

Original Article

Repeated application of glucocorticoids exacerbate pruritus via inhibition of prostaglandin D₂ production of mast cells in a murine model of allergic contact dermatitis

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ABSTRACT — Rebound is known to occur most typically when topical glucocorticoids are abruptly discontinued; however, its frequency and severity are poorly characterized. We previously created a novel murine model of topical glucocorticoid-induced pruritus; however, the mechanism underlying pruritus in this model has not been elucidated. Using this murine model, we aimed to determine the cause of augmentation of pruritus with a focus on the production of prostaglandin (PG) D₂. BALB/c mice with chronic allergic contact dermatitis induced by 5 weeks of repeated application of 2,4,6-trinitro-1-chlorobenzene (TNCB) were treated topically with dexamethasone for 5 weeks immediately after the elicitation of dermatitis and after ear-swelling and scratching behavior were measured. RBL-2H3 mast cells were used to investigate the effect of dexamethasone on degranulation or PGD₂ production in IgE/antigen-stimulated mast cells. The scratching behavior induced by TNCB was augmented by topical application of dexamethasone, but dexamethasone did not have any effect on scratching bouts in mice that had not been treated with TNCB. Topical dexamethasone reduced the PGD₂ level, which increase in TNCB-treated mice, to the baseline level. Moreover, dexamethasone significantly decreased the PGD₂ production in IgE/antigen-stimulated RBL-2H3 mast cells; however, the same concentration of dexamethasone did not have any effect on the degranulation of stimulated mast cells. Topical glucocorticoids may exacerbate pruritus in a mouse model of allergic contact dermatitis via inhibition of PGD₂ production in antigen-mediated activated mast cells in the skin.

Key words: Topical glucocorticoids, Pruritus, Contact dermatitis, Prostaglandin D₂, Mast cells

INTRODUCTION

Atopic dermatitis (AD) is one of the most common chronic inflammatory and pruritic skin disorders and is marked by alternating periods of relapse and remission (Rudikoff and Lebwohl, 1998; Barnetson and Rogers, 2002). Topical exposure to haptens, such as some drugs and other low molecular-weight chemical compounds, can cause allergic contact dermatitis (ACD) (Karlberg *et al.*, 2008). A high prevalence of ACD, about 20% of the population, has been observed in Western Europe and North America and does not seem to be dependent on age, race, or geographic origin (Thyssen *et al.*, 2007).

In dermatology, topical glucocorticoids are the most widely used therapy for AD and ACD. Topical glucocorti-

coid therapy can induce numerous cutaneous side effects. The potency and in particular the duration of the therapy determine the occurrence and severity of these adverse effects. The most important cutaneous side effects of glucocorticoid treatment are skin atrophy and disturbance of wound healing (Schäcke *et al.*, 2002). Another possible concern with the use of topical corticosteroids in the treatment of AD and ACD is rebound, wherein disease recurs after the topical corticosteroid is discontinued with a greater severity than during the pretreatment baseline (Hengge *et al.*, 2006). Although rebound is known to occur most typically when topical corticosteroids are abruptly discontinued, its frequency and severity are poorly characterized (Menter *et al.*, 2009). Therefore, we considered that this phenomenon warrants further inves-

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tigation.

In patients with AD or ACD, itch-associated scratching damages the integrity of the skin and increases inflammation, which in turn intensifies the itching and causes further damage. Consequently, a vicious itch-scratch cycle easily develops and remains refractory (Pfenninger and Zainea, 2001; Mahtani *et al.*, 2005). Itching can also occur as a rebound phenomenon after withdrawal of glucocorticoids, and it seems to be important for exacerbation of symptoms. Therefore, we created a novel experimental model of glucocorticoid-induced pruritus in mice with chronic ACD to investigate the mechanism of itching after withdrawal of glucocorticoid treatment (Yamaura *et al.*, 2011).

Nerve growth factor (NGF) released by keratinocytes induces sensory nerve fiber sprouting in the epidermis and subsequently decreases the pruritus threshold in atopic skin (Tominaga *et al.*, 2007; Tanaka and Matsuda, 2005). It has recently been suggested that glucocorticoids may exacerbate irritant chemical-triggered scratching through an increase in NGF levels and nerve fiber density at the application site (Fujii *et al.*, 2010). However, the involvement of NGF in the exacerbation of pruritus was not significant in our animal model of glucocorticoid-induced pruritus (Yamaura *et al.*, 2011). Therefore, in the search for another factor that may be involved in our pruritus model, we focused on prostaglandin (PG) D₂ in the present study. PGD₂, which is produced primarily by skin mast cells (Lewis *et al.*, 1982; Peters *et al.*, 1984) and macrophages (Cao *et al.*, 2008), acts as an endogenous anti-pruritic agent (Arai *et al.*, 2004; Honma *et al.*, 2005) by suppressing histamine release from mast cells (Chan *et al.*, 2000; Hashimoto *et al.*, 2005).

To our knowledge, this is the first study to show that a topical glucocorticoid, dexamethasone, increases pruritic activity by reducing PGD₂ levels in mast cells. This pruritic-augmenting activity may be involved in the mechanism of glucocorticoid-induced rebound caused by long-term treatment. Our animal model may be useful for establishing safer and more effective use of topical glucocorticoids.

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MATERIALS AND METHODS

Animals

Female BALB/c mice (6 weeks old) were obtained from Japan SLC Inc. (Shizuoka, Japan), and housed under controlled light (07:00-19:00) and temperature (24°C) conditions with food and water available *ad libitum*. All experiments and procedures were approved by the Chiba University Institutional Animal Care and Use Committee.

Materials

Dexamethasone, indomethacin, monoclonal anti-dinitro-phenol (DNP) IgE, DNP bovine serum albumin (DNP-BSA), and *p*-nitrophenyl-N-acetyl-β-D-glucosaminide were purchased from Sigma Chemical Company (St Louis, MO, USA) and 2, 4, 6-trinitro-1-chlorobenzene (TNCB) was purchased from Tokyo Chemical Company (Tokyo, Japan).

TNCB-induced chronic ACD

TNCB and dexamethasone were dissolved in acetone at 1.0% and 0.03% (w/v), respectively, based on our previous study (Yamaura *et al.*, 2011). The experimental protocols are illustrated in Fig. 1. The abdominal region of the mice was shaved with a hair clipper 1 day before sensitization. On day -7, animals were sensitized with 100 μl TNCB solution applied to the shaved abdomen, and on day 0 the animals were challenged with the application of 10 μl TNCB solution to each ear. TNCB solution was then applied repeatedly to each ear three times a week

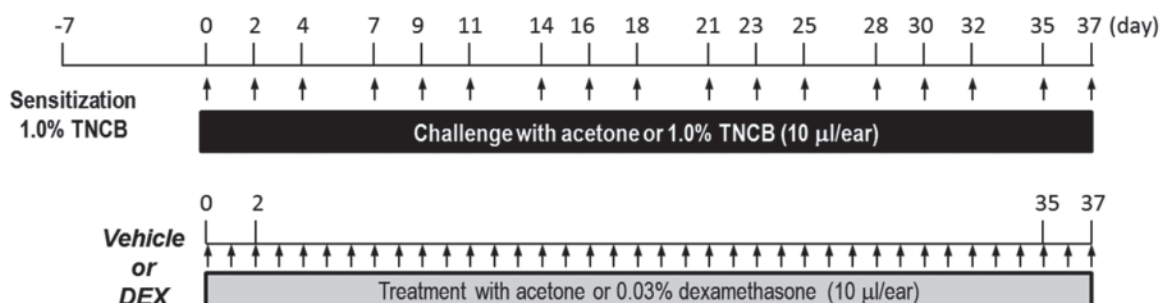


Fig. 1. Schedule for the elicitation of chronic allergic contact dermatitis and application of reagents. DEX, dexamethasone at 0.003 mg/ear/day.

until day 37. For assessment of the influence of glucocorticoids, dexamethasone was applied topically to both ears every day from day 0 to day 37 at a volume of 10 μ l/ear (0.003 mg/ear) 30 min before TNCB application. Acetone was applied as vehicle control for TNCB non-treatment (Nil) and dexamethasone non-treatment (Vehicle)

Measurement of ear thickness

Right and left ear thickness was measured with a micrometer (Mitsutoyo Corporation, Kanagawa, Japan) under light ether anesthesia 24 hr after each challenge. Ear swelling was calculated as the average of the thickness values for both ears.

Measurement of scratching behavior

The number of bouts of scratching behavior was counted for 2 hr immediately after TNCB challenge on days 2 and 35. Pruritus was evaluated by automatic counting of the scratching bouts using MicroAct (Neuroscience Inc., Tokyo, Japan), as reported previously (Inagaki *et al.*, 2003).

Expression of NGF mRNA in mouse ears

Ears samples were obtained 3 hr after the last TNCB challenge. Specimens were homogenized, and the total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA was prepared from RNA by reverse transcription using a PrimeScript RT reagent kit (TAKARA Bio INC., Shiga, Japan). Real-time quantitative PCR was performed on a Step One TM Real Time PCR System (Applied Biosystems Inc., Carlsbad, CA, USA) using SYBR Premix Ex Taq for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and mouse NGF in accordance with the manufacturer's instructions (TAKARA Bio Inc.). Results are expressed as the mRNA level relative to GAPDH mRNA as an internal control.

Measurement of the skin levels of PGD₂

Mice were intravenously injected with indomethacin (10 mg/kg) to prevent further production of PGD₂, and ear samples were obtained. Samples were minced and homogenized in 1.5 ml ice-cold acetone containing 10 μ M indomethacin with a polytron tissue homogenizer for 20 s, and then the precipitate was removed by centrifugation at 2,000 g for 10 min at 4°C. The supernatant was poured into a test tube and evaporated to dryness under a stream of nitrogen and resuspended in enzyme immunoassay buffer. The concentrations of PGD₂ were measured using the Prostaglandin D₂-MOX EIA Kit (Cayman Chemical Co., Ann Arbor, MI, USA), according to the manufacturer's protocol.

Cell culture

RBL-2H3 mast cells, a rat basophilic leukemia cell line, were obtained from RIKEN Bio Resource Center (Tsukuba, Japan). In brief, RBL-2H3 cells were maintained in Eagle's minimal essential medium (EMEM) (Nissui Seiyaku., Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS) (Equitech-Bio., Kerrville, TX, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO₂.

Mast cell degranulation assay

RBL-2H3 mast cells in EMEM containing 10% FBS were seeded onto 24-well culture plates at a density of 1×10^5 cells/well and incubated for 24 hr at 37°C under a humidified atmosphere containing 5% CO₂ with monoclonal anti-DNP IgE (50 ng/ml) and dexamethasone (from 10^{-12} to 10^{-6} M). IgE-sensitized cells were washed with phosphate-buffered saline. The IgE-sensitized cells were stimulated with 20 ng/ml DNP-BSA in EMEM at 37°C for 30 min. Degranulation was assessed by measuring β -hexosaminidase release. In brief, 50 μ l supernatant or cell lysates and 50 μ l 2mM *p*-nitrophenyl-N-acetyl- β -D-glucosaminide (in 0.4 M citrate and 0.2 M phosphate buffer, pH 4.5) were added to each well of a 96-well plate, and incubated for 2 hr at 37°C. The enzyme reaction was terminated by adding 100 μ l 0.25 M glycine buffer at pH 10.4. Absorbance at 405 nm was measured using a Multiscan JX microplate reader (Thermo Lab Systems, Kanagawa, Japan). The release of β -hexosaminidase was calculated using the following equation; % of release = (activity of β -hexosaminidase in supernatant) / (total activity of β -hexosaminidase in supernatant and cell lysates) $\times$ 100.

Measurement of PGD₂ release

Detection of PGD₂ in the supernatants of mast cells was performed using the Prostaglandin D₂-MOX EIA Kit, according to the manufacturer's protocol.

Statistical analysis

All data are presented as mean $\pm$ S.E.M. Statistical significance between two means was analyzed using either paired or unpaired Student's *t*-test, depending on the design of the experiment. Statistical significance of two or more mean values from a single control data set was analyzed using Dunnett's method. Differences at $p < 0.05$ were considered statistically significant. All statistical analyses were conducted using StatLight software (Yukms Co., Ltd. Tokyo, Japan).

RESULTS

Effect of topical application of dexamethasone on ear thickness

Repeated exposure of mouse ears to TNCB induced progressive and chronic ACD characterized by skin swelling. Ear thickness of TNCB-treated control mice significantly increased on day 36 compared with that on day 3 (Fig. 2). Topical dexamethasone inhibited the increase in ear thickness to the level of the TNCB non-treated vehicle controls on both days 3 and 36.

Effect of topical application of dexamethasone on scratching behavior

Repeated challenge with TNCB evoked an increase in the number of scratching bouts from day 2 to 35. Repeated topical application of dexamethasone significantly increased the scratching bouts to 176.8 in TNCB-treated mice on day 35 compared with 87.3 on day 2 (Fig. 3A). By contrast, repeated topical dexamethasone did not affect scratching behavior in TNCB non-treated controls.

Time course analysis of the scratching bouts revealed that topical dexamethasone on day 35 caused a higher level of scratching bouts throughout the 2-hr measurement period after challenge with TNCB compared with vehicle control (Fig. 3B).

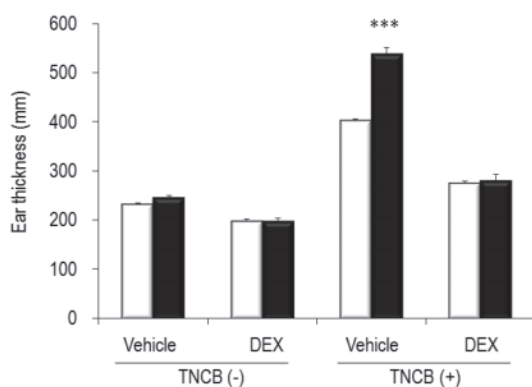


Fig. 2. Effect of dexamethasone on increased ear thickness induced by repeated application of TNCB. The ears of TNCB-sensitized mice were repeatedly challenged with TNCB three times a week from day 0 to day 37. Dexamethasone was applied topically everyday from day 0 to day 37. Ear thickness was measured 24 hr after TNCB challenge on days 2 and 35. Vehicle, acetone; DEX, dexamethasone. Values represent the mean $\pm$ S.E.M. for $n = 4-7$ mice. *** $p < 0.001$ vs the corresponding day 3 group (paired t -test).

Effect of topical dexamethasone on the expression of NGF mRNA

NGF is an important factor in the development of pruritus. We investigated the effect of dexamethasone on the levels of NGF mRNA in lesional skin. The level of NGF in lesioned ears sampled at day 37 was the same as in TNCB non-treated controls. Topical dexamethasone increased the level of NGF mRNA 1.4-fold relative to the vehicle control group, but the increase was not significant (Fig. 4).

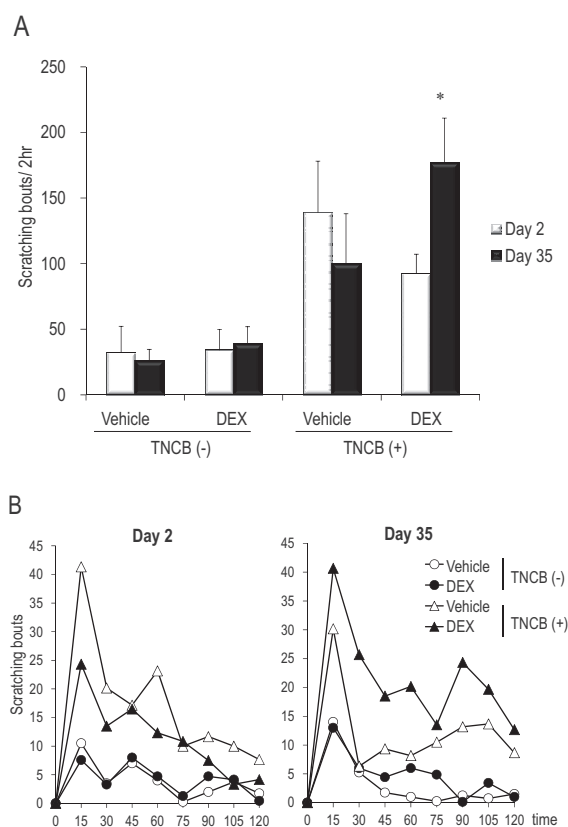


Fig. 3. Effect of dexamethasone on scratching behavior induced by repeated application of TNCB. The ears of TNCB-sensitized mice were repeatedly challenged with TNCB three times a week from day 0 to day 37. Dexamethasone was applied topically everyday from day 0 to day 37. Immediately after the TNCB challenge, scratching bouts were counted for 2 hr using MicroAct (A, total scratching bouts for 2 hr; B, time course analysis of scratching bouts). Vehicle, acetone; DEX, dexamethasone. Values represent the mean $\pm$ S.E.M. for $n = 4-7$ mice. * $p < 0.05$ vs the corresponding day 2 group (paired t -test).

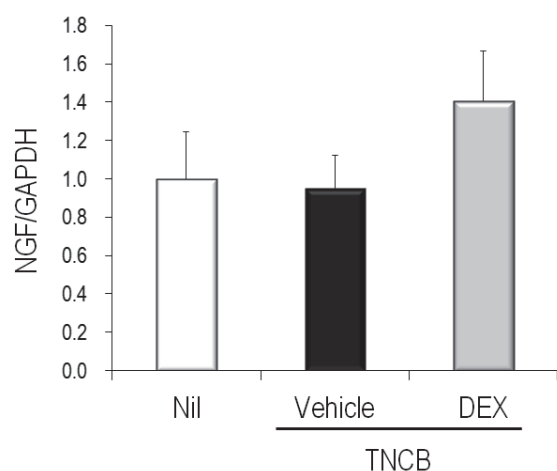
Glucocorticoids exacerbate pruritus via inhibition of PGD₂ production

Fig. 4. Effect of topical dexamethasone on the expression of NGF mRNA induced by repeated application of TNCB. The ears of TNCB-sensitized mice were repeatedly challenged with TNCB three times a week from day 0 to day 37. Dexamethasone was applied topically everyday from day 0 to day 37. Total RNA was extracted from each ear 3 hr after the last TNCB challenge. Real-time quantitative PCR was conducted using specific primers for NGF. Nil, TNCB non-treatment/acetone; Vehicle, acetone; DEX, dexamethasone. Values represent the mean $\pm$ S.E.M. for $n = 4-7$ mice.

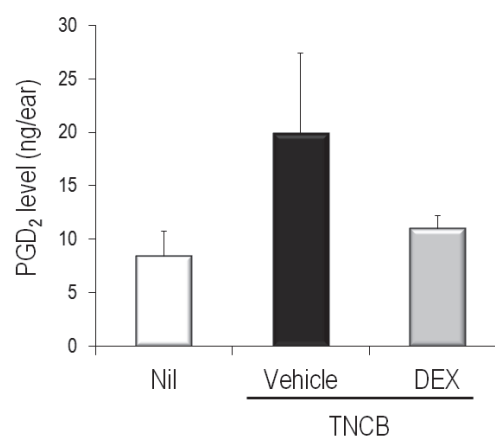


Fig. 5. Effect of topical dexamethasone on PGD₂ levels in mouse ears induced by repeated application of TNCB. The ears of TNCB-sensitized mice were repeatedly challenged with TNCB three times a week from day 0 to day 37. Dexamethasone was applied topically everyday from day 0 to day 37. Samples were obtained from each ear 3 hr after the last TNCB challenge and homogenized. Total PGD₂ levels in homogenized ear tissues were determined using an enzyme-linked immunosorbent assay. Nil, TNCB non-treatment/acetone; Vehicle, acetone; DEX, dexamethasone. Values represent the mean $\pm$ S.E.M. for $n = 4-7$ mice.

Effect of topical dexamethasone on PGD₂ levels in mouse ears

To investigate the effect of dexamethasone on an endogenous anti-pruritic agent, we measured the levels of PGD₂ in homogenized ear tissue. PGD₂ is released primarily from activated mast cells. We found that the level was increased by repeated challenge with TNCB more than 2-fold compared with the TNCB non-treated control at day 36, but the increase was not significant. Topical dexamethasone reduced this increase in TNCB-treated mice to the baseline level (Fig. 5).

Effect of dexamethasone on antigen-mediated PGD₂ production and degranulation of mast cells

Stimulation of sensitized RBL-2H3 mast cells with DNP-BSA antigen led to the production of 10.5 ng/ml PGD₂ from mast cells. Dexamethasone showed significant inhibition of PGD₂ production by approximately 60-70% in RBL-2H3 cells after treatment with the antigen (Fig. 6A). To further determine the mechanism involved, the mast cell degranulation marker β -hexosaminidase was analyzed from the same supernatants. Stimulation of

mast cells with antigen led to the release of 27.6% of the β -hexosaminidase. Dexamethasone did not suppress this degranulation in mast cells (Fig. 6B).

DISCUSSION

Repeated challenge with TNCB not only increases the serum level of IgE but also induces a shift in the cutaneous cytokine milieu from a T-helper type (Th) 1 cell to a Th2 cell profile (Kitagaki *et al.*, 1997), followed by the development of chronic ACD that is clinically, histologically and immunologically similar to AD. We have previously shown that long-term topical application of dexamethasone exacerbates the pruritic response in this TNCB-induced model of dermatitis (Yamaura *et al.*, 2011). In the present study, we first examined the pruritus-enhancing response of topical dexamethasone using control mice in addition to animals with experimentally induced ACD to clarify the pruritogenic effect of dexamethasone in normal skin. Dexamethasone exacerbated the scratching behavior in animals with experimentally induced ACD mice. In contrast, it had no significant

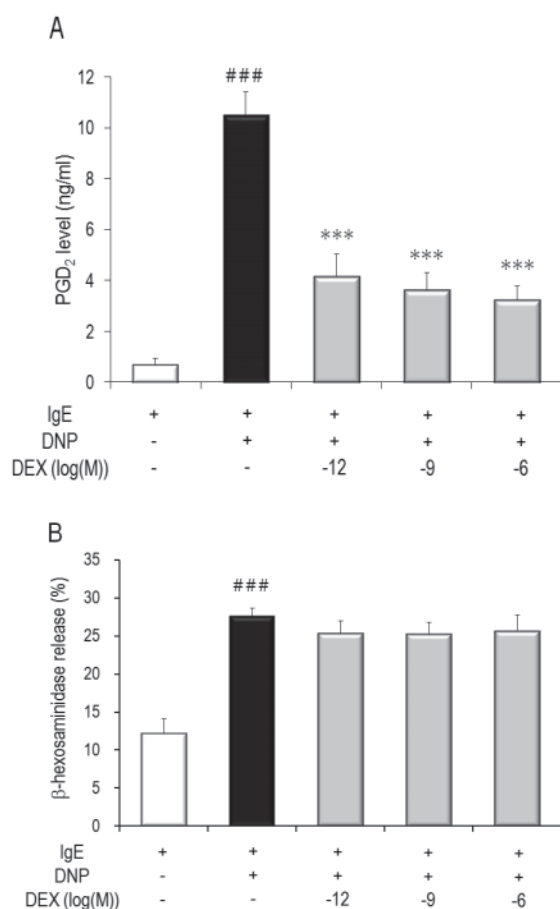


Fig. 6. Effect of dexamethasone on antigen-mediated PGD₂ production and degranulation of mast cells. IgE-sensitized RBL-2H3 mast cells were pre-incubated with the indicated concentrations of dexamethasone for 24 hr and then stimulated with DNP-BSA antigen for 30 min. (A) Supernatants collected after 30 min of antigen treatment were used to assess PGD₂ levels by enzyme-linked immunosorbent assay. (B) The supernatants and pellets were assayed to evaluate the release of the granule marker β-hexosaminidase as an indicator of mast cell degranulation. DEX, dexamethasone; DNP, DNP-BSA antigen. Values represent the mean ± S.E.M. for n = 3 (A) or n = 4 (B) experiments. ****p* < 0.001 vs IgE/DNP (Dunnett's multiple comparisons). ###*p* < 0.001 vs IgE alone (Student's *t*-test).

effect on control mice (Fig. 3A).

Skin atrophy due to long-term topical application of glucocorticoids was seen, and it is possible that thinning skin might perpetuate itching by lowering the threshold to stimulation of the cutaneous sensory nerves. Therefore, skin atrophy may be involved in the dexamethasone-

induced pruritus in our model. However, dexamethasone did not enhance the scratching behavior in mice with normal skin, suggesting that skin atrophy is not involved in this model.

By analyzing the time course of scratching bouts, we found that topical dexamethasone enhanced scratching behavior throughout the 2-hr analysis period after challenge with TNCB. Therefore it appears that dexamethasone inhibits some responses that might play a role in reducing pruritus accompanying the dermatitis.

As an endogenous anti-pruritic agent, we focused on PGD₂ which is released primarily from activated mast cells. PGD₂ is a metabolite of arachidonic acid released from membrane phospholipids by phospholipase A₂ (PLA₂), and produced by the action of cyclooxygenase and prostaglandin D synthase (PGDS) (Larsen and Henson, 1983). In the biosynthesis pathway, two synthases, lipocalin-type PGDS (L-PGDS) and hematopoietic PGDS (H-PGDS), catalyze the isomerization of PGH₂ to PGD₂ (Kanaoka and Urade, 2003). L-PGDS is expressed in the brain and testis. In contrast, H-PGDS is expressed and localized in immune and inflammatory cells such as mast cells (Urade *et al.*, 1990), antigen-presenting cells (Urade *et al.*, 1989) and Th2 cells (Tanaka *et al.*, 2000). The effects of PGD₂ are mediated by a high-affinity interaction with D prostanoid receptor (DP) 1 and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (Pettipher, 2008). Both these DPs belong to the G protein-coupled receptor family. The two receptors vary in their cellular expression: the DP1 receptor is found in mast cells, whereas the CRTH2 receptor is expressed in a number of cells, such as Th2 cells and basophils (Park and Christman, 2006).

Mast cells stimulated through the high-affinity IgE receptor, FcεRI, promote the release of PGD₂ (Hewson *et al.*, 2011). Topical dexamethasone attenuated the increase in the total serum IgE level caused by repeated application of TNCB (Yamaura *et al.*, 2011), suggesting that the decreasing level of PGD₂ by topical dexamethasone as shown in Fig. 5 is likely to be, at least in part, associated with this reduction in IgE (data not shown). Furthermore, we aimed to investigate the direct effect of dexamethasone on PGD₂ production in IgE/antigen-stimulated RBL-2H3 mast cells. Dexamethasone significantly inhibited the PGD₂ production of activated mast cells, which suggests that the decreasing level of PGD₂ by topical dexamethasone is due to the direct effect of dexamethasone on mast cells.

The mechanism by which dexamethasone inhibits PGD₂ production in antigen-mediated activated mast cells is thought to be due to glucocorticoids suppression of PG

synthesis by inhibiting the release of arachidonic acid by PLA₂ (Parente and Solito, 2004). This inhibition is due to the induction of annexin 1 in inflammatory cells which specifically targets cytosolic PLA₂ by both direct enzyme inhibition and suppression of cytokine-induced activation of the enzyme. Pruritus and scratching in combination with cutaneous hyperreactivity and a reduced threshold for pruritus in barrier-disrupted skin underlie the vicious circle of continuous mechanical stimulation and dysregulated cytokine release by keratinocytes. It is assumed that PGD₂ produced in the skin might also play a physiological role in recovery from disruption of the cutaneous barrier (Honma *et al.*, 2007).

During inflammation, mast cells release mediators that potentially bind to and activate high-affinity receptors on sensory neurons, which subsequently causes itching (Paus *et al.*, 2006). Degranulation of mast cells leading to the release of pre-formed mediators, such as histamine and tryptase as well as rapidly metabolized lipid-derived factors such as prostaglandins and leukotrienes (Hewson *et al.*, 2011), has been confirmed as one of the most important factors inducing pruritus and an important target for treating chronic itch (Yamashita *et al.*, 2007). We showed that dexamethasone did not affect the degranulation of mast cells at the same concentration at which PGD₂ production was significantly inhibited. Therefore, instead of affecting an itch mediator in the skin, topical dexamethasone may inhibit an anti-pruritic agent.

It has been reported that an enhancement of the NGF level is associated with an increase in the scratching count in animals receiving topical application of glucocorticoids (Fujii *et al.*, 2010). However, in the present study, the level of NGF mRNA was only moderately increased by topical application of dexamethasone to the ear.

In conclusion, we found that a topical glucocorticoid may exacerbates pruritus in a mouse model of ACD via inhibition of PGD₂ production in antigen-mediated activated mast cells in the skin.

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