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Cutaneous Biology

Effects of olopatadine hydrochloride, an antihistamine drug, on skin inflammation induced by repeated topical application of oxazolone in mice

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Summary

Background Olopatadine hydrochloride (olopatadine) is one of the second-generation antihistamines, which is prescribed for allergic disorders such as rhinitis, urticaria and eczema dermatitis.

Objectives To investigate the possible anti-inflammatory effect of olopatadine on the chronic contact hypersensitivity response to repeated topical application of oxazolone in mice.

Methods The preventive and therapeutic effects of oral olopatadine were quantified by measurements of ear swelling, cytokine protein and mRNA expression in the ear lesion, and were compared with those of topical betamethasone 17-valerate (betamethasone).

Results The ear receiving repeated applications of oxazolone exhibited erythema, oedema and abrasion. Both preventive and therapeutic administration of olopatadine (10 mg kg^{-1} day^{-1}) significantly inhibited the ear swelling and the increased production of interleukin (IL)-4, IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF) and nerve growth factor. In the histopathological analysis, olopatadine ameliorated epidermal hyperplasia and infiltration of inflammatory cells. Consistent with these results, olopatadine significantly reduced the increased expression of interferon-γ and IL-4 mRNA. Although betamethasone (0·012 mg ear^{-1} day^{-1}) showed similar activities to olopatadine against these responses, it caused atrophy of the ear skin.

Conclusions These results indicate that olopatadine is an antihistamine agent having inhibitory activities against chronic inflammatory dermatitis, possibly resulting from its diminishing effect on elevated cytokines.

Key words: animal model, antihistamine, atopic dermatitis, olopatadine hydrochloride

Atopic dermatitis (AD), allergic contact dermatitis, and psoriasis vulgaris are the most common skin diseases. AD is a chronically relapsing inflammatory skin disease characterized by episodes of intense pruritus, multiple lesions with erythema, excoriation, erosions, lichenification, papules, dry skin and susceptibility to cutaneous infection. The first-line treatment regimen for the eczematous lesions of AD is the topical application of steroids and emollients.[1,2] Antihistamines have long been prescribed for AD as an adjunct therapy with topical agents, in the belief that they reduce pruritus by blocking the action of histamine in the skin.[3]

A complex interrelation of genetic, environmental, skin barrier, pharmacological, psychological and immunological factors plays an important part in the pathogenesis of AD.[4] The lesioned skin in inflammatory dermatitis hyper-expresses several proinflammatory cytokines and chemokines, including interferon (IFN)-γ, interleukin (IL)-4, IL-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α, IL-8 and eotaxin.[5–9] Moreover, the

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number of mast cells increases and the activated mast cell also produces the inflammatory mediators, such as histamine, IL-4, GM-CSF and nerve growth factor (NGF), and substance P.[10–12] Therefore, these cytokines have been assumed to contribute to the lesions in AD.

The contact sensitivity response in mice has been used widely to explore antigen presentation and T-lymphocyte activation. Cytokine profiles, at both the protein and the mRNA level, have been evaluated in several mouse models of contact sensitivity. These models have utilized several skin sensitizers, including oxazolone, trinitrochlorobenzene and dinitrofluorobenzene. Webb *et al.* and Kitagaki *et al.* have examined the cutaneous cytokine profile of mice exposed to repeated application of oxazolone or 2,4,6-trinitro-1-chlorobenzene (TNCB) and observed the shift in the local cytokine pattern from a T-helper (Th)1 to a Th2 type profile.[13–16] As most of these findings are also observed in patients with AD, the mouse models appear to mimic many, if not all, events occurring within the lesioned skin of patients with AD.

Olopatadine hydrochloride (olopatadine; Allelock®, Kyowa Hakko Kogyo Co., Ltd, Japan) is an antiallergic agent with histamine H1-receptor antagonistic action that is indicated for the signs and symptoms of allergic rhinitis, chronic urticaria, eczema dermatitis, prurigo, pruritis cutaneous, psoriasis vulgaris and erythema exsudativum multiform.[17] Olopatadine exhibits potent antihistamine activity *in vivo* following its systemic administration. In addition to its potent antihistaminic effect, previous studies have demonstrated that olopatadine inhibits the release of tachykinin,[18] IL-6, IL-8,[19] and TNF-α.[20] The purposes of the present study were, first, to determine the possible preventive and therapeutic effects of olopatadine in a mouse model of chronic inflammatory dermatitis induced by repeated challenge of oxazolone to the skin, and, second, if olopatadine proved to be effective, to investigate the proinflammatory cytokine levels and the mRNA expression in the lesioned skin.

Materials and methods

Animals

Male 6-week-old Balb/c mice were purchased from Charles River Japan (Kanagawa, Japan). The animals were kept in the specific pathogen-free animal facility that maintained a temperature of 19–25 °C, humidity of 30–70%, and a 12-h day/night cycle, and were 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 in Kyowa Hakko Kogyo Co., Ltd. (Shizuoka, Japan).

Drugs and materials

Olopatadine hydrochloride (olopatadine) was synthesized in our Sakai Plant (Osaka, Japan). Betamethasone 17-valerate (betamethasone) and 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Olopatadine was dissolved in distilled water. Betamethasone and oxazolone were dissolved in acetone.

The oxazolone-induced chronic contact hypersensitivity response

The Balb/c mice were sensitized and challenged with oxazolone according to the method of Webb *et al.*[13] and Kitagaki *et al.*[14] Using four or eight mice in each group, the same skin site of the right ear was sensitized by a single application of 10 μL of 0·5% w/v oxazolone in acetone 7 days before the first challenge (day 0), and 10 μL of 0·5% w/v oxazolone in acetone was repeatedly applied to the sensitized right ear three times per week. In the nonsensitized animals, acetone alone was applied to the right ear. Olopatadine was orally administered at a volume of 1 mL per 100 g body weight. Betamethasone was applied at a volume of 10 μL per ear. Each drug was administered once daily. On the day of each challenge, each drug was administered 1 h before the challenge.

Experimental protocol

To investigate whether olopatadine prevents the chronic dermatitis, we employed two experimental protocols.

The preventive treatment protocol. Olopatadine was orally administered at 3 or 10 mg kg^{-1} day^{-1}, and betamethasone was topically applied at 0·012 mg ear^{-1} day^{-1} to the right ear, once daily from days 0 to 16. At 24 h after the final challenge, blood was collected for measurement of serum IgE, and the animals in each group were sacrificed to remove the ears. A 7·5-mm diameter punch biopsy specimen was taken from each ear and the swelling was assessed in terms of the weight. Each ear sample was homogenized and centrifuged, and then the

supernatant was stored at − 80 °C until the cytokine assay. In another series of experiments, the changes in the ear thickness were measured at 24-h intervals with a dial thickness gauge (PEACOCK; Model G-1A, Ozaki Corp., Tokyo, Japan). At 6 h after the final challenge, the animals in each group were sacrificed to remove the ears for semiquantification of cytokine mRNA expression. Moreover, for assessment of skin atrophy, olopatadine or betamethasone was administered to normal mice, once daily for 17 days, as described in the above preventive treatment protocol. At 24 h after the final administration, eight mice in each group were sacrificed and the ears were removed to assess the skin atrophy in terms of weight.

The therapeutic treatment protocol. Olopatadine (10 mg kg^{-1} day^{-1}) and betamethasone (0·012 mg ear^{-1} day^{-1}) were administered from days 21 to 30. The changes in ear thickness were measured at 24-h intervals with a dial thickness gauge. In addition, blood was collected for measurement of serum IgE, and the ears were removed at 24 h after the final challenge. A 7·5-mm diameter punch biopsy specimen was taken from each ear and the swelling was assessed in terms of weight. Each ear sample was homogenized and centrifuged, and then the supernatant was stored at − 80 °C until the cytokine assay.

Measurement of interferon-γ, interleukin-4, interleukin-1β, granulocyte-macrophage colony-stimulating factor and nerve growth factor in ears

Individual mouse ears were homogenized in phosphate-buffered saline (PBS; pH 7·4; ICN Biomedicals, Aurora, OH, U.S.A.) containing the protease inhibitor (Complete™; Roche Diagnostics, Mannheim, Germany) and centrifuged, and then the supernatant was used for the measurement of IFN-γ, IL-4, IL-1β, GM-CSF and NGF by specific enzyme-linked immunosorbent assay (ELISA). IL-1β and GM-CSF levels were determined using the commercial kits of sandwich ELISA from R&D SYSTEMS (Minneapolis, MN, U.S.A.). IL-4 and IFN-γ levels were determined using the commercial kits of sandwich ELISA from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). NGF levels were determined using the commercial kit of sandwich ELISA from Promega (Madison, WI, U.S.A.). The assays were performed according to the manufacturer's instructions. The optical density of each well was determined by using the microplate reader THERMOmax™ (Molecular Devices, Sunnyvale, CA, U.S.A.).

Measurement of serum IgE

Serum harvested from each blood sample was used for IgE quantification. Serum IgE levels were determined using the commercial kit of sandwich ELISA from BD Science (San Diego, CA, U.S.A.) according to the manufacturer's instructions.

Histopathological examinations

Four mice in each group were treated according to the preventive treatment protocol. The oxazolone-challenged skin of each animal was removed 24 h after the final challenge. The specimens were fixed in 10% vol. neutral buffered formalin, and then embedded in

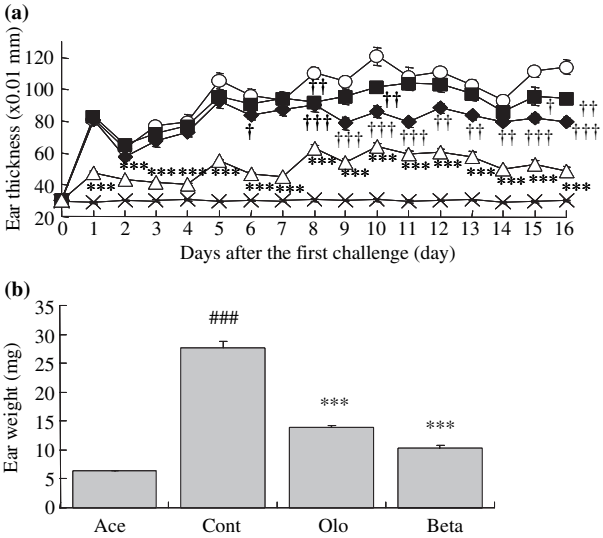

Figure 1. Preventive effects of olopatadine hydrochloride (olopatadine) and betamethasone 17-valerate (betamethasone) on the ear swelling induced by repeated application of oxazolone. Mice were sensitized on the ear with oxazolone 7 days before the first challenge, and were repeatedly challenged on the sensitized ear with oxazolone three times per week until the end of the experimental period. Olopatadine (3 and 10 mg kg^{-1} day^{-1}) was administered orally and betamethasone (0·012 mg ear^{-1} day^{-1}) was applied topically under the preventive treatment protocol, as described in Materials and methods. (a) Ear thickness was measured 24 h after each oxazolone challenge. ×, Acetone; ○, oxazolone challenge; ■, olopatadine at 3 mg kg^{-1} day^{-1}; ◆, olopatadine at 10 mg kg^{-1} day^{-1}; △, betamethasone at 0·012 mg ear^{-1} day^{-1}. Each point represents the mean ± SEM of eight mice. †$P < 0·05$; ††$P < 0·01$; †††$P < 0·001$: significantly different from the control group (Dunnett test or Steel test). ***$P < 0·001$: significantly different from the control group (Student's *t*-test or Aspin–Welch test). (b) Ear weight was measured 24 h after the final oxazolone challenge. Ace, Acetone; Ox, oxazolone challenge; Olo, olopatadine at 10 mg kg^{-1} day^{-1}; Beta, betamethasone at 0·012 mg ear^{-1} day^{-1}. Each column represents the mean + SEM of eight mice. ###$P < 0·001$: significantly different from the acetone group (Aspin–Welch test). ***$P < 0·001$: significantly different from the control group (Aspin–Welch test).

paraffin wax. Tissue sections were stained with haematoxylin and eosin for the light microscopic observation. For the identification of mast cells and eosinophils, the sections were stained with toluidine blue and fast green FCF, respectively. The preparation was observed under a light microscope (Model BX60; Olympus Optical, Tokyo, Japan).

Measurement of mRNA for interferon-γ, interleukin-4, and glyceraldehyde-3-phosphate dehydrogenase

Eight mice in each group were treated according to the preventive treatment protocol. At 6 h after the final challenge, the skin of the challenged ear was collected, and total RNA was extracted with the RNeasy® Total RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted RNA was suspended in 50 μL of RNase-free water; its concentration was determined spectrophotometrically at a wavelength of 260 nm, and the quality of the RNA was checked by electrophoresis. cDNA was synthesized by incubating 0·3 μg of total RNA with 20 μL of reaction mixture containing 0·2 μg of oligo (dT) primer using the Superscript First-strand Synthesis System (Invitrogen, New York, NY, U.S.A.). All primers and probes used in this study were designed with ABI PRISM Primer Express software (Applied Biosystems, Foster City, CA, U.S.A.) and were synthesized at Invitrogen and Qiagen, respectively. The probes were labelled with the reporter fluorescent dye, FAM (6-carboxyfluorescein), at the 5′-end and the fluorescent dye quencher, Tamra (tetramethylrhodamine), at the 3′-end. The resulting cDNA samples were then amplified by polymerase chain reaction (PCR), using the GeneAmp RNA PCR kit (Perkin Elmer, Foster City, CA, U.S.A.). Primer sets and positive cDNA controls for murine IFN-γ, IL4 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were purchased from Clontec Laboratories (Palo Alto, CA, U.S.A.). The levels of cytokine mRNA were then normalized against those of G3PDH mRNA. The primers used were IFN-γ forward primer (F) 5′-CTC AAG TGG CAT AGA TGT GGA AGA-3′, reverse primer (R) 5′-GCT CTG CAG GAT TTT CAT GTC A-3′, probe 5′-TCT CTT CTT GGA TAT CTG GAG GAA CTG GC-3′; IL4 (F) 5′-ATG TGC CAA ACG TCC TCA CA-3′, (R) 5′-AAG CAC CTT GGA AGC CCT ACA-3′, probe 5′-AAC GAA GAA CAC CAC AGA GAG TGA GCT CG-3′; G3PDH (F) 5′-TCG CTC CTG GAA GAT GGT GAT-3′, (R) 5′-CAG TAT GAC TCT ACC CAC GGC AAG-3′, probe 5′-TCA ACG GCA CAG TCA AGG CTG

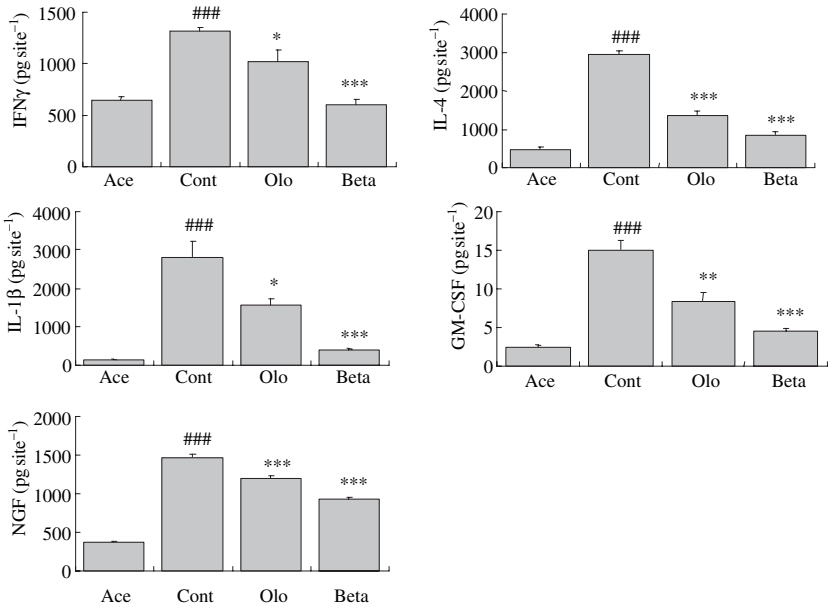

Figure 2. Preventive effects of olopatadine hydrochloride (olopatadine) and betamethasone 17-valerate (betamethasone) on the levels of interferon (IFN)-γ, interleukin (IL)-4, IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF) and nerve growth factor (NGF) in the lesioned skin subjected to repeated application of oxazolone. IFN-γ, IL-4, IL-1β, GM-CSF and NGF levels in the homogenized ear tissue were measured 24 h after the final oxazolone challenge as described in Materials and methods. Ace, Acetone; Ox, oxazolone challenge; Olo, olopatadine at 10 mg kg^{-1} day^{-1}; Beta, betamethasone at 0·012 mg ear^{-1} day^{-1}. Each column represents the mean + SEM of eight mice. ###$P < 0·001$: significantly different from the acetone group (Student's *t*-test or Aspin–Welch test). *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$: significantly different from the control group (Student's *t*-test or Aspin–Welch test).

AGA ATG-3′. Quantitative PCR was performed with reverse transcription products, TaqMan Universal PCR Master Mix (Applied Biosystems), forward primer (final, 1 μmol L^{-1}), reverse primer (final, 1 μmol L^{-1}), probe (final, 0·2 μmol L^{-1}), and distilled water in a total volume of 30 μL. PCR was performed at 50 °C for 2 min, at 95 °C for 10 min and then for 45 cycles at 95 °C for 15 s and at 60 °C for 1 min on the ABI PRISM 7700 detection system (Applied Biosystems). Because in the preliminary experiments the mRNA expression of these cytokines was found to be increased by repeated challenge with oxazolone and to peak at 6 h after the final challenge, the effect of olopatadine was evaluated at 6 h after the final challenge.

Statistical analysis

Data are presented as mean ± SEM. The Aspin–Welch test or Student's *t*-test following the *F*-test was used for analysis of differences between two groups. Multiple comparisons among treatment groups were assessed by one-way analysis of variance, followed by the Dunnett's test or the Steel test. Values of $P < 0{\cdot}05$ were considered statistically significant. All statistical calculations were performed with the Statistical Analysis System (SAS: Release 8.2; SAS Institute, Cary, NC, U.S.A.).

Results

The preventive effect on the ear swelling

Figure 1a shows the effect of olopatadine on the ear thickness response to repeated challenge with oxazolone. In the oxazolone-challenged group, the ear thickness significantly increased from day 1 throughout the experimental period. Olopatadine at 3 and 10 mg kg^{-1} day^{-1} significantly suppressed the increase in ear thickness. Betamethasone also significantly inhibited the increase in ear thickness. In the control group, the ear weight significantly increased by 4·4-fold compared with that in the acetone-treated group after the final challenge (Fig. 1b). When olopatadine was administered orally at 10 mg kg^{-1} day^{-1} from days 0 to 16, it significantly suppressed the increase in ear weight at 24 h after the final oxazolone challenge by 64·3%. Betamethasone at a dose of 0·012 mg ear^{-1} day^{-1} also significantly inhibited the increase in ear weight by 81·2%.

Because topical steroids can cause skin atrophy, we examined whether olopatadine and betamethasone caused skin atrophy in the normal ear. Repeated application of betamethasone significantly decreased the weight (acetone, 6·3 ± 0·1 mg; betamethasone, 5·4 ± 0·0 mg). On the other hand, olopatadine had no effect on the normal ear (normal, 6·7 ± 0·1 mg; olopatadine, 6·8 ± 0·1 mg).

The preventive effect on the levels of interferon-γ, interleukin-4, interleukin-1β, granulocyte-macrophage colony-stimulating factor and nerve growth factor in the lesioned ear

To elucidate the mechanism by which olopatadine inhibits the development of ear swelling, we investigated its effect on the levels of IFN-γ, IL-4, IL-1β,

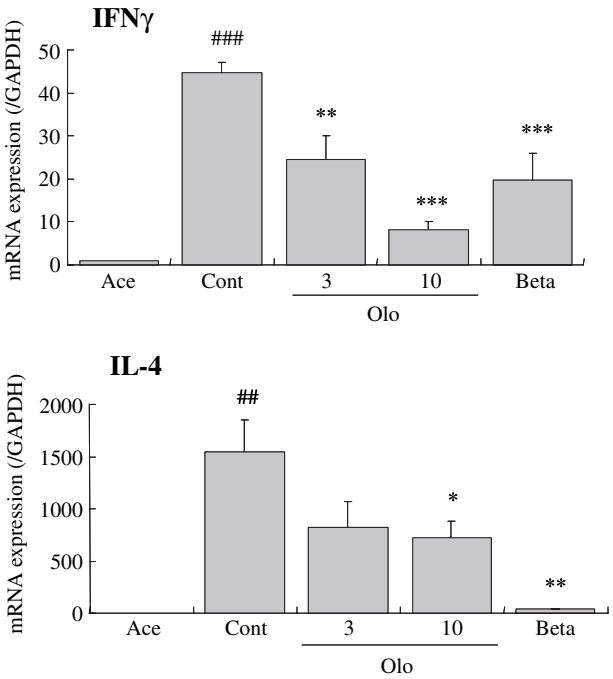

Figure 3. Preventive effects of olopatadine hydrochloride (olopatadine) and betamethasone 17-valerate (betamethasone) on the expression of interferon (IFN)-γ and interleukin (IL)-4 mRNAs in the lesioned skin subjected to repeated application of oxazolone. Olopatadine was administered orally and betamethasone was applied topically according to the preventive treatment protocol, as described in Materials and methods. Total RNA was extracted from the lesioned sites at 6 h after the final oxazolone challenge. Reverse transcription–polymerase chain reaction was performed by using specific primers for IFN-γ or IL-4. Each column represents the mean + SEM of eight mice. Ace, Acetone; Ox, oxazolone challenge; Olo 3, olopatadine at 3 mg kg^{-1} day^{-1}; Olo 10, olopatadine at 10 mg kg^{-1} day^{-1}; Beta, betamethasone at 0·012 mg ear^{-1} day^{-1}. ##$P < 0{\cdot}01$; ###$P < 0{\cdot}001$: significantly different from the acetone group (Aspin–Welch test). *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$: significantly different from the control group (Student's *t*-test or Aspin–Welch test).

GM-CSF and NGF in the lesioned ear. As shown in Figure 2, the levels of IFN-γ, IL-4, IL-1β, GM-CSF and NGF in the ears taken at 24 h after the final challenge (day 17) were significantly increased compared with those in the acetone-treated ear. The levels of IFN-γ (Th1) and IL-4 (Th2) in the lesioned ear increased by 2·1- and 6·1-fold, respectively, compared with those in the acetone-treated ear, resulting in Th2 dominance. Preventively administered olopatadine significantly inhibited the increased levels of IFN-γ, IL-4, IL-1β, GM-CSF and NGF by 43·8%, 63·9%, 47·1%, 52·8% and 24·9%, respectively. Betamethasone also significantly inhibited the increased levels of these cytokines.

The preventive effect of olopatadine on the expression of cytokine mRNAs in the lesioned ear

To investigate the possible inhibitory action of olopatadine against the cytokine increase, we determined the effect of olopatadine on the expression of IFN-γ and IL-4 mRNAs in the lesioned sites. The amount of cytokine mRNAs in the lesioned site under the preventive treatment protocol was expressed as the value relative to the amount of G3PDH mRNA. As shown in Figure 3, the expression of IFN-γ (Th1) and IL-4 (Th2) mRNAs in the lesioned site increased by 44·6- and 1931-fold, respectively, compared with those in the acetone-treated site, resulting in Th2 dominance. Preventive administration of olopatadine at 3 mg kg^{-1} day^{-1} significantly suppressed the expression of IFN-γ mRNA by 44·8%. Olopatadine at 10 mg kg^{-1} day^{-1} significantly suppressed the expression of IFN-γ and IL-4 mRNAs by 81·8% and 53·4%, respectively, resulting in the attenuated tendency toward the Th2 dominance. Betamethasone also significantly suppressed their expression.

The preventive effect on the histological change of the lesioned ear

In the pathological examination on day 17, the lesioned ear swelled markedly (Fig. 4). The epidermis exhibited prominent epidermal hyperplasia, moderate inflammatory cell infiltration (lymphocytes, neutrophils and eosinophils mainly), abscess, crust formation and erosion. In the dermis, severe inflammatory cell infiltration (neutrophils, eosinophils, monocytes and mast cells mainly), slight oedema and moderate fibrosis were observed. Olopatadine and betamethasone ameliorated these pathological changes. In the sections stained by toluidine blue or fast green FCF, it was shown that the number of mast cells and eosinophil infiltration were decreased by these drugs.

The therapeutic effect on the ear swelling

Figure 5 shows the therapeutic effect of olopatadine on the ear swelling response when it was administered orally at 10 mg kg^{-1} day^{-1} from days 21 to 30. Olopatadine significantly suppressed the increases in ear thickness (Fig. 5a) and ear weight (Fig. 5b) by

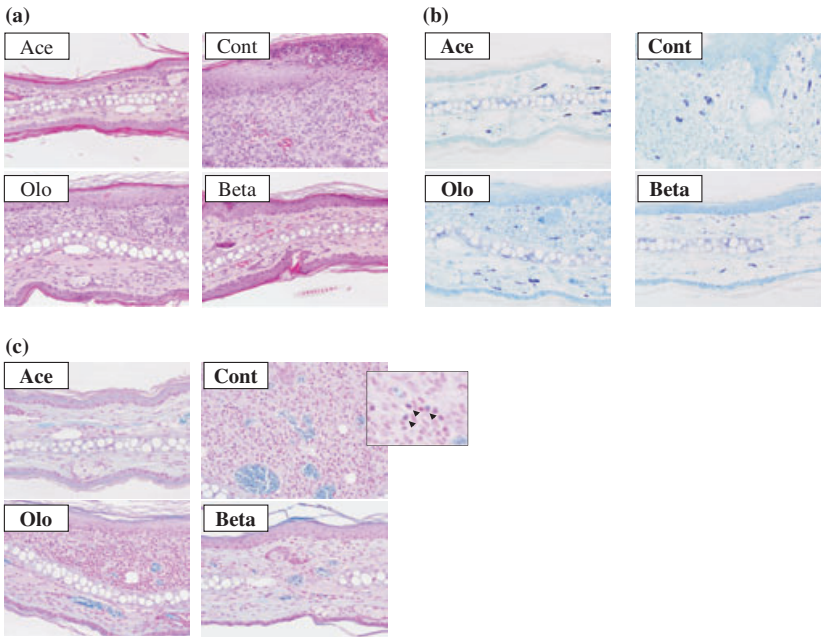

Figure 4. Photographs of the skin following repeated application of acetone or oxazolone. Olopatadine was administered orally and betamethasone was applied topically according to the preventive treatment protocol, as described in Materials and methods. Ears were excised 24 h after the last application of oxazolone. Skin sections were stained with haematoxylin and eosin (A), toluidine blue (B) and fast green FCF (C). Ace, Acetone; Ox, oxazolone challenge; Olo, olopatadine at 10 mg kg^{-1} day^{-1}; Beta, betamethasone at 0·012 mg ear^{-1} day^{-1}. Magnification for large panels: ×200; small panel: ×800. Arrowheads point to eosinophils.

18·9% and 19·4%, respectively, at 24 h after the final challenge. Betamethasone also significantly inhibited the increases in ear thickness and ear weight by 47·6% and 68·5%, respectively.

The therapeutic effect on the levels of interferon-γ, interleukin-4, interleukin-1β, granulocyte-macrophage colony-stimulating factor and nerve growth factor in the lesioned ear

As shown in Figure 6, the levels of IL-4, IL-1β, GM-CSF and NGF in the ear taken at 24 h after the final challenge (day 31) were increased compared with those in the acetone-treated ear; in contrast, the IFN-γ level was significantly decreased, resulting in Th2 dominance. Therapeutically administered olopatadine significantly reduced the increased levels of IL-4, IL-1β, GM-CSF and NGF by 33·9%, 53·2%, 43·8% and 48·4%, respectively. Betamethasone also significantly reduced these increased levels. Olopatadine and betamethasone had no effect on the decreased level of IFN-γ.

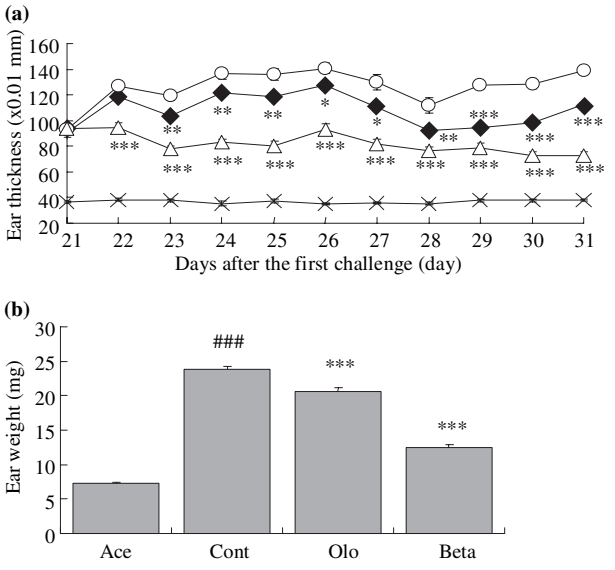

Figure 5. Therapeutic effects of olopatadine hydrochloride (olopatadine) and betamethasone 17-valerate (betamethasone) on the ear swelling induced by repeated application of oxazolone. Mice were sensitized on the ear with oxazolone 7 days before the first challenge, and were repeatedly challenged on the sensitized ear with oxazolone three times per week until the end of the experimental period. Olopatadine (10 mg kg^{-1} day^{-1}) was administered orally and betamethasone (0·012 mg ear^{-1} day^{-1}) was applied topically under the therapeutic treatment protocol, as described in Materials and methods. (a) Ear thickness was measured 24 h after each oxazolone challenge. ×, Acetone; ○, oxazolone challenge; ◆, olopatadine at 10 mg kg^{-1} day^{-1}; Δ, betamethasone at 0·012 mg ear^{-1} day^{-1}. Each point represents the mean ± SEM of eight mice. $^{*}P < 0·05$; $^{**}P < 0·01$; $^{***}P < 0·001$: significantly different from the control group (Student's *t*-test or Aspin–Welch test). (b) Ear weight was measured 24 h after the final oxazolone challenge. Ace, Acetone; Ox, oxazolone challenge; Olo, olopatadine at 10 mg kg^{-1} day^{-1}; Beta, betamethasone at 0·012 mg ear^{-1} day^{-1}. Each column represents the mean + SEM of eight mice. $^{###}P < 0·001$: significantly different from the acetone group (Aspin–Welch test). $^{***}P < 0·001$: significantly different from the control group (Student's *t*-test).

The effect on the serum IgE level

The total serum IgE levels were quantified under both the preventive and the therapeutic protocol. The total IgE level was increased by repeated challenge with oxazolone. Neither olopatadine nor betamethasone affected the increase in total serum IgE levels in spite of their inhibiting the development of ear swelling response (data not shown).

Discussion

The present study demonstrated that olopatadine, administered either preventively or therapeutically, mitigated the cutaneous inflammation in a mouse model of chronic inflammatory dermatitis induced by repeated challenge of oxazolone to the ear. Olopatadine suppressed the histological changes such as severe oedema, inflammatory cell infiltration and epidermal hyperplasia. Moreover, olopatadine attenuated the increased levels of IFN-γ, IL-4, IL-1β, GM-CSF and NGF, and suppressed the expression of IFN-γ and IL-4 mRNAs in the lesioned site. Thus, olopatadine may offer a novel regimen for treating chronic inflammatory dermatitis such as AD by inhibiting the inflammatory cytokines.

The present study demonstrated that the oxazolone-repeated challenge increased levels of both Th1 and Th2 cytokines in the lesioned skin and the more continuous oxazolone exposure led to Th2 dominance. This is in accordance with the previous study by Kitagaki *et al.*, who demonstrated that Th2 cytokine mRNA was abundant in lesioned skin in mice with the chronic contact hypersensitivity response.[14] There has been accumulating evidence indicating that both Th1 and Th2 cytokines are present in the lesioned skin of AD.[21–23] In particular, Th2 cells accumulated in the lesioned skin are thought to play a major role in the pathogenesis of dermatitis.[22] The Th2 cytokine IL-4 affects a broad spectrum of different cell types including T cells, B cells, mast cells, monocytes/macrophages, endothelial cells, fibroblasts, dendritic cells, Langerhans

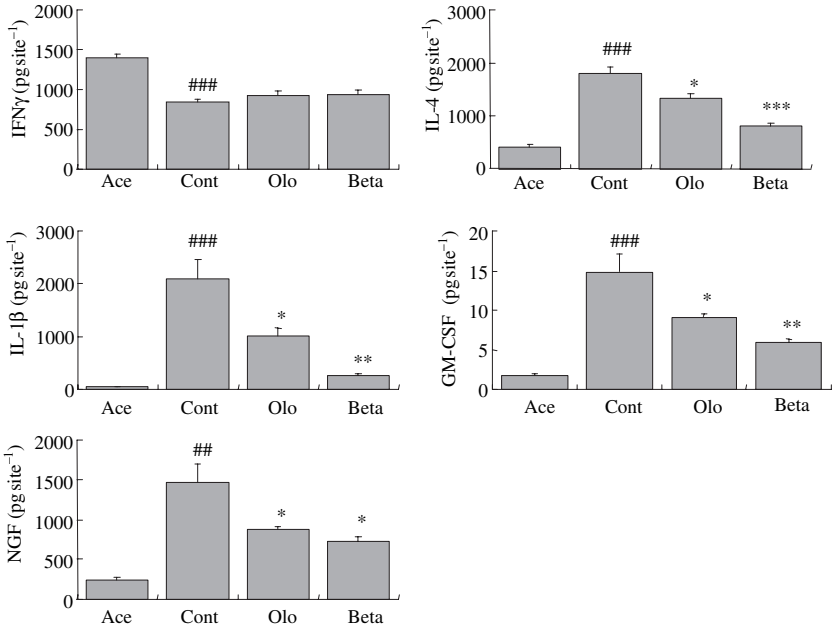

Figure 6. Therapeutic effects of olopatadine hydrochloride (olopatadine) and betamethasone 17-valerate (betamethasone) on the levels of interferon (IFN)-γ, interleukin (IL)-4, IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF) and nerve growth factor (NGF) in the lesioned skin induced by repeated application of oxazolone. IFN-γ, IL-4, IL-1β, GM-CSF and NGF levels in the homogenized ear tissue were measured 24 h after the final oxazolone challenge as described in Materials and methods. Ace, Acetone; Ox, oxazolone challenge; Olo, olopatadine at 10 mg kg^{-1} day^{-1}; Beta, betamethasone at 0·012 mg ear^{-1} day^{-1}. Each column represents the mean + SEM of eight mice. ##P < 0·01; ###P < 0·001: significantly different from the acetone group (Student's t-test or Aspin–Welch test). *P < 0·05; **P < 0·01; ***P < 0·001: significantly different from the control group (Student's t-test or Aspin–Welch test).

cells and keratinocytes, and regulates the immune response in a number of ways, thus suggesting a crucial role of IL-4 in the pathogenesis of AD.[24,25] On the other hand, the Th1 cytokine IFN-γ has been reported to be important for the development of skin hypertrophy in a murine model of dermatitis. Indeed, the dermal thickening of ovalbumin-sensitized skin was reduced in IFN-γ-deficient mice.[26] These observations suggest that the Th2 cytokines, especially IL-4, play major roles and the Th1 cytokine is also involved in the development of oxazolone-induced dermatitis in mice. The present mouse model of oxazolone-induced dermatitis is likely, at least in part, to mimic AD in humans.

In the present study, olopatadine significantly suppressed the increased levels of Th1 and Th2 cytokines, and suppressed the expression of IFN-γ (Th1) and IL-4 (Th2) mRNAs under the upregulated Th1 and Th2 responses. Moreover, olopatadine attenuated the tendency toward the Th2 dominance. Keratinocytes are one of the important sources of cytokines, and express the histamine receptor, which is one of the factors that regulate cytokine production.[27] Accordingly, the blockade by olopatadine of histamine receptors of keratinocytes may be involved in the suppression of cytokines, resulting in the improvement of the allergic dermal inflammation.

The present histopathological examination showed that the oxazolone-repeated challenge induced the increased number of dermal mast cells in the lesioned skin and that olopatadine decreased the number of dermal mast cells in the lesioned skin. Mast cells, which participate in the inflammatory cascade, serve as an abundant source of Th1 and Th2 cytokines, as well as inflammatory mediators.[10–12] On the other hand, IL-4 is known to be a potent activator of mast cells. In fact, olopatadine has been shown to inhibit the expression of IL-4 mRNA in RBL-2 mast cells,[28] suggesting a possible involvement of the inhibition of IL-4 mRNA expression in the amelioration of the lesioned skin. Thus, the mast cell is likely to be another plausible target for olopatadine to ameliorate the inflammatory responses in the present model of dermatitis.

Eosinophil infiltration has been well documented in the dermal layer of AD.[29] In this study, olopatadine inhibited the eosinophil infiltration into the lesioned skin sites. The inhibition by olopatadine of eosinophil infiltration might be due to inhibition of IL-4-mediated immunological events that promote eosinophil infiltration, such as endothelial expression of cell adhesion molecules.[30] Indeed, olopatadine is capable of inhibiting histamine-induced E-selectin and intercellular adhesion molecule-1 expression on human umbilical vein endothelial cells.[31] Moreover, olopatadine has been shown to suppress eosinophil infiltration into the airway in guinea pigs.[32] Thus, the inhibitory effect of olopatadine on eosinophil infiltration may have contributed to the amelioration of the dermatitis in this experimental model.

The increase in NGF level was attenuated by olopatadine in this study. NGF potentiates the proliferation of sensory neurones, resulting in augmented itch

sensation in inflammatory skin diseases.[33] It has been assumed that olopatadine inhibits pruritus by reducing the tachykinin release from peripheral sensory nerve endings[18] as well as by blocking the action of histamine on its receptors in the skin. The present observation suggests that the inhibition of NGF, in addition to the previously postulated mechanisms, is involved in the amelioration by olopatadine of pruritis and itch sensation.

Betamethasone is a steroid used clinically in the treatment of AD and other skin disorders based on its potent anti-inflammatory effects. The potent anti-inflammatory effect of betamethasone was confirmed in the present model of dermatitis. However, its topical use can cause intense skin atrophy, one of the side-effects limiting its chronic use for skin diseases. In the present study, indeed, repeated application of betamethasone caused the skin atrophy in the mouse ear. Betamethasone may have suppressed the ear swelling through atrophogenic effects in addition to its anti-inflammatory effects. In contrast, olopatadine exhibited the anti-inflammatory effects without the skin atrophy.

In conclusion, olopatadine was demonstrated to be an antihistamine agent having inhibitory activities against chronic inflammatory dermatitis, possibly resulting from its diminishing effect on elevated cytokines in the lesioned skin. Olopatadine may be useful as an add-on therapy, or used in combination with topical steroids may result in better efficacy or reduction of the dose of the topical steroids.

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