Topical Application of Josamycin Inhibits Development of Atopic Dermatitis-Like Skin Lesions in NC/Nga Mice

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ABSTRACT - Background: Patients with atopic dermatitis (AD) have superficial skin colonization by *Staphylococcus aureus* and an increased number of T helper type 2 (Th2) cells in their peripheral blood. Our previous study showed that josamycin, a macrolide antibiotic, had excellent bactericidal activity against *S. aureus* strains isolated from AD patients and simultaneously inhibited Th1 and Th2 cell development mediated by Langerhans cells. The purpose of the present study was to evaluate the effect of topical application of josamycin on AD-like skin lesions in NC/Nga mice. **Methods:** Josamycin (0.1%) was topically administered to NC/Nga mice with AD-like skin lesions induced by 2, 4, 6-trinitrochlorobenzene (TNCB). The therapeutic effects of josamycin were assessed by measurement of the skin severity scores, histological changes in the lesioned skin, serum levels of total IgE, and expression of interferon (IFN)-γ and interleukin (IL)-4 in lymph nodes and skin lesions. **Results:** Topical treatment with josamycin significantly suppressed the increase in the skin severity score in NC/Nga mice. This suppressive effect was equal to that of betamethasone, and was associated with a decrease in the density of cellular infiltration into the dermis, the mast cell count in the dermis and the serum IgE level. Furthermore, topical application of josamycin reduced the expression of IFN-γ and IL-4 in auricular lymph node cells and the skin lesions. **Conclusion:** The present results show that topical application of josamycin inhibits the development of AD-like skin lesions in NC/Nga mice. This suggests that topical application of josamycin to AD lesions colonized by *S. aureus* would be beneficial for control of AD by acting on superficially located *S. aureus* and by inhibiting the development of Th1 and Th2 cells.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with immunopathologic features that vary depending on the duration of the lesions. The majority of AD patients show superficial skin colonization by *Staphylococcus aureus* and increased expression of T helper type 2 (Th2) cytokines such as interleukin (IL)-4, IL-5 and IL-13 in their peripheral blood mononuclear cells (1). *S. aureus* can be isolated from 96-100% of skin lesions of AD patients, whereas only 0-10% of healthy individuals show skin colonization by this organism (2, 3). We have also found that the incidence of *S. aureus* detection in the lesioned skin of AD patients is higher than that in non-lesioned skin, and that the *S. aureus* bacterial cell count in lesioned skin is significantly higher than that in non-lesioned skin (3). Furthermore, our recent studies have demonstrated that chronic skin colonization with *S. aureus* may augment Th2 cell development in AD patients (4-6). Therefore, treatment with antibiotics has a beneficial effect in AD patients, not only those with impetiginized AD but also those without clinical signs of superinfection.

Langerhans cells (LCs) are a subpopulation of bone marrow-derived dendritic cells (DCs). They are antigen-presenting cells (APCs), capable of internalizing and processing antigen (7). Because they reside in skin epithelium, they become the primary response cells for antigens entering the skin (8). After antigen uptake, LCs migrate to regional lymph nodes where peptides, in the context of major histocompatibility complex (MHC) class II molecules, are presented to naïve Th cells bearing appropriate Th cell receptors. This initial signal delivered to naïve Th cells, together with a second signal, delivered in part by interaction between the CD80 and CD86 molecules on LCs and CD28 on Th cells, results in activation of the Th cells (9, 10). Furthermore, LCs work as the primary orchestrators in the polarization of the immune response towards

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Th1 or Th2. The nature of this polarization is influenced by a number of factors, and the development of Th2 cells, producing type 2 cytokines such as IL-4, IL-5 and IL-13, plays a particularly pivotal role in inducing allergic inflammation (11). Therefore, allergic inflammation might be controllable through regulation of LCs. In our previous study (12), we found that a macrolide antibiotic, josamycin, had excellent bactericidal activity against *S. aureus* strains isolated from skin lesions of AD patients and simultaneously inhibited LC-mediated development of Th1 and Th2 cells. These findings suggested that topical application of josamycin to skin lesions of AD patients might be beneficial for control of AD. Although josamycin is usually used for the treatment of a wide range of infections, including respiratory infections, skin infections, otorhinological infections, urinary tract infections, therapeutic effects for skin diseases including AD are unknown. Therefore, in the present study, we evaluated the effect of topical application of josamycin on AD-like skin lesions in NC/Nga mice.

MATERIALS AND METHODS

Mice

Female specific pathogen-free NC/Nga mice were obtained from Japan SLC (Hamamatsu, Japan) and used at the age of 6 weeks. They were housed in plastic cages with sterilized paper bedding in a clean, air-conditioned room at 24 °C and allowed free access to a standard laboratory diet and water. All procedures performed on the mice were in accordance with the guidelines of "Animal Care and Use Committee of Meiji Pharmaceutical University" and were approved by the committee.

Reagents

2, 4, 6-Trinitrochlorobenzene (TNCB) and betamethasone were purchased from Tokyo Chemical Industry (Tokyo, Japan). Josamycin was provided by Astellas Pharma Inc. (Tokyo, Japan). White petrolatum including 5% (w/w) liquid paraffin was used as the vehicle, and 0.1% (w/w) betamethasone ointment and 0.1% (w/w) josamycin ointment were prepared.

Induction of AD-Like Skin Lesions

The abdominal hair of NC/Nga mice was shaved, then 100 µL of 5% TNCB dissolved in an ethanol and acetone mixture (4:1) was applied topically to the abdominal skin and hind footpads. Four days after sensitization, the dorsal side of the ears and the shaved dorsal skin were challenged with 100 µL of 0.8% TNCB dissolved in an olive oil and acetone mixture (4:1). After the first challenge, 0.8% TNCB was repeatedly applied to the same area of the skin a further 5 times at intervals of 1 week. The design of the experimental schedule is summarized in Fig. 1.

Measurement of Skin Severity Score

The severity of dermatitis was assessed macroscopically according to the scoring system described below. One skin lesion on each ear, and one on the back, were scored on the basis of the following criteria (13). The dermatitis score (minimum 0; maximum 30 [= 3 regions × 2 points × 5 symptoms]) was defined as the sum of the individual scores for the three regions, and graded as 0 (no symptoms), 1 (less than 1/3 of the skin area) or 2 (1/3 and more of the skin area), for each of the following 5 symptoms: redness / scratch marks, edema / lichenification / thickening, hemorrhage / scabbing, erosion, and desquamation.

Topical Application of Betamethasone and Josamycin to NC/Nga Mice

After 4 days of the second 0.8% TNCB challenge, vehicle, 0.1% betamethasone ointment and 0.1% josamycin ointment were applied topically to the dorsal side of the ears and the dorsal skin (50 mg/body) in each group once per day, except Sunday and the day of 0.8% TNCB challenge, for a total of 29 days. The design of the experimental schedule is summarized in Fig. 1.

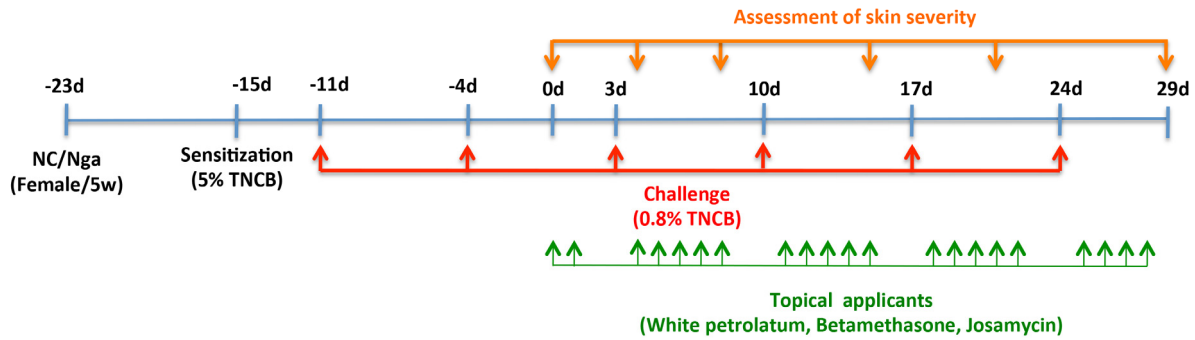

Figure 1. Experimental schedule for induction of AD-like dermatitis in NC/Nga mice and treatment with betamethasone and josamacin ointment.

Histopathological Observations

The dorsal skin was removed 29 days after assessment of skin severity, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 5 μm. Tissue sections were then stained with haematoxylin-eosin (HE) and toluidine blue (TB), respectively. The number of mast cells was counted in 5 high-power fields (HPF) selected randomly at ×400 magnification.

Measurement of Serum Total IgE Levels

Blood specimens were collected from the heart 29 days after assessment of skin lesion severity. The concentration of total IgE in serum was measured by enzyme-linked immunosorbent assay (ELISA) using a mouse IgE ELISA kit (Cedarlane, Ontario, Canada).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To determine the levels of expression of interferon (IFN)-γ and IL-4 mRNA in auricular lymph node cells and dorsal skin lesions, total RNA was extracted from each at 29 days after assessment of skin severity by the single-step method using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA). cDNA was then synthesized from 2 μg of total RNA using a first-strand cDNA synthesis kit (Takara, Shiga, Japan). PCR was performed using the following primers: β-actin (540 bp): 5' primer, 5'-GTGGGCCGCTCTAGGCACCAA-3' and 3' primer, 5'-CTCTTTGATGTCACGCACGATTTC-3'; IFN-γ (405 bp): 5' primer, 5'-GCTACACACTG CATCTTGGCTTTG-3' and 3' primer, 5'-CACT CGGATGAGCTCATTGAATGC-3'; IL-4 (400 bp): 5' primer, 5'-AGTTGTCATCCTGCTCTTCTT TCTC-3' and 3' primer, 5'-CGAGTAATCCAT TTGCATGATGCTC-3'. Each PCR was performed using a GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, CT, USA) in 25 μL of reaction mixture comprising 1.5 μL cDNA (corresponding to 200 ng total RNA starting material), 200 μM deoxynucleotide triphosphate mixture, 400 nM each PCR primer and 25 U/mL Ex Taq DNA polymerase (Takara, Shiga, Japan). The reaction conditions were as follows: one 4-min cycle at 94 °C, 35 cycles comprising 45 s at 94 °C, 45 s at 61 °C and 2 min at 72 °C, followed by one 7-min cycle at 72 °C, and the PCR products were separated on a 2% agarose gel containing ethidium bromide.

Quantification of Th1 and Th2 Cytokine Production from T Lymphocytes in Lymph Nodes

Auricular lymph node cells were harvested on the 29th day of assessment of skin severity, and adjusted to 1×10^6 cells/mL in RPMI 1640 medium with L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Sigma-Aldrich), 25 mM Hepes (Sigma-Aldrich), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco RBL, Grand Island, NY, USA). The cultures (0.2 mL/well) were incubated in 96-well culture plates (Nunc, Roskilde, Denmark) in the presence of Dynabeads® Mouse T-Activator CD3/CD28 (Life Technologies, Oslo, Norway) at 37°C in a humidified atmosphere with 5% CO_2. The culture supernatants were collected after incubation for 48 h, and the IFN-γ and IL-4 concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits for quantification of murine IFN-γ and IL-4, respectively (R & D Systems, Minneapolis, MN, USA).

Immunohistological Analysis

Immunohistochemical staining for IFN-γ and IL-4 was performed on sections of dorsal skin at 29 days after assessment of skin severity. The skin specimens were embedded and frozen with OCT compound (Miles, Elkhard, IN, USA) at –80 °C. Frozen sections cut at a thickness of 6 μm were fixed in cold acetone (–20 °C) for 10 min, treated with blocking buffer (10% normal rat serum in PBS), and incubated with biotinylated rat anti-mouse monoclonal antibodies against IFN-γ (clone XMG1.2, IgG1) or IL-4 (clone BVD6-24G2, IgG1) (Bio-Rad, Hercules, CA, USA) at a dose of 2 μg/mL. After being washed with PBS, the skin sections were incubated with peroxidase-conjugated streptavidin (Dako, Carpenteria, CA, USA). Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in PBS. Endogenous biotin was blocked by sequential incubations with avidin (Vector Laboratories, Burlingame, CA) and biotin (Sigma-Aldrich). The tissue sections were stained by incubation in a solution of 3, 3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and treated with methyl green solution for visualization of nuclei.

STATISTICAL ANALYSIS

The data were expressed as means (± SD). The results in each group were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Therapeutic Effects of Topical Application of Josamycin on TNCB-Induced AD-Like Skin Lesions

Assessment of skin lesion severity in NC/Nga mice sensitized with 5% TNCB was started 4 days after the second challenge with 0.8% TNCB (Fig. 1). The clinical skin lesion severity in the TNCB-sensitized mice increased gradually with time (Fig. 2). All mice in the positive control group (TNCB only) exhibited AD-like skin lesions comprising redness/scratch marks, edema / lichenification / thickening, hemorrhage / scabbing, erosion and desquamation (Fig. 3). However, in the negative control mice (untreated), no superficial lesions were observed throughout the experimental period. After 8 days of assessment, the therapeutic efficacy of 0.1% josamycin ointment became apparent and persisted throughout the experimental period, its efficacy being approximately equal to that of 0.1% betamethasone ointment (Figs. 2 and 3). However, topical application of vehicle only had no effect on the development of dermatitis.

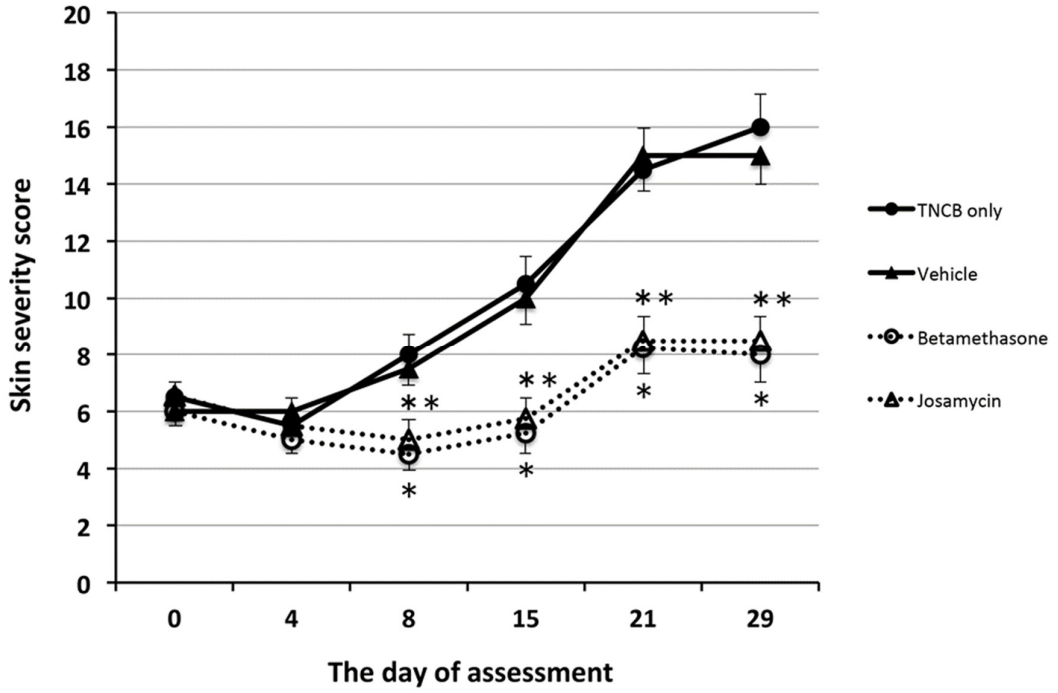

Figure 2. Effects of topical application of betamethasone and josamacin on skin severity score. The results for each experimental group are expressed as means ± SD (n = 6). **P*<0.01, ***P*<0.01 versus TNCB only.

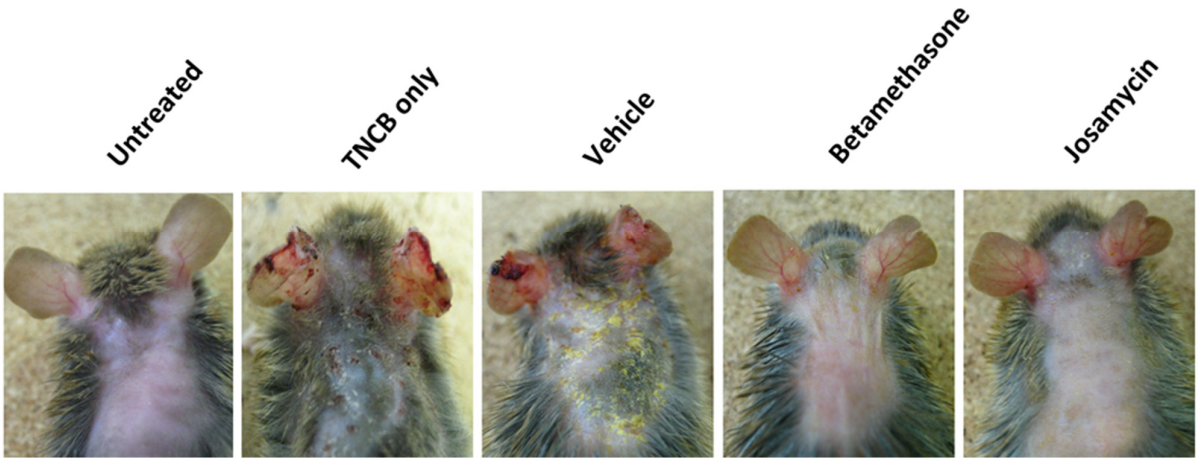

Figure 3. Macroscopic features of AD-like skin lesions in NC/Nga mice. The photograph shows skin lesions on the 29th day of assessment.

Effects of Topical Application of Josamycin on Histopathological Changes in Dorsal Skin

As shown in Fig. 4A, in the dorsal skin of negative control mice (untreated), no abnormal histopathological changes were observed. However, positive control mice (TNCB only) showed epidermal hyperplasia and dense infiltration of inflammatory cells such as mast cells, eosinophils and lymphocytes, similar to that in skin lesions of AD patients. , topical application of vehicle only had no influence on these histopathological changes, topical application of 0.1% josamycin ointment had an inhibitive effect. Furthermore, the level of inhibition elicited by josamycin was equal to that of betamethasone. Specifically, there was a clear increase in the number of mast cells in positive control mice, and this was significantly reduced by application of josamycin and betamethasone, but not vehicle only (Fig. 4B and Fig. 5).

Effects of Topical Application of Josamycin on Serum Total IgE Levels

Serum total IgE levels on the 29th day of assessment of skin lesion severity were significantly elevated in positive control mice (TNCB only) (Fig. 6). Although the increased concentration of IgE in serum was not reduced by topical application of vehicle, it was reduced significantly by application of betamethasone and josamycin, respectively.

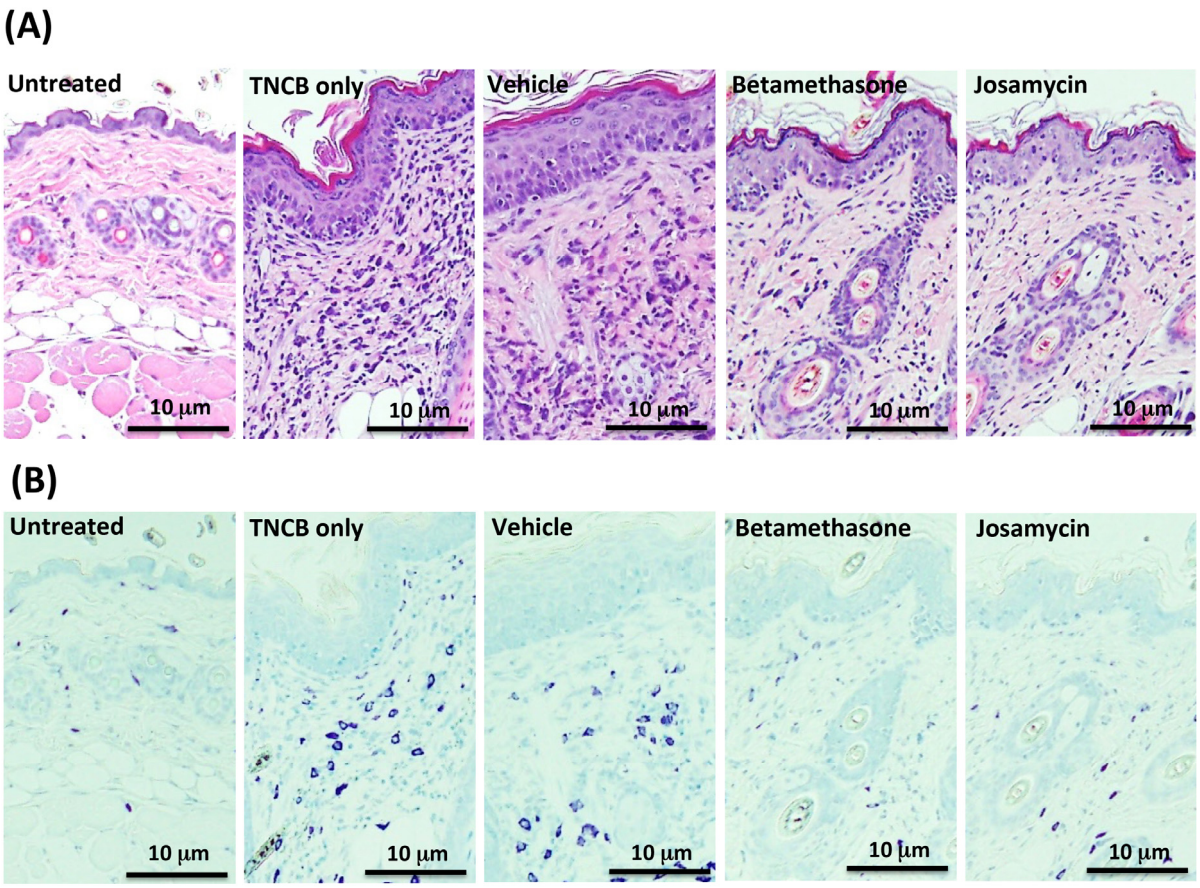

Figure 4. Histopathological analysis of AD-like skin lesions in NC/Nga mice. Skin sections were stained with haematoxylin-eosin (A) and toluidine blue (B) and observed at ×100. The photograph shows sections of skin lesions on the 29th day of assessment.

Effects of Topical Application of Josamycin on Expression of IFN-γ and IL-4

The expression of mRNA for IFN-γ and IL-4 in auricular lymph node cells and dorsal skin of negative control mice (untreated) was below the detection limit (Fig. 7A, B). However, positive control mice (TNCB only) showed expression of IFN-γ and IL-4 mRNA in both lymph node cells and skin. Although topical application of vehicle only had no influence on this mRNA expression, topical application of 0.1% josamycin ointment inhibited it. Similar inhibition of IFN-γ and IL-4 mRNA

expression was also observed in mice treated with 0.1% betamethasone. In addition, using ELISA and immunohistological analysis, we examined whether expression of mRNA for IFN-γ and IL-4 in lymph node cells and skin lesions was correlated with the synthesis of cytokine protein. ELISA for IFN-γ and IL-4 was carried out using culture supernatants of lymph node cells stimulated through surface CD3/CD28 molecules for 48 h. Fig. 7C shows that IFN-γ and IL-4 production from lymph node cells was significantly increased by TNCB treatment and suppressed by treatment with josamycin as well as betamethasone, but not by vehicle. Fig. 7D shows that TNCB treatment induced cells that were positive for IFN-γ and IL-4 protein in the dermis, and that josamycin suppressed the infiltration of these cells to a similar extent as betamethasone, but not vehicle. HE staining of the skin suggested that the possible source of each cytokine was mainly T lymphocytes (data not shown).

DISCUSSION

Th1/Th2 immune balance is closely related to various immunological diseases, including allergy. Many investigators have demonstrated that Th2 immunity is responsible for allergic immune responses and subsequent pathogenesis of allergic inflammation diseases (14-16). AD is one such allergy-related disease, and AD patients show a marked increase in the number of Th2 cells in both peripheral blood and acute skin lesions (1). Therefore, it has been proposed that the Th2 immune response plays a key pathogenetic role in AD, and this is supported by the presence of blood eosinophilia and enhanced serum IgE levels in most AD patients (17, 18). However, no immunoregulatory method for prevention of Th2 cell development in AD patients has yet been established.

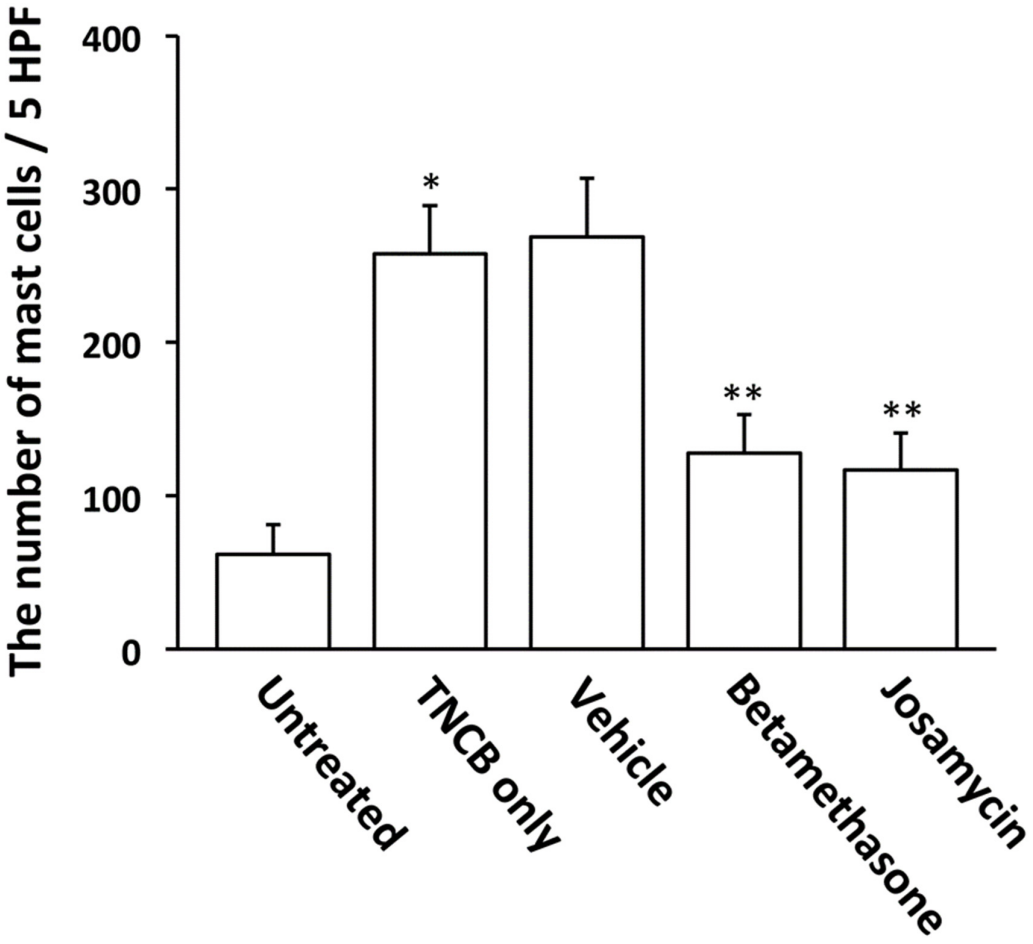

Figure 5. Quantification of mast cells in the dermatitis of NC/Nga mice with AD-like skin lesions. Dermal sections of skin lesions on the 29th day of assessment were observed at ×400 and the number of mast cells was counted in 5 randomly chosen visual fields. The results for each experimental group are expressed as means ± SD (n = 6) of the number of mast cells per 5 high-power fields (HPF). *P<0.01 versus untreated, **P<0.01 versus TNCB only.

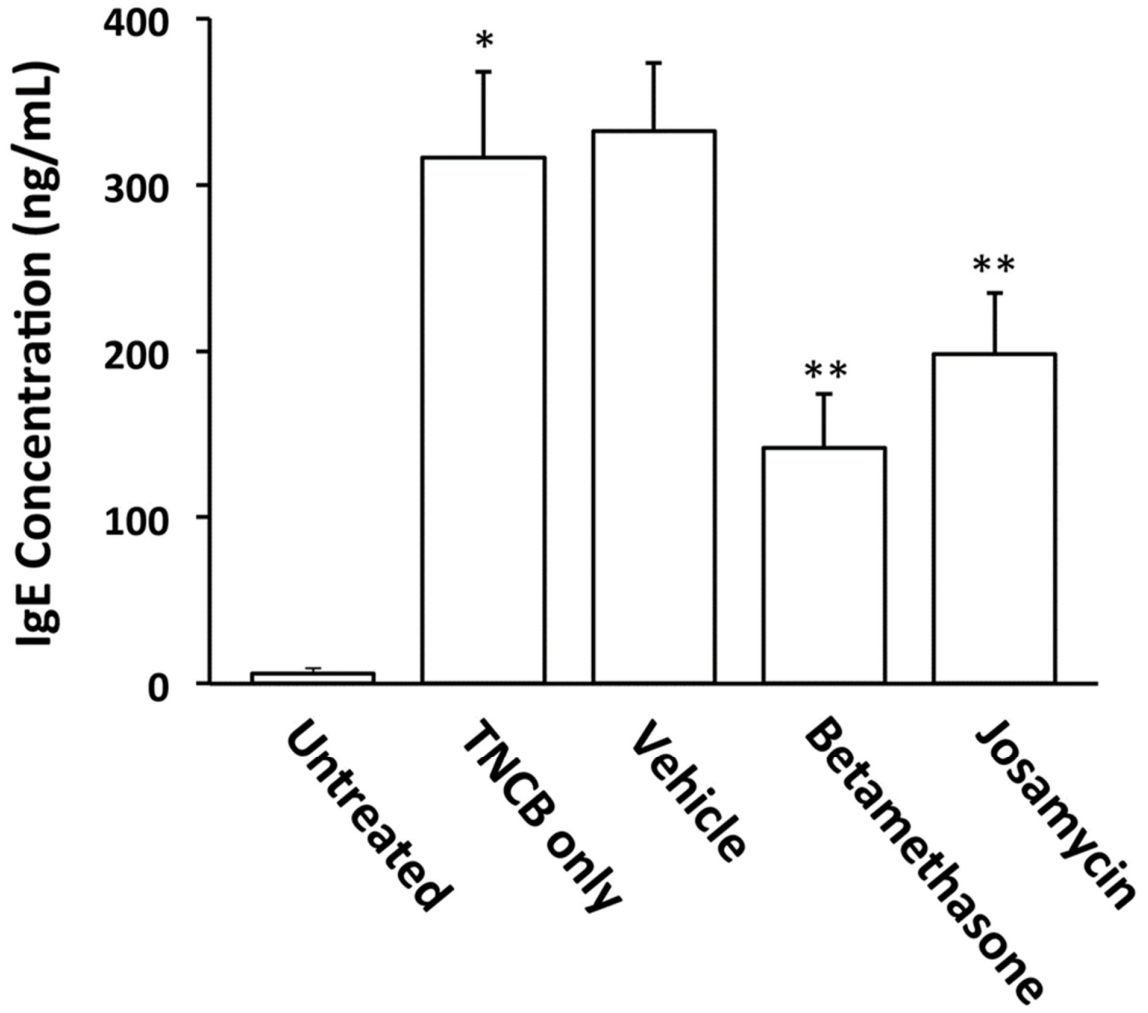

Figure 6. Quantification of IgE concentration in the serum of NC/Nga mice with AD-like skin lesions. The serum total IgE levels on the 29th day of assessment were measured by ELISA. The results for each experimental group are expressed as means ± SD (n = 6). **P*<0.01 versus untreated, ***P*<0.01 versus TNCB only.

In the present study, we demonstrated that topical application of josamycin markedly ameliorated AD-like skin lesions in NC/Ng mice. In comparison to the vehicle control group, skin lesion severity assessed macroscopically in terms of redness / scratch marks, edema / lichenification / thickening, hemorrhage/scabbing, erosion and desquamation was significantly decreased by application of 0.1% josamycin ointment as well as 0.1% betamethasone ointment, the latter having been used widely in Japan for topical treatment of AD. These findings were also supported by histopathologic analysis. Topical application of josamycin inhibited epidermal hyperplasia and dense infiltration of inflammatory cells such as mast cells, eosinophils and lymphocytes as effectively as betamethasone. The decrease in the number of mast cells was particularly noticeable. Our previous study (12) showed that LCs treated with josamycin inhibited Th2 cell development in lymph nodes through down-regulation of Jagged 1 and T-cell immunoglobulin and mucin domain-containing protein (TIM)-4 expression in LCs. Therefore, it was thought that topical application of josamycin to skin lesions of NC/Nga mice targeted LCs in the epidermis, which then moved to lymph nodes, where Th2 cell development and subsequent IL-4 production were down-regulated. Furthermore, TNCB treatment induced expression of the Th1 cytokine, IFN-γ, in the lymph nodes of NC/Nga mice, and its expression was inhibited by the topical application of josamycin. This might also explain the results of our previous study (12) in which LCs treated with josamycin showed inhibition of not only Th2 cell development but also Th1 cell development in lymph nodes through down-regulation of CD86.

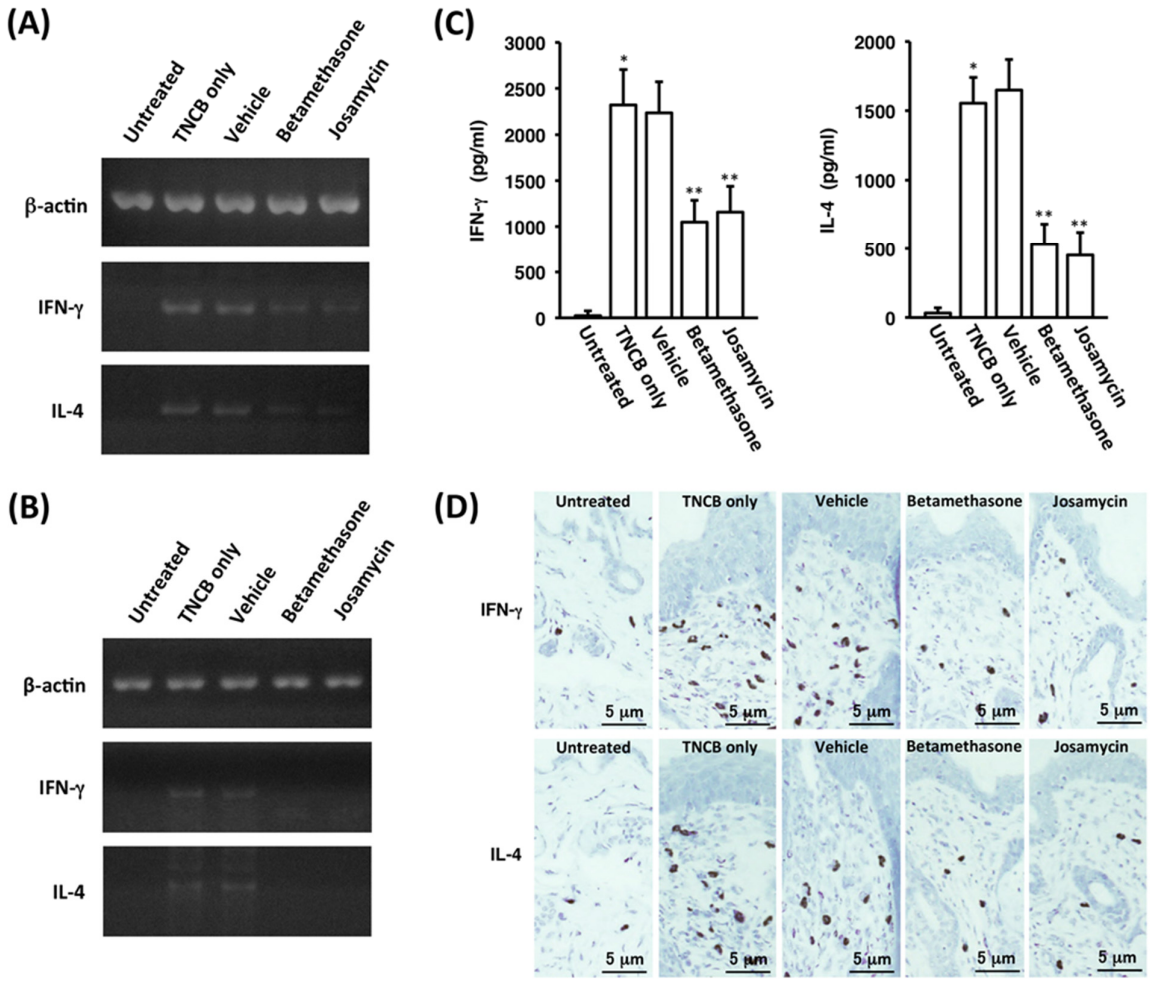

Figure 7. Effects of topical application of betamethasone and josamacin on IFN-γ and IL-4 expression in auricular lymph node cells and skin lesions of NC/Nga mice with AD-like skin lesions. mRNA was extracted from auricular lymph node cells (A) and skin lesions (B) of NC/Nga mice on the 29th day of assessment of skin severity, reverse-transcribed and amplified by PCR using primer sets for β-actin, IFN-γ and IL-4. The data shown are representative results of six independent experiments. (C) Auricular lymph node cells on the 29th day of assessment of skin severity were stimulated through their surface CD3/CD28 molecules, and the IFN-γ and IL-4 concentrations in the culture supernatants were determined by ELISA. Each culture was prepared in triplicate, and the mean value was obtained as a representative result for one experiment. The results are expressed as means ± SD (n = 6). **P*<0.01 versus untreated, ***P*<0.01 versus TNCB only. (D) Skin sections on the 29th day of assessment of skin severity were stained with biotinylated monoclonal antibodies against IFN-γ and IL-4, and observed at ×100. Positive staining of cells is indicated by dark brown coloration.

On the other hand, elevation of IFN-γ and IL-4 expression in TNCB-treated NC/Nga mice was observed in skin lesions, similarly to human AD lesions (17), and topical application of josamycin inhibited the expression of both IFN-γ and IL-4. It seems that the levels of these cytokines might mirror those in lymph nodes, as the Th1 cell marker, CCR5, and its ligand CCL3/macrophage inflammatory protein (MIP)-1α, are strongly expressed in skin lesions of NC/Nga mice (19, 20). Furthermore, the Th2 cell marker, CCR4, and its ligands CCL17/thymus and activation-regulation chemokine (TARC) and CCL22/monocyte-derived chemotactic cytokine (MDC), are also highly expressed in AD skin lesions. Therefore, Th1 and Th2 cells in the skin lesions of TNCB-treated NC/Nga mice would be derived from lymph node cells. It is known that the Th2 cytokine response is dominant in the acute phase of AD, and also in the late phase the Th1 cytokine response is increased in addition to the Th2 cytokine response, contributing to chronic inflammation (1, 17, 21). These facts indicate that topical application of josamycin can regulate both acute and chronic inflammation, and this would contribute to amelioration of AD-like skin lesions in NC/Ng mice.

Patients with AD show increased serum levels of IgE (70-80% of patients), which are associated with disease severity (1). Elevation of serum IgE levels was also observed in TNCB-treated NC/Nga mice, and topical application of josamycin significantly reduced the serum IgE concentration as effectively as betamethasone. IL-4 receptor-mediated signaling in B cells is essential for induction of IgE synthesis (22). Therefore, elevation of serum IgE levels in TNCB-treated NC/Nga mice and its inhibition by topical application of josamycin could be explained by the degree of IL-4 expression in lymph nodes.

The data from this study suggest that josamycin may have excellent ability to inhibit Th1 and Th2 cell development in AD patients. Furthermore, our previous study showed that *S. aureus* strains isolated from the lesional skin of AD patients were susceptible to josamycin (12). Since the skin of most AD patients shows superficial *S. aureus* colonization and barrier disruption due to a decrease of filaggrin (23), bacterial products such as staphylococcal enterotoxins, lipoteichoic acid and peptidoglycan would be expected to penetrate the skin and induce the production of Th2 cells and chemokines, which in turn would induce a Th2 immune response and augment skin inflammation (1, 4-6, 24-26). Therefore, topical application of josamycin to the lesioned skin of AD patients appears to exert an excellent effect involving a bactericidal action against *S. aureus* and inhibition of the Th2 immune response in lesioned skin by inhibiting the development of LC-mediated allergen-specific Th2 cells, unlike immunosuppressants such as tacrolimus, or steroids (12). Since it is thought that topical application of josamycin to the skin has few side effects, it might be possible to increase its concentration in ointment to a level that would more strongly inhibit Th2 cell development in AD patients and the subsequent Th2 immune response in AD lesions.

CONCLUSUIONS

Our results demonstrated that topical application of josamycin inhibits the development of AD-like skin lesions and the Th1/Th2 immune response in NC/Nga mice. Thus, topical administration of josamycin might be beneficial and preferable to betamethasone as a new therapeutic strategy for AD lesions with superficial *S. aureus* colonization.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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