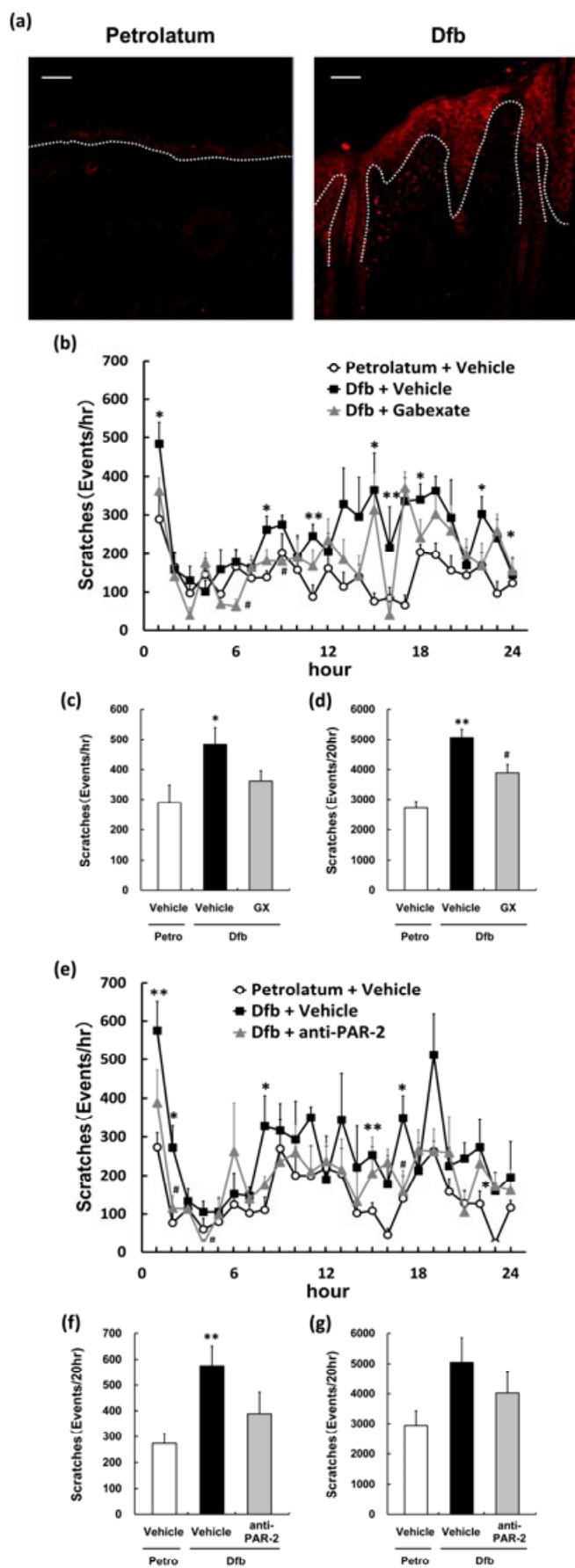


Figure 4