The Atopic Dermatitis-Like Symptoms Induced by MC903 Were Alleviated in JNK1 Knockout Mice

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Atopic dermatitis (AD) is a common allergic disease, imposing large social and economic burdens worldwide. Atopic dermatitis is characterized by eczematous skin lesions and immunoglobulin E (IgE) hypersecretion. We investigated the role of JNK1 on the development of AD in mice. The vitamin D3 analogue MC903, a psoriasis therapeutic drug, was used to induce AD-like symptoms in wild-type (WT) and JNK1−/− mice. The symptoms of AD were less severe in JNK1−/− mice compared with WT mice. JNK1−/− mice showed less ear thickening and infiltration of eosinophils and mast cells in AD-like lesions than did WT mice when treated with MC903. MC903-treated JNK1−/− mice also showed significantly lower level of serum IgE, which was elevated in MC903-treated WT mice. Splenocytes isolated from MC903-treated WT and JNK1−/− mice were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies. Splenocytes from JNK1−/− mice produced lower levels of T-helper (Th2) cytokines (interleukin-4 and -13) and transcription factor GATA-binding protein 3, and produced increased levels of the Th1 cytokines interferon-γ and transcription factor T-box expressed in T cells. Our results indicate that JNK1 plays an important role in the pathogenesis of AD and may be a useful target for therapies to ameliorate AD.

Key Words: atopic dermatitis; JNK1; IgE; cytokines; GATA-3; T-bet.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by itchy eczematous skin lesions (Leung et al., 2004). Although the underlying causes of AD are complex and incompletely understood, genetic predisposition, nutrition, and a combination of allergic and nonallergic factors appear important in determining disease expression (Watson and Kapur, 2011). Skin lesions associated with AD involve severe rash, edema, hemorrhage, and desquamation (Leung and Bieber, 2003). Pathological changes associated with AD include epidermal thickening and marked infiltration of inflammatory cells, such as eosinophils and mast cells (Imokawa et al., 1991).

At least 2 forms of AD have been delineated to date: an “extrinsic” form associated with immunoglobulin E (IgE)-mediated sensitization that affects 70%–80% of patients, and an “intrinsic” form lacking IgE-mediated sensitization that affects 20%–30% of patients (Leung et al., 2004; Zheng and Flavell, 1997). In extrinsic AD, memory T cells expressing the skin homing receptor cutaneous lymphocyte-associated antigen (CLA) produce increased levels of T-helper 2 (Th2) cell cytokines, including interleukin (IL)-4 and IL-13, which induce isotype switching to IgE synthesis, and IL-5, which plays an important role in eosinophil development and survival (Leung et al., 2004). These CLA+ T cells also produce abnormally low levels of interferon (IFN)-γ, a Th1 cytokine known to inhibit Th2 cell function (Leung et al., 2004). Production of IL-4 and IL-13 is lower in intrinsic than extrinsic AD (Leung et al., 2004).

Intracellular signaling via mitogen-activated protein kinases has been implicated in a variety of cellular events, including the activation and differentiation of T-helper cells (Glimcher and Murphy, 2000). JNK, also known as stress-activated protein kinase, phosphorylates the transcription factor c-Jun and increases transcription activity of activator protein-1, which reportedly plays an important role in regulating cytokine genes in Th1 and Th2 cells (Dong et al., 1998; Rincon et al., 1997; Rooney et al., 1995). Dong et al. (1998) generated JNK1-deficient mice and found that they exhibit deficiencies in Th differentiation characterized by...
an exaggerated Th2 response (Dong et al., 1998). Whether JNK1 regulates the Th responses in the development of AD has not been elucidated.

Topical application of either vitamin D3 or its low-calcemic analogue calcipotriol MC903 reportedly induces synthesis of thymic stromal lymphopoietin in mouse keratinocytes, triggering an AD-like syndrome (Li et al., 2006). In the present study, we used JNK1−/− mice to investigate whether JNK1 is associated with MC903-induced development of AD. We also aimed to identify Th cytokines and transcription factors regulated by JNK1 in AD-like symptoms.

MATERIALS AND METHODS

Animals. Female wild-type (WT) and JNK1−/− C57BL/6J mice (12–15 weeks old) were purchased from Jackson Laboratory (Bar Harbor, Maine). Mice were housed in individual ventilated cages under specific pathogen-free conditions at 22±2°C with a 12-h light-dark cycle. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University, Seoul, Korea. To induce AD-like skin lesions in mice, 1 nmol MC903 (Sigma, St. Louis, Missouri) in ethanol was applied topically to both ears daily for 14 days.

Blood and tissue sampling. Blood samples were collected from anesthetized mice by cardiac puncture on the last day of the experiment and serum obtained from whole blood was stored at −80°C until use. After blood collection, the mice were sacrificed and the ears were excised for histologic evaluation. The spleen was aseptically removed to evaluate cytokine and transcription factor production by splenocytes.

Histological examination. The ear tissues from WT and JNK1−/− mice were fixed with 4% paraformaldehyde, embedded in paraffin, and 4-µm-thick sections were cut and transferred onto slides. Deparaffinized skin sections were stained with hematoxylin-eosin, toluidine blue, and Congo red. Tissue sections were examined using an Olympus AX70 light microscope (Olympus, Tokyo, Japan) to assess histological changes and to count the number of eosinophils and mast cells per 0.025 mm² of ear skin at ×400 magnification. The number of eosinophils and mast cells per square millimeter was calculated and presented in the Results.

Measurement of ear thickness. After MC903 treatment, the ears of mice became swollen, so we could confirm the severity of AD by measuring the ear thickness. The measurements were performed by an investigator who was blinded to the experimental conditions. During the 14-day MC903 treatment, ear thickness was measured and recorded 3 times per week using a vernier caliper (Mitutoyo, Kanagawa, Japan).

Determination of total serum IgE. The total level of IgE in the serum was determined using an ELISA kit (Shibayagi, Gunma, Japan), according to the manufacturer’s instructions.

Th1 and Th2 cytokine production in splenocytes. The spleen was aseptically removed from each animal. Red blood cells (RBC) were lysed using RBC lysis buffer (Qiagen, Hilden, Germany), and the lysate was centrifuged at 1500 × g for 10 min at 4°C. The prepared splenocytes (5 × 10⁶ cells/ml) were stimulated with plate-bound anti-CD3 (1 µg/ml) and anti-CD28 (3 µg/ml) monoclonal antibodies (mAbs; BD Bioscience, San Jose, California) at 37°C in 5% CO₂ for 48 h, at which time the culture supernatant was collected and stored at −80°C until use. The levels of IL-4, IL-13, and IFN-γ produced by the splenocytes were determined using a multiplex MCYTOMAG-70K assay (Millipore, Billerica, Massachusetts), according to the manufacturer’s instructions. The levels of GATA-binding protein 3 (GATA-3) and T-box expressed in T cells (T-bet) were determined using Western blot analysis.

Western blot analysis. Western blotting was performed as described previously (Jung et al., 2008). The protein concentration of each sample was determined with a dye-binding protein assay kit (Bio-Rad Laboratories, Hercules, California), as described in the manufacturer’s manual. Cell lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, New Jersey). The membrane was blocked with 5% skim milk and incubated with specific primary antibodies against GATA-3 and T-bet (Abcam, Cambridge, United Kingdom) diluted 1000x in Tris-Buffered Saline with Tween 20, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using a chemiluminescence detection kit (Amersham Pharmacia Biotech).

Statistical analysis. Data are expressed as the mean and SEM, and the significance of differences was determined using the Student’s t test. Probability values of < .05, .01, and .001 were used as criteria for statistical significance.

RESULTS

The Severity of MC903-Induced AD-Like Symptoms Was Lower in JNK1−/− Mice

To investigate the role of JNK1 on AD-like symptoms, JNK1−/− and WT mice were treated daily with the vitamin D3 analogue calcipotriol (MC903) by topical application to the ear for 14 days to induce AD-like symptoms. Ethanol was used as vehicle control. After 14 days of treatment, the ears of MC903-treated WT mice became dry, scaly, red, swollen, and lichenified (Fig. 1A). In contrast, the ears of MC903-treated JNK1−/− mice showed less-severe AD-like symptoms. We found no marked differences in ear histology between the vehicle-treated JNK1−/− and vehicle-treated WT mice.

The ear thickness of MC903-treated and vehicle-treated JNK1−/− and WT mice was also measured over the course of the 14 days treatment period. The ears of MC903-treated WT mice were significantly thicker (0.37±0.01 mm) than those of vehicle-treated WT mice (0.24±0.00 mm) (Fig. 1B). In contrast, the ears of MC903-treated JNK1−/− mice were thinner (0.32±0.01 mm) than those of MC903-treated WT mice (0.37±0.00 mm). We found no significant difference in ear thickness between vehicle-treated JNK1−/− mice (0.23±0.01 mm) and vehicle-treated WT mice (0.24 mm±0.00 mm). Overall, MC903-induced AD-like symptoms were suppressed in JNK1−/− mice, suggesting that JNK1 plays an important role in the development of AD symptoms.

MC903-Induced Infiltration of Eosinophils and Mast Cells in AD-Like Skin Lesions Was Reduced in JNK1−/− Mice

To investigate whether the infiltration of eosinophils and mast cells into AD-like skin lesions is modulated in JNK1−/− mice, tissue sections were stained with Congo red and toluidine blue to discriminate eosinophils and mast cells, respectively. No difference was observed with respect to the number of eosinophils in the skin of the ears of vehicle-treated WT (0.43±0.30 cells per 0.025 mm²) and vehicle-treated JNK1−/− (0.29±0.29 cells per 0.025 mm²) mice (Figs. 2A and B). Repeated MC903 administration over the course of 14 days induced significant
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Therefore, we examined whether JNK1 is associated with elevation of serum IgE in MC903-treated mice. The serum concentration of IgE in MC903-treated WT mice was 4372 ± 790 ng/ml (Fig. 3), whereas that of MC903-treated JNK1−/− mice was significantly lower (483 ± 68 ng/ml), suggesting that JNK1 is involved in the elevation of serum IgE in AD.

The Level of Serum IgE Was Reduced in JNK1−/− Mice

Elevation in the serum IgE level is a hallmark of AD (Kotani et al., 2000; Matsuda et al., 1997); therefore, we examined whether JNK1 is associated with elevation of serum IgE in MC903-treated WT and JNK1−/− mice were stimulated with anti-CD3 and anti-CD28 mAbs. An ELISA specific for IL-4, IL-13, and IFN-γ was performed using splenocyte supernatants. As shown in Figures 4A and 4B, splenocytes derived from JNK1−/− mice produced lower levels of the Th2 cytokines IL-4 (883 ± 163 pg/ml) and IL-13 (54 ± 8 pg/ml) than splenocytes derived from WT mice (IL-4, 1625 ± 253 pg/ml; IL-13, 86 ± 27 pg/ml). In contrast, splenocytes derived from JNK1−/− mice produced a higher level of the Th1 cytokine IFN-γ (8953 ± 138 pg/ml) than those from WT mice (7768 ± 405 pg/ml) (Fig. 4C).

Levels of the Immunomodulatory Transcription Factors GATA-3 and T-bet Were Altered in JNK1−/− Splenocytes

The level of the immunomodulatory transcription factor GATA-3, which is selectively expressed in Th2 cells (Zheng and Flavell, 1997), was significantly lower in splenocytes from JNK1−/− mice compared with those from WT mice (Figs. 5A and B). The level of T-bet, which is selectively expressed in Th1 cells (Szabo et al., 2000), was significantly higher in splenocytes from JNK1−/− mice compared with those from WT mice (Figs. 5A and C). These data indicate that JNK1 plays an important role in regulating the levels of transcription factors that balance Th1 and Th2 cytokine production.

Discussion

Dong et al. (1998) implicated JNK signaling pathways in immune responses and reported that JNK1−/− T cells hyperproliferate, exhibit decreased activation-induced cell death, and preferentially differentiate into Th2 cells. In contrast to results by Dong et al. (1998), our results clearly showed that MC903-induced Th2 responses are alleviated in JNK1−/− mice.

The systemic balance between Th1 and Th2 responses is critical for proper cellular and humoral immune function, and is normally
maintained by immunoregulatory cytokines (Oberholzer et al., 2000). Imbalances in Th1 and Th2 responses often cause pathological conditions (Singh et al., 1999). Compared with normal skin or uninvolved skin, acute skin lesions associated with AD have a significantly greater number of cells expressing IL-4, IL-5, and IL-13 messenger RNAs, but fewer cells expressing IFN-γ or IL-12 messenger RNAs (Leung et al., 2004). We found that splenocytic production of the Th2 cytokines IL-4 and IL-13 was lower in MC903-treated JNK1−/− mice than in WT mice, but production of the Th1 cytokine IFN-γ was higher (Fig. 4), suggesting that JNK1 is involved in IL-4 and IL-13 production and in maintaining a low IFN-γ level in AD.

One of the most important factors that influence differentiation of Th cells is the transcription factor GATA-3 and T-bet (Chakir et al., 2003). Some transcription factors are expressed selectively in Th1 or Th2 cells and thus are identified as Th1 or Th2 specific (Murphy and Reiner, 2002). For example, GATA-3 is selectively expressed in Th2 cells and activates transcription of the genes encoding IL-4 and IL-13 (Murphy and Reiner, 2002; Ouyang et al., 2000; Zheng and Flavell, 1997). In contrast, T-bet is selectively expressed in Th1 cells and activates transcription of the IFN-γ gene (Szabo et al., 2000). Our study showed that GATA-3 expression was downregulated, and T-bet expression was upregulated in MC903-treated JNK1−/− mice.
compared with WT mice (Fig. 5), suggesting that JNK1 plays an important role in regulating GATA-3 and T-bet levels in AD.

One of the main characteristics of AD in humans is elevated serum IgE (Bruynzeel-Koomen et al., 1986; Wollenberg and Bieber, 2000). Topical application of MC903 reportedly increases serum IgE levels (Li et al., 2006). We found that the serum IgE level was significantly lower in MC903-treated JNK1−/− mice than in WT mice (Fig. 3). The Th2 cytokines, particularly IL-4 and IL-13, regulate IgE synthesis by B cells (Leung et al., 2004), suggesting that JNK1 might be involved in increasing serum IgE by stimulating IL-4 and IL-13 production.

In the skin, Th2 cytokines induce proliferation and activation of eosinophils (Kapp, 1993). Eosinophils defend against parasites and mediate allergic reactions, and are thus found in large numbers in many AD patients (Liu et al., 2011; Weller, 1997). We observed extensive eosinophil infiltration in affected tissues of MC903-treated WT mice but not in MC903-treated JNK1−/− mice (Figs. 2A and B). Mast cells can be activated by allergen-sensitized IgE (Williams and Galli, 2000). We found that the number of mast cells in AD-like skin lesions was significantly reduced in MC903-treated JNK1−/− mice.

In conclusion, we found that MC903-induced AD-like symptoms are less severe in JNK1−/− mice compared with WT mice. JNK1−/− mice showed alleviated Th2 responses against MC903. Our results clearly suggest that JNK1 plays an important role in the pathogenesis of AD, and the inhibition of the JNK1 signaling pathway may be helpful in treating AD.

**FIG. 3.** Serum IgE level in MC903-treated WT and JNK1−/− mice. The level of serum IgE was measured using an ELISA. Blood was collected from WT and JNK1−/− mice after 14 days of treatment with MC903 (1 nmol). Data are the means ± SEM (n = 4). *, p < .05, MC903-treated WT versus JNK1−/− mice. Abbreviation: WT, wild type.

**FIG. 4.** IL-4, IL-13, and IFN-γ levels in splenocytes derived from MC903-treated WT and JNK1−/− mice. Splenocytes were collected from WT and JNK1−/− mice after 14 days of treatment with MC903 (1 nmol) and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. The levels of IL-4 (A), IL-13 (B), and IFN-γ (C) were measured using a multiplex assay. Data are the means ± SEM (n = 5). *, p < .05, MC903-treated WT versus JNK1−/− mice. Abbreviations: IL, interleukin; IFN, interferon; mAb, monoclonal antibody; WT, wild type.
Overall, we propose that new therapeutic strategies to treat AD be developed that target JNK1.

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**REFERENCES**


**FIG. 5.** Levels of GATA-3 and T-bet in splenocytes derived from vehicle or MC903-treated WT and JNK1−/− mice. Splenocytes were collected from WT and JNK1−/− mice after 14 days of treatment with vehicle or MC903 (1 nmol) and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. The levels of GATA-3, T-bet, and GAPDH were determined with Western blotting (A). The ratio of GATA-3 (B) and T-bet (C) to GAPDH was determined with densitometry and normalized to the levels in WT mice. Data are the means ± SEM (n = 5). ##, p < .01, vehicle-treated versus MC903-treated WT mice. ***, p < .01, MC903-treated WT versus JNK1−/− mice. Abbreviations: T-bet, T-box expressed in T cells; WT, wild type.
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