Effects of Josamycin on Scratching Behavior in NC/Nga Mice with Atopic Dermatitis-Like Skin Lesions

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Our previous study showed that chronic skin colonization by Staphylococcus aureus exacerbated atopic dermatitis (AD) and that control of such skin colonization using antibiotic ointment might relieve AD-related skin inflammation. However, the role of S. aureus colonization in the pruritus accompanying AD was not elucidated. The aim of the present study was to evaluate the effect of topically applied josamycin, a macrolide antibiotic, on the scratching behavior of NC/Nga mice with AD-like skin lesions. Josamycin (0.1%) was topically administered to NC/Nga mice with AD-like skin lesions induced by a mite antigen, Dermatophagoides farinae extract, and the therapeutic effects of josamycin were assessed by measurement of the skin severity score, S. aureus colonization, scratching count, and interleukin (IL)-31 mRNA expression in the skin lesions. Topical treatment with josamycin ointment significantly suppressed the increase of the skin severity score in NC/Nga mice. This suppressive effect was associated with decreases in the S. aureus count on the lesioned skin, scratching behavior of mice and IL-31 mRNA expression in the lesions. The present results show that the severity of AD-like skin inflammation in NC/Nga mice is correlated with the level of S. aureus colonization and subsequent IL-31 production in the skin. Therefore, topical application of josamycin to AD lesions colonized by S. aureus would be beneficial for control of AD by eliminating superficially located S. aureus and by suppressing the IL-31-induced scratching behavior.

Key words josamycin; atopic dermatitis; Staphylococcus aureus; interleukin (IL)-31; pruritus

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with immunopathologic characteristics that change depending on the lesional stage, and pruritus is one of significant symptoms. The sustained scratching behavior elicits an itch-scratch cycle in which the skin barrier is destroyed and skin inflammation is exacerbated. Thus, regulation of this itch-scratch cycle can be an effective strategy for preventing exacerbation of AD. Furthermore, most AD patients show superficial skin colonization by Staphylococcus aureus and increased expression of T helper type 2 (Th2) cytokines in their peripheral blood mononuclear cells.1) S. aureus is isolated from 96–100% of AD skin lesions, whereas only 0–10% of healthy subjects show skin colonization by this bacterium.2,3) We have also observed that the rate of S. aureus detection on the lesional skin of AD patients is higher than that on non-lesioned skin, and that the S. aureus cell count on lesional skin is significantly higher than that on non-lesioned skin.3) In addition, our recent studies have shown that chronic skin colonization by S. aureus augments Th2 cell development in AD patients.4–6) Therefore, it is expected that antibiotic treatment would be beneficial not only for patients with impetiginized AD but also those without clinical signs of bacterial infection.

We have previously found that a macrolide antibiotic, josamycin, exerts strong bactericidal activity against S. aureus strains isolated from AD skin lesions and simultaneously inhibits Th2 cell development mediated by Langerhans cells (LCs).7) It is well known that Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-31 play a key pathogenetic role in AD, and this is supported by the presence of excess serum immunoglobulin E (IgE) levels, blood eosinophilia and strong pruritus, respectively, in most AD patients.1,8) Therefore, our findings suggested that topical application of josamycin to lesioned skin of AD patients would be therapeutically beneficial. Josamycin is already used for treatment of a wide range of bacterial infections, including those of the respiratory tract, skin, nose and throat, and urinary tract. However, the therapeutic effects of josamycin for pruritus in AD have not been verified. In the present study, we evaluated the effect of topically applied josamycin on AD-like skin lesions of NC/Nga mice focusing on control of S. aureus colonization of the lesioned skin and scratching behavior.

MATERIALS AND METHODS

Mice Female 6-week-old specific pathogen-free NC/Nga mice were purchased from Japan SLC (Hamamatsu, Japan) and used at the age of 7 weeks. They were kept in plastic cages with sterilized paper bedding in a clean, air-conditioned room at 24°C and provided with a standard laboratory diet and tap water. All procedures performed on the mice were in accordance with the guidelines of the Animal Care and Use Committee of Meiji Pharmaceutical University, Tokyo.

Reagents A mite antigen, Dermatophagoides farinae extract (Biostir AD), was purchased from Biostir Inc. (Osaka, Japan). Josamycin was purchased from Adipogen (Liestal, Switzerland). White petrolatum including 5% (w/w) liquid paraffin was used as the vehicle, and 0.1% (w/w) josamycin ointment was prepared. A recombinant murine IL-31 was obtained from Peprotech (Rocky Hill, NJ, U.S.A.).

Induction of AD-Like Skin Lesions The abdominal hair of NC/Nga mice was shaved off with a hair clipper, then removed with a depilatory cream, and the skin barrier was disrupted by topical application of 150 µL of 4% sodium do-

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schedule is summarized in Fig. 1. 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 previously. Briefly, one skin lesion on each ear (1 cm² skin) and one on the back (8 cm² skin) were scored on the basis of the following criteria. 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: 1) redness/scratch marks, 2) edema/lichenification/thickening, 3) hemorrhage/scabbing, 4) erosion, and 5) desquamation.

Topical Application of Josamycin to NC/Nga Mice Eight days after the first mite antigen sensitization, vehicle and 0.1% josamycin ointment were applied topically to the dorsal side of the ears and the dorsal skin (50 mg/body [=50 mg/1 cm² skin]) in each group once per day, except Sunday, for a total of 21 d. The design of the experimental schedule is summarized in Fig. 1.

Detection of S. aureus Skin Colonization Bacterial isolates were obtained from each skin lesion by applying a “Film Stamp” for 10 s to the affected dorsal skin (8 cm² area [=2 × 4 cm]). After an overnight culture at 37°C, the colonies grown on the tryptic soy agar plates were characterized according to their color and diameter, and the colony numbers were expressed as colony forming units (CFUs) per 8 cm² skin area. Microscopic examination of Gram-stained colonies and the PS Latex (Eiken Chemical, Tokyo, Japan) slide agglutination test were also carried out to identify these organisms. The final identification of S. aureus was based on the reaction profile obtained from the 20 biochemical tests included in the API STPH system (Biomérieux, Marcy-l’Etoile, France).

Measurement of Scratching Behavior of NC/Nga Mice Scratching behavior of NC/Nga mice was assessed for 24 h (17:00 on the day before assessment of skin severity–17:00 on the day of assessment) to judge the therapeutic effect of josamycin ointment on AD-associated pruritus. For individual assessment of the scratching count, a small magnet (1.0 mm diameter, 3.0 mm length) was implanted subcutaneously into the instep of both hind paws. The mouse was then placed in an observation chamber surrounded by a round coil. The electric current induced in the coil by movement of the magnets in the hind paws, which is associated with scratching behavior, was amplified and recorded. Scratching behavior (over 1.0 s duration) was automatically detected and objectively evaluated using MicroAct (Neuroscience, Tokyo, Japan).

Relative Expression of IL-31 mRNA Total RNA was extracted from the affected dorsal skin of each mouse by the single-step method using TRI-Reagent (Molecular Research Center, Cincinnati, OH, U.S.A.). The cDNA was then synthesized from the RNA using a first-strand cDNA synthesis kit (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). Relative real-time PCR was performed with SYBR Green Master Mix using an Applied Biosystems 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). The sequences of the primers were as follows: β-actin, 5’-TGA CAGGATGCAAGAAGA-3’ and 5’-GCTGGAGGTTGGACAAG-3’; IL-31, 5’-ATA CAGCTGCGGAGTTC-3’ and 5’-GGCATTACCCACGAA-3’.

Relative expression levels were calculated by the relative standard curve method as outlined in the manufacturer’s technical handbook. A standard curve was generated by using the fluorescence data from four-fold serial dilutions of cDNA samples. This was then used to calculate the relative amounts of IL-31 mRNA in skin samples, which were normalized to the corresponding β-actin mRNA in each sample.

Detection of IL-31 Protein in the Skin The skin specimen (1 × 1 cm) was homogenized in 200 µL phosphate buffered saline (PBS) and a extract was obtained. The concentration of IL-31 in the extract was measured using an enzyme-linked immunosorbent assay (ELISA) kit for quantification of murine IL-31 (Abcam, Cambridge Biomedical Campus, Cambridge, U.K.).

Statistical Analysis The data were expressed as means (± standard error of the mean (S.E.M.)), and differences between means were analyzed by the Tukey–Kramer multiple comparison test. Differences at $p < 0.05$ were considered to be statistically significant.
RESULTS

Therapeutic Effects of Topically Applied Josamycin on Mite Antigen-Induced AD-Like Skin Lesions  
Assessment of lesion severity in NC/Nga mice sensitized with a mite antigen and medication with ointment was started 8 d after the first sensitization with the antigen (Fig. 1). The clinical severity of the skin lesions increased gradually with time (Fig. 2). All mice in the positive control (non-treatment) group exhibited AD-like skin lesions comprising redness/scratch marks, edema/lichenification/thickening, hemorrhage/scabbing, erosion and desquamation (Fig. S1). After 8 d of assessment, the therapeutic effect of 0.1% josamycin ointment became clear and persisted throughout the experimental period. However, topical application of vehicle only had no effect on the development of dermatitis. Histopathological analysis on the 21st day of assessment of skin severity showed that topical application of 0.1% josamycin ointment inhibited dense infiltration of inflammatory cells in the dermis (Fig. S2a). This inhibitory effect was specifically remarkable in terms of the number of mast cells (Fig. S2b).

The CFU counts of S. aureus per 8 cm² area of dorsal skin in the above NC/Nga mice are shown in Fig. 3. The S. aureus CFU count on the lesioned skin of the mite antigen-sensitized mice increased gradually along with the skin severity score (Fig. 2). After 8 d of assessment, topical application of 0.1% josamycin ointment to the mite antigen-sensitized mice continued to significantly suppress the skin CFU counts. However, topical application of vehicle only had no effect on the skin CFU values for the mite antigen-sensitized mice.

Effects of Topically Applied Josamycin on Scratching Behavior of Mite Antigen-Sensitized Mice  
To estimate the involvement of scratching behavior in the development of skin lesions, scratching counts were also assessed. The 24 h-scratching counts in the mite antigen-sensitized mice increased gradually with time along with the increase in the skin severity score (Fig. 2), whereas topical application of 0.1% josamycin ointment suppressed these parameters up to the 21st day of assessment (Fig. 4). However, topical application of vehicle only had entirely no effect on the scratching behavior.

Next, to investigate the correlation between S. aureus colonization of lesioned skin and scratching behavior, IL-31 gene expression was evaluated by relative real-time PCR. In the lesioned skin of the mite antigen-sensitized mice, the IL-31 transcripts showed a daily increase (Fig. 5). After 8 d of assessment, topical application of 0.1% josamycin ointment showed a tendency to suppress the expression of IL-31 mRNA in the skin, and this suppressive effect was significant from the 15th day of assessment. However, topical application of vehicle only had no effect on IL-31 mRNA expression in the mite antigen-sensitized mice. In addition, to examine whether the level of IL-31 mRNA expression in the skin is correlated with that of the synthesis of IL-31 protein, the level of IL-31 production in the skin on the 21st day of assessment was measured. As shown in Fig. 6, it was confirmed that the level of IL-31 mRNA expression in the skin reflected the amount of...
Furthermore, recombinant IL-31 was used for intradermal injection to obtain a direct evidence showing a relationship between the inhibitory activity of josamycin on IL-31 production in the skin and the scratching behavior of mouse. On the day before 21st days of assessment, 20µL of murine recombinant IL-31 (100 ng/mL) was injected into the dermis around the base of both ears and scratching behavior for 24 h was observed. As shown in Fig. 7, administration of IL-31 to the josamycin-treated NC/Nga mice completely restored the suppressed scratching behavior.

DISCUSSION

*S. aureus* colonization of the lesional skin in AD is closely related to exacerbation of the condition. Many investigators have demonstrated that *S. aureus* on the skin is involved in the induction of allergic immune responses and subsequent pathogenesis of AD. As AD is an allergic condition, affected patients show a notable increase of Th2 cells in both the peripheral blood and skin lesions. Therefore, it has been suggested that the Th2 immune response plays an important pathogenetic role in AD, and this notion is supported by the fact that most AD patients show blood eosinophilia, increased serum IgE levels and elevated IL-31 production. IL-31 is known to be one of the Th2 cytokines causing the itch-associated scratching behavior of AD patients as well as NC/Nga mice, an model animal of AD. However, it is unclear what augments IL-31 production in AD patients, and the relationship between *S. aureus* colonization and scratching behavior in AD patients has not yet been clarified. Our present results suggest that *S. aureus* skin colonization might induce IL-31 production in AD patients. We previously demonstrated that peptidoglycan from *S. aureus* stimulated LCs in the epidermis, then augmented the Th2 cell development in the second lymphoid tissues and the subsequent infiltration of Th2 cells into the dermis. Consequently, it can be said that IL-31 production in the skin induced by *S. aureus* would be derived from Th2 cells. Furthermore, some studies of IL-31 support our hypothesis that *S. aureus* colonization of lesional skin in AD patients may induce scratching behavior through IL-31 production. Therefore, eradication of *S. aureus* on the lesional skin would likely suppress the scratching behavior of AD patients and subsequent destruction of skin barrier function, thus reducing skin inflammation.

Our previous study showed that LCs treated with josamycin inhibited Th2 cell development in lymph nodes. Therefore, it was thought that topical application of josamycin to skin lesions of NC/Nga mice would target LCs in the epidermis. These LCs would then move to lymph nodes, where Th2 cell development and subsequent IL-31 production would be down-regulated. In fact, increased expression of IL-31 was observed in the skin lesions of mite-antigen-treated NC/Nga mice, similarly to human AD lesions, and topical application of josamycin inhibited the expression of IL-31. 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-like skin lesions of NC/Nga mice.\(^\text{21}\) Therefore, IL-31 expression in the skin lesions of mite antigen-treated NC/Nga mice would reflect the pattern of Th2 cytokine expression in their lymph nodes.

We previously showed that most \textit{S. aureus} strains isolated from the lesioned skin of AD patients were susceptible to josamycin.\(^\text{22}\) Since the skin of most AD patients shows \textit{S. aureus} colonization and barrier disruption due to a decrease of filaggrin,\(^\text{23}\) 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.\(^\text{1,4,6,19,23,24}\) Therefore, topical application of josamycin to the lesioned skin of AD patients appears to exert a beneficial effect involving a bactericidal action against \textit{S. aureus} and inhibition of the Th2 immune response by inhibiting the development of LC-mediated allergen-specific Th2 cells, unlike immunosuppressants such as tacrolimus, or steroids.\(^\text{7}\) Moreover, the microbiota on the skin surface would eventually be predominant with coagulase-negative staphylococci strains that show a capacity to produce antimicrobials as seen in healthy subjects and exclude a further \textit{S. aureus} colonization.\(^\text{25}\) Josamycin has a molecular weight of 824 and can penetrate the barrier-disrupted skin such as AD skin lesions, but not the normal skin. Therefore, it is likely that topical application of josamycin to the skin would have few side effects, it would be possible to increase its concentration in ointment to a level that would more strongly inhibit IL-31 expression and subsequent pruritus in AD patients.

CONCLUSION

Our present results demonstrate that topical application of josamycin inhibits the development of AD-like skin lesions in NC/Nga mice through regulation of \textit{S. aureus} skin colonization and scratching behavior. Since this scratching behavior is associated with expression of the Th2 cytokine, IL-31, in the skin, control of skin colonization by \textit{S. aureus} would likely inhibit the Th2-prone immune response and subsequent pruritus. Thus, if \textit{S. aureus} strains isolated from the lesional skin of AD patients are susceptible to josamycin, topical administration of josamycin might be a promising new therapeutic strategy for those patients.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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