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 glucocorticoid 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-
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) D\textsubscript{2} in the present study. PGD\textsubscript{2} 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 PGD\textsubscript{2} 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.

**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.](image-url)
Glucocorticoids exacerbate pruritus via inhibition of PGD₂ production

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 μl/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 (Imagaki 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 a 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 μM 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 × 10⁵ 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 10⁻⁵ 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 μl supernatant or cell lysates and 50 μl 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 μl 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)) × 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 ± 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).

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. 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, ace- tone; DEX, dexamethasone. Values represent the mean ± S.E.M. for n = 4-7 mice. **p < 0.001 vs the corresponding day 3 group (paired t-test).

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 ± S.E.M. for n = 4-7 mice. *p < 0.05 vs the corresponding day 2 group (paired t-test).
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
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

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).
Glucocorticoids exacerbate pruritus via inhibition of PGD₂ production

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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REFERENCES


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