

Inhibitory effect of arazyme on the development of atopic dermatitis-like lesions in BALB/c and Nc/Nga mice

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Abstract. Arazyme is a metalloprotease released by *Aranicola proteolyticus* that was shown to inhibit cytokine release in HaCaT and endothelial cells. However, the regulatory effects of arazyme in atopic dermatitis remain to be fully understood. In the present study, the anti-inflammatory effects of arazyme in BALB/c and Nc/Nga mice induced with 2,4-dinitrochlorobenzene (DNCB) were investigated. BALB/c mice were sensitized with DNCB and were subsequently administered arazyme for 4 weeks either orally, dorsally or orally/dorsally. Arazyme administration significantly reduced epidermal thickening and infiltration of inflammatory cells into the dermis compared with the DNCB group. However, serum immunoglobulin E (IgE) levels were not altered by arazyme treatment. Additionally, the level of secretion of interleukins (IL)-4, -5 and -13 in the splenocytes of BALB/c mice was elevated following stimulation with concanavalin A, while the increase of IL-4 and IL-13 was inhibited by arazyme. Administration of arazyme (25 mg/kg in phosphate-buffered saline) to Nc/Nga mice that had been sensitized with DNCB for 6 weeks reduced the skin severity score compared with that in the DNCB group and inhibited the histological manifestations of atopic dermatitis-like skin lesions. In addition, the serum IgE levels were reduced in the arazyme-treated NC/Nga mice relative to the DNCB group. Collectively, these results indicated that arazyme attenuates the development of atopic dermatitis-like lesions via lowering the levels of IgE and inflammatory cytokines. The results of the present study will aid in the development of effective therapeutic strategies for the treatment of allergic diseases, including atopic dermatitis.

Introduction

Atopic dermatitis (AD) is a chronic pruritic and inflammatory skin disease that generally occurs in children, and the incidence of AD is increasing annually. AD is caused by a variety of genetic and environmental factors and characterized by inflammation and tissue damage in the skin (1,2). Previous studies have reported that AD is associated with increased expression of immunoglobulin E (IgE), secretion of T helper (Th) 2 cytokines and eosinophil count in the serum (3,4). However, the pathogenesis of AD remains to be fully elucidated. As a result, patients with AD are not treated with drugs specific to AD, but are administered with anti-inflammatory or immunosuppressive drugs. Certain drugs used for the long-term treatment of AD have been reported to cause severe side effects, including immunosuppression and dysfunction of the epidermal barrier (5,6).

Arazyme is an extracellular metalloprotease produced by *Aranicola proteolyticus*, which is an aerobic Gram negative bacterium isolated from the intestine of the spider *Nephila clavata* (7,8). A previous study demonstrated that arazyme inhibits the secretion of inflammatory cytokines and increases the expression of skin barrier proteins (9). In addition, arazyme has been reported to suppress the inflammatory response induced by lipopolysaccharides in endothelial cells (10). In the present study, the anti-inflammatory effects of arazyme were investigated in AD-like animal models, BALB/c and Nc/Nga mice.

Materials and methods

Enzyme purification. Arazyme was purified from extracellular fractions of *S. proteamaculan* HY-3 (KCTC2390; Korean Collection for Type Culture, Daejeon, Korea) as previously described (8). In brief, extracellular fractions were collected by centrifugation of the Luria-Bertani (LB) culture medium (Sigma-Aldrich Korea, Seoul, Korea) at 5,000 x g for 10 min, or by filtration using a 0.2-μm membrane filter (Pall Life Sciences, Port Washington, NY, USA). Chromatography was subsequently performed on a DEAE-cellulose column (GE Healthcare Life Sciences, Little Chalfont, UK)

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equilibrated with 50 mM potassium phosphate buffer (pH 7.6; Sigma-Aldrich Korea). Bound proteins were then eluted with a 0.1–0.5 M sodium chloride (Sigma-Aldrich Korea) gradient at a flow rate of 400 ml/h and following this, each fraction was concentrated using a 10-kD cassette membrane (Pall Life Sciences). The protein solution was loaded onto a Sephadex G-75 column (GE Healthcare Life Sciences) equilibrated with 50 mM potassium phosphate buffer (pH 7.8) at a flow rate of 20 ml/h and fractions with proteolytic activity were concentrated with a 10-kD cassette membrane and stored at -20°C . Proteolytic activity was determined spectrophotometrically by measuring absorbance at 405 nm (SpectroQuest UV-2800, cat. no. S90424; Thermo Fisher Scientific, Inc., Waltham, MA USA), as previously described (8).

Induction of allergic dermatitis in BALB/c and Nc/Nga mice. A total of 40 five-week-old female BALB/c mice (weight, 17–19 g) and 30 NC/Nga mice (weight, 19–21 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and acclimated for one week prior to the start of the experiments. Animals were housed in an air-conditioned animal unit at $23\pm 2^{\circ}\text{C}$ and a humidity of $50\pm 10\%$. Mice were provided with solid feed (Rodfeed; Daehan Biolink Co., Ltd., Eumsung, Korea). A schematic diagram of the experimental procedure is provided in Fig. 1. Induction of AD was performed with 2,4-dinitrochlorobenzene (DNCB; Sigma-Aldrich, St. Louis, MO, USA) in BALB/c and NC/Nga mice. In brief, 1% DNCB was dissolved in an acetone-olive oil mixture (acetone/olive oil, 3:1; Sigma-Aldrich Korea). The dorsal hair of the mice was removed with an electric razor and no skin damage (e.g. chafed skin or hemorrhage) was observed. A total of 0.15 ml 1% DNCB solution was applied to the same area of dorsal skin. Following sensitization with 1% DNCB, the BALB/c mice were dorsally treated with 0.3% DNCB three times/week for 4 weeks, then once/week for 4 weeks. NC/Nga mice were treated with 0.3% DNCB three times/week for 4 weeks and then twice per week for 6 weeks. The protocol for the care and treatment of the mice was approved by the Institutional Animal Care and Use Committee of Eulji University (Daejeon, Republic of Korea).

Arazyme administration. BALB/c mice were divided into the following eight groups ($n=5$ in each group): Untreated; DNCB; oral arazyme (10 mg/kg); 25 mg/kg dorsal arazyme; combined treatment with 5 mg/kg oral and 12.5 mg/kg dorsal arazyme; 5 mg/kg oral dexamethasone; 5 mg/kg dorsal dexamethasone; and combined treatment with 2.5 mg/kg oral and 2.5 mg/kg dorsal dexamethasone, which were all purchased from Sigma-Aldrich Korea. NC/Nga mice were divided into the following six groups ($n=5$ in each group): Untreated; DNCB; 25 mg/kg arazyme; 50 mg/kg arazyme; 125 mg/kg arazyme; 5 mg/kg dexamethasone. The DNCB, arazyme and dexamethasone groups were dorsally administered with 1% DNCB and subsequently dorsally treated with 0.3% DNCB. The DNCB, arazyme and dexamethasone groups were treated with phosphate-buffered saline (PBS; Sigma-Aldrich Korea), arazyme and dexamethasone via gastric inoculation with a mouse-feeding needle (Cadence, Inc., Staunton, VA, USA) and/or application to the same area of the dorsal skin. The untreated group was treated with PBS without administration of DNCB, arazyme or dexamethasone.

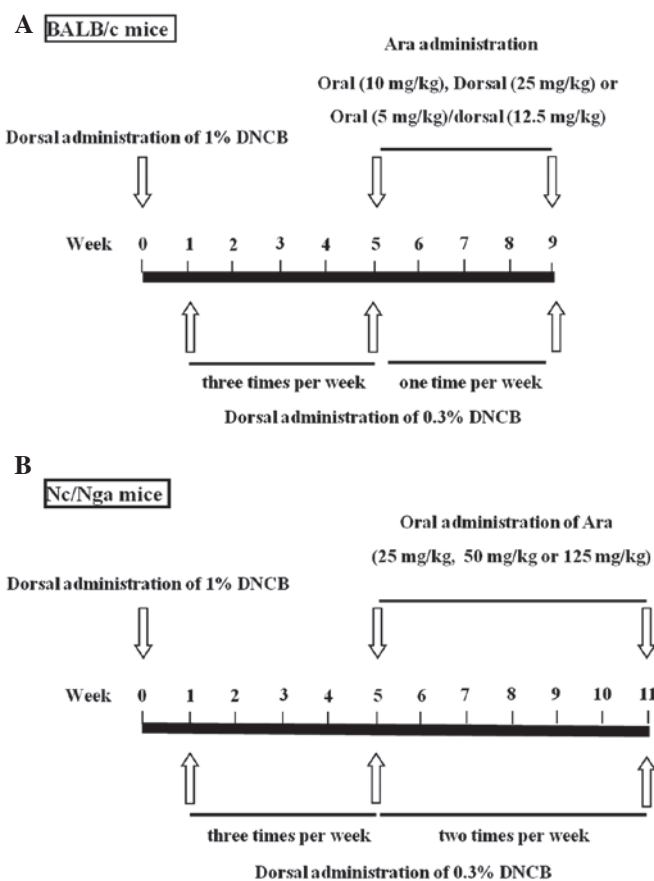


Figure 1. Experimental schedule for the induction of AD lesions. AD was induced by DNCB in (A) BALB/c and (B) NC/Nga mice. Following initial sensitization with 1% DNCB, the BALB/c mice were dorsally treated with 0.3% DNCB three times/week for 4 weeks and then once/week for 4 weeks. NC/Nga mice were treated with 0.3% DNCB three times/week for 4 weeks and then twice/week for 6 weeks. The BALB/c and NC/Nga mice were treated orally, dorsally or orally/dorsally with Ara. AD, atopic dermatitis; DNCB, 2,4-dinitrochlorobenzene; Ara, arazyme.

Histological analysis. Subsequent to sacrifice of the mice by CO_2 asphyxiation, the dorsal skin was removed, fixed in Carnoy's solution (Sigma-Aldrich Korea), embedded in paraffin (Sigma-Aldrich Korea) and sectioned (5 μm -thick). The sections were then stained with hematoxylin-eosin solution (Sigma-Aldrich Korea) and subsequently examined by light microscopy (Leica Microsystems, Wetzlar, Germany) for histological evaluation. Specifically, the epidermis was evaluated for hypertrophy and infiltration by inflammatory cells, while the dermis was evaluated for infiltration by inflammatory cells.

Measurement of serum IgE. Blood was collected from the tail of the mice every week. The serum was obtained by centrifugation and then stored at -70°C until required. Total IgE levels in the serum were measured using sandwich ELISA kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

Splenocyte preparation. The BALB/c mice were sacrificed and subsequently their spleens were removed under aseptic conditions. Splenocytes were then isolated from the spleens as previously described (11), after which the red blood cells were

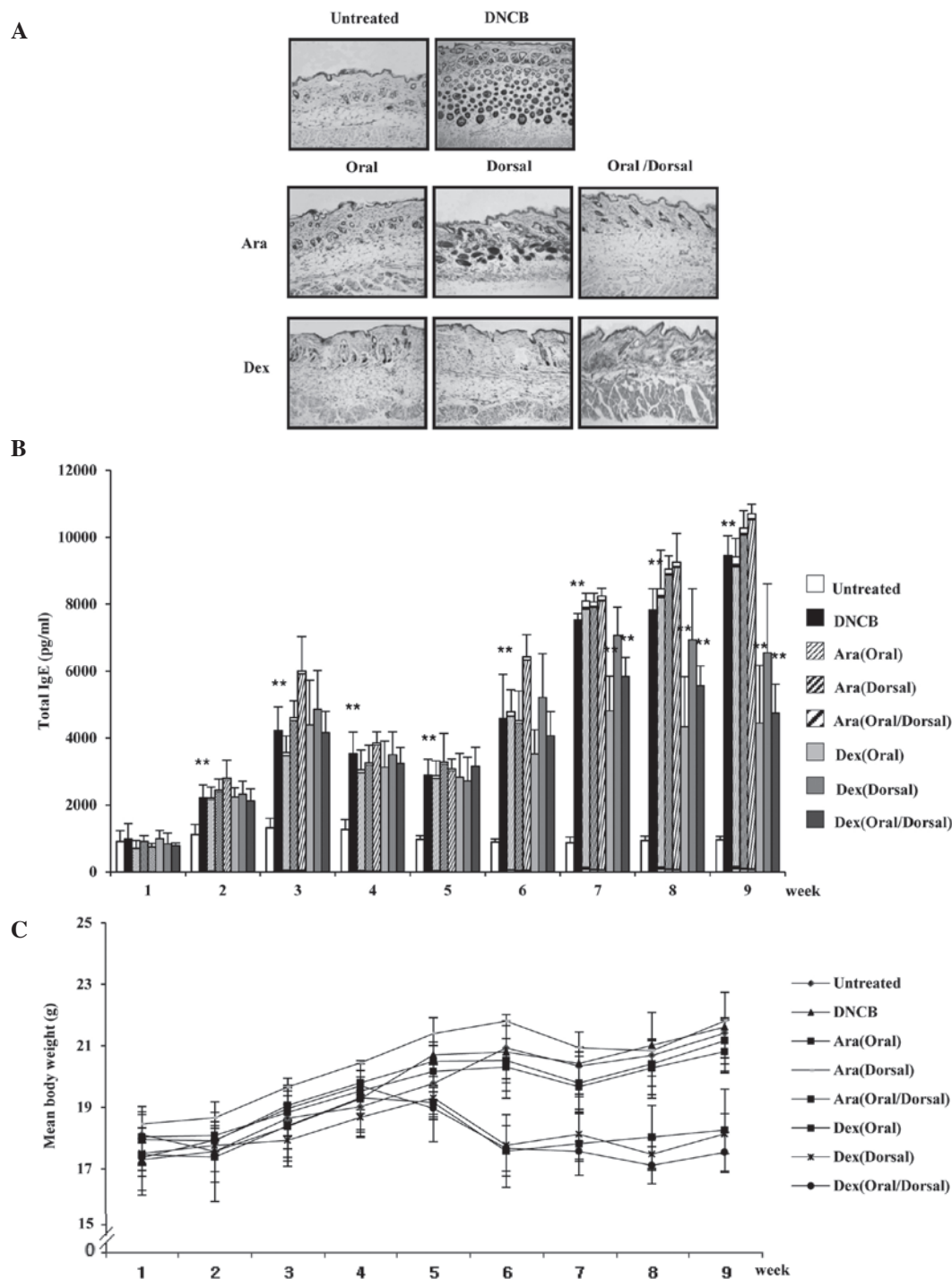


Figure 2. Arazyme reduces the aggravation of atopic-like skin lesions, but has no effect on the serum levels of IgE in DNCB-induced BALB/c mice. Mice were divided into the following four groups: Untreated, DNCB, Ara and Dex. (A) For histological analysis, the dorsal skin was fixed and embedded in paraffin, sectioned, stained with hematoxylin and eosin solution and examined by light microscopy (magnification, x200). (B) Serum was collected from the blood of the NC/Nga mice weekly and total IgE levels in the serum were measured using sandwich ELISA kits. (C) The mean body weight of mice was measured using an electric scale. Values are presented as the mean \pm standard deviation. ** $P < 0.01$, untreated vs. DNCB group and DNCB vs. drug-treated group. IgE, immunoglobulin E; DNCB, 2,4-dinitrochlorobenzene; Ara, arazyme; Dex, dexamethasone.

hemolyzed using red blood cell lysis solution (Sigma-Aldrich). Splenocytes were seeded in a 24-well plate at a concentration of 5×10^6 cells/ml in RPMI-1640 medium with 1% penicillin-streptomycin and 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA).

ELISA. Splenocytes were pretreated in the absence or presence of arazyme and then stimulated with $1 \mu\text{g/ml}$ concanavalin A

(Sigma-Aldrich Korea) for 24 h. The cell supernatants were collected and the concentrations of interleukin (IL)-4, IL-5 and IL-13 were measured in the supernatant by a sandwich ELISA [OptEIA™ Human IL-4 and IL-5 sets; BD Biosciences; and Human IL-13 DuoSet kit, R&D Systems, Inc. (Minneapolis, MN, USA)] according to the manufacturer's instructions. The concentration of each protein was calculated from the standard curves.

Evaluation of skin severity. The severity of dermatitis was assessed macroscopically in a blinded experiment. The four indicators of skin lesions were: i) Erythema/hemorrhage, ii) edema/swelling, iii) excoriation/erosion and iv) dryness. Scoring was performed as follows: 0 (no symptoms), 1 (mild), 2 (moderate) and 3 (severe) (12).

Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The concentrations of ALT and AST were measured using the Reitman-Frankel method (13) in the serum of BALB/c mice using ALT and AST assay kits (Asan Pharm Co., Seoul, Korea) according to the manufacturer's instructions.

Statistical analysis. Values are expressed as the mean \pm standard deviation. Data were analyzed using Student's t-test using SPSS software, version 10.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Arazyme reduces the aggravation of atopic-like skin lesions but has no effect on the serum IgE levels in DNCB-induced BALB/c mice. The therapeutic effects of arazyme were investigated in mice with DNCB-induced dermatitis by histological evaluation. Histological analysis of the skin of mice in the untreated group revealed that the tissue was normal, whereas mice in the DNCB group exhibited epidermal hypertrophy, hyperkeratosis of the epidermis and infiltration of inflammatory cells (Fig. 2A). Oral and oral/dorsal administration of arazyme markedly ameliorated the histopathological alterations as compared with those in the dexamethasone group, while dorsal administration of arazyme resulted in a small reduction in these alterations. Mice in the group administered oral/dorsal dexamethasone exhibited atrophy of the skin. Since IgE is known to act as an important pathogenic factor in AD (3,4), it was investigated whether the anti-inhibitory effects of arazyme were involved in the alteration of IgE production. Although the serum levels of IgE in the DNCB group were markedly upregulated compared with those in the untreated group, arazyme was not observed to effectively reduce these elevated IgE levels (Fig. 2B). The body weight of mice in the arazyme group was comparable to that of mice in the untreated and DNCB groups (Fig. 2C), while a reduction in body weight was observed in the dexamethasone group after 5 weeks.

Arazyme suppresses cytokine levels in mouse splenocytes. Inflammatory cytokines, particularly Th2 cytokines, serve essential roles in allergic diseases such as AD (3,4); thus, the effects of arazyme on cytokine production were investigated. Splenocytes isolated from the spleens of BALB/c mice were treated with arazyme for 1 h and subsequently with concanavalin A for 24 h. The synthesis of IL-4, IL-5 and IL-13 was observed to increase in the supernatant of splenocytes following stimulation with concanavalin A for 24 and 48 h (Fig. 3). Pretreatment with arazyme inhibited the increased secretion of IL-4 and IL-13. IL-5 release remained unchanged in the arazyme-treated splenocytes.

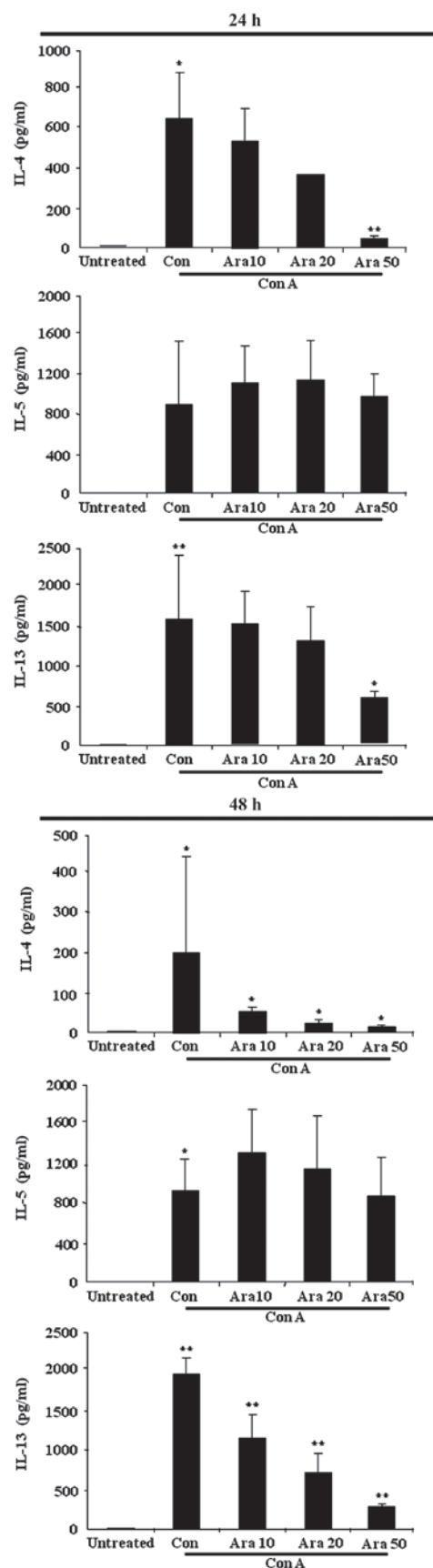


Figure 3. Arazyme suppresses cytokine synthesis in BALB/c mouse splenocytes pretreated with Ara at the indicated concentrations (Ara 10, 10 mg/ml; Ara 20, 20 mg/ml; Ara 50, 50 mg/ml) for 1 h. The cytokines were analyzed by ELISA. Values are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$ and ** $P < 0.01$, untreated vs. control group and control vs. arazyme-treated group. Ara, arazyme; Con, control; Con A, concanavalin A; IL, interleukin.

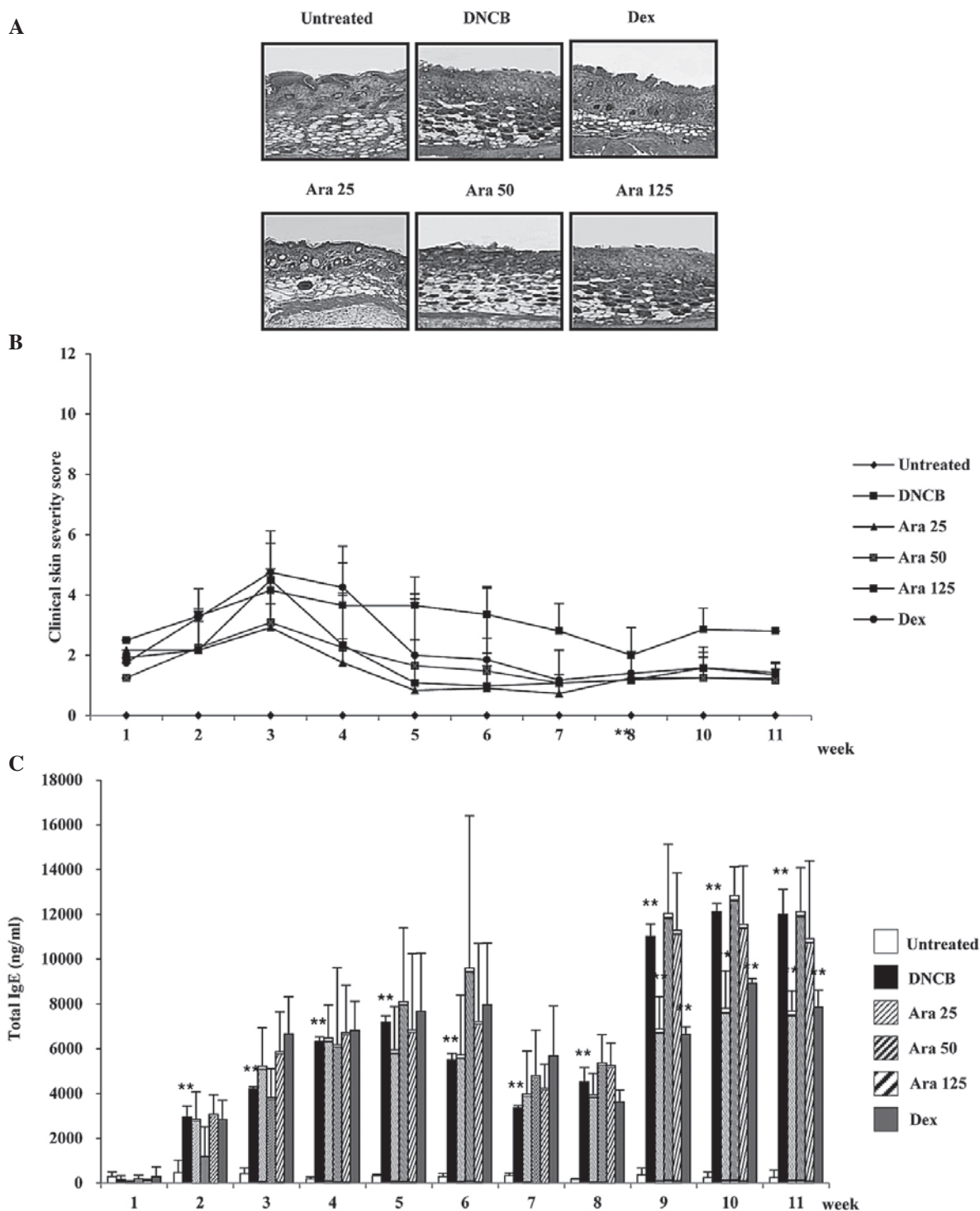


Figure 4. Arazyme suppresses the severity of dermatitis and IgE levels in DNCB-induced AD using NC/Nga mice. The mice were divided into the following four groups: Untreated, DNCB, Ara and Dex. The DNCB, Ara and Dex groups were dorsally administered with 1% DNCB and then dorsally treated with 0.3% DNCB. Ara was administered orally at the following concentrations: Ara 25, 25 mg/kg; Ara 50, 50 mg/kg; Ara 125, 125 mg/kg. Dex was administered orally at 5 mg/kg. (A) For histological analysis, the dorsal skin was fixed and embedded in paraffin, sectioned, stained with hematoxylin and eosin solution and examined by light microscopy (magnification, x200). (B) The severity of dermatitis was assessed macroscopically in a blinded experiment and (C) total IgE levels in the serum were measured using sandwich ELISA kits. Values are presented as the mean \pm standard deviation. ** $P < 0.01$, untreated vs. DNCB group and DNCB vs. drug-treated group. Ara, arazyme; IgE, immunoglobulin E; DNCB, 2,4-dinitrochlorobenzene; AD, atopic dermatitis; Dex, dexamethasone.

Arazyme suppresses the severity of dermatitis and IgE levels in DNCB-induced AD using NC/Nga mice. As the oral treatment of arazyme proved effective at inhibiting histopathological features in AD-like BALB/c mice, the effects of oral administration of arazyme on Nc/Nga mice were investigated as an additional AD-like model. The DNCB group

exhibited epidermal hyperplasia, hyperkeratosis and inflammation (Fig. 4A). Oral administration of arazyme resulted in suppression of the histological phenomena associated with AD at low concentrations (25 mg/kg), while treatment with high concentrations (50 and 100 mg/kg) of arazyme resulted in either a weak or absent effect. The severity of dermatitis

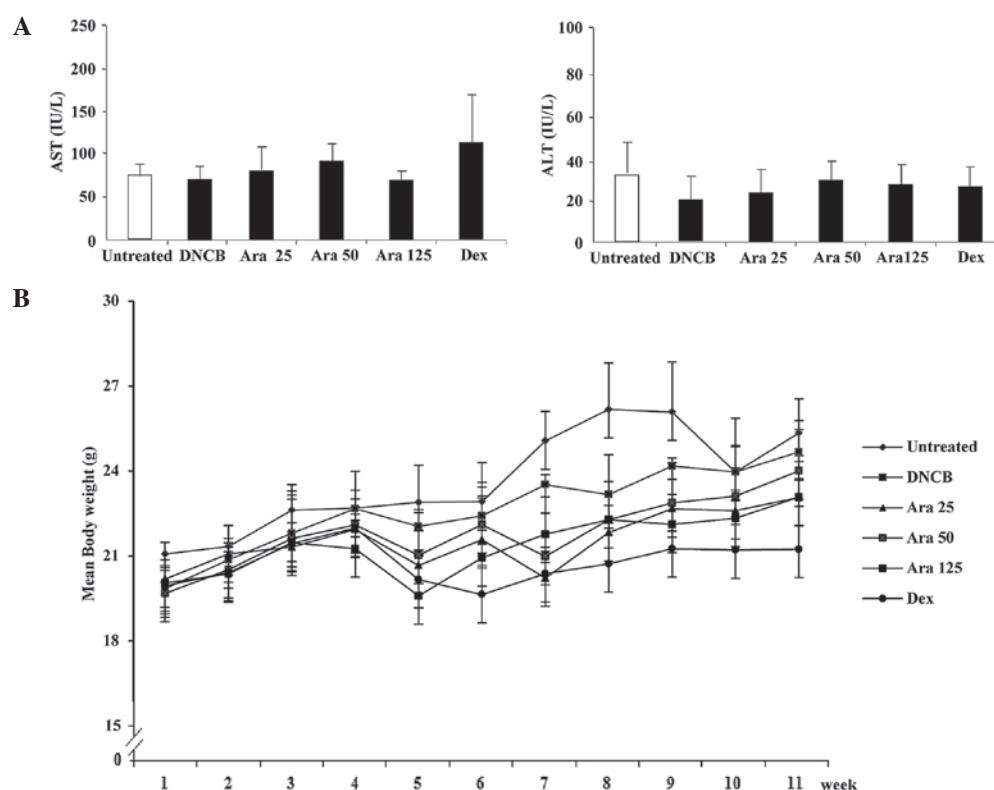


Figure 5. Effects of Arazyme on transaminase levels and body weight of mice. (A) AST and ALT levels in serum were measured by the Reitman-Frankel method. (B) The mean body weight of the mice was measured using an electric scale. Values are presented as the mean \pm standard deviation of three independent experiments. AST, aspartate aminotransferase; ALT, alanine aminotransferase; DNCB, 2,4-dinitrochlorobenzene; Ara, arazyme; Dex, dexamethasone.

was evaluated every week. Clinical signs and symptoms of AD developed subsequent to dorsal treatment with DNCB and these symptoms were observed to worsen with time following the initial treatment. As demonstrated in Fig. 4B, the DNCB group exhibited thick skin with severe erythema, hemorrhage, edema, erosion and excoriation. However, oral application of arazyme at low concentrations inhibited the development of these skin conditions. In addition, 25 mg/kg arazyme was observed to significantly reduce ($P < 0.01$) the elevated IgE levels in the serum at weeks 9, 10 and 11 and the suppressive effect of arazyme was comparable to that produced by treatment with dexamethasone (Fig. 4C). However, high concentrations of arazyme had no inhibitory effect on the alterations in IgE levels. To confirm the cytotoxicity of arazyme, the alterations in body weight, ALT and AST levels in the serum were investigated. The levels of ALT and AST were unchanged by administration of arazyme (Fig. 5A). Body weight was only identified to be altered in the dexamethasone group (Fig. 5B).

Discussion

Arazyme isolated from *Aranicola proteolyticus* has been previously observed to serve a protective role in hepatic injury (8,14). Arazyme has been identified to exert an inhibitory effect on the inflammatory response in human umbilical vein endothelial cells induced by lipopolysaccharides and on cytokine expression in inflammatory cells; furthermore, an arazyme-induced upregulation of skin barrier protein levels has been observed in keratinocytes (9,10). Thus, the present

study investigated whether arazyme alleviates the clinical features of AD. Mouse models of AD are commonly classified into three groups: i) Those that require epicutaneous administration of sensitizers, ii) skin gene-defective transgenic models and iii) models involving the spontaneous development of AD (4,15). In the present study, BALB/c and Nc/Nga mice were selected for use as AD-like mouse models and DNCB was employed as the sensitizer. In BALB/c mice, arazyme was identified to inhibit the histological features associated with AD. Furthermore, oral administration of arazyme was observed to be more effective than dorsal treatment. Arazyme additionally suppressed the histopathological appearance and clinical severity score in Nc/Nga mice. In contrast to BALB/c mice, arazyme lowered the serum IgE levels in Nc/Nga mice. This difference may have been due to the use of different mouse species. It is well known that immunological responses of humans and mice are distinctly different; therefore, caution should be taken prior to conducting a clinical trial. In the present study, the effects of arazyme on the severity of AD were not dose-dependent. To determine an appropriate concentration of arazyme and overcome the different results obtained using different animal models, future studies employing another model such as a mite-induced or transgenic model would be beneficial.

The pathogenesis and progression of AD is caused by various factors associated with immune dysregulation or hypersensitivity and the balance of T helper (Th) 1/Th2 cytokines (16-18). IL-4 results in the differentiation of naive Th0 cells to Th2 cells and induces chronic inflammation (15). Additionally, it was recently reported that IL-4 regulates

alternative macrophage activation (19). The function of IL-13 is similar to that of IL-4 (15). In the present study, arazyme suppressed the secretion of IL-4 and IL-13, but had no effect on that of IL-5. Inhibition of IL-4 and IL-13 may be correlated with alleviation of histopathological features associated with AD. Although increased circulation of IgE in AD is positively correlated to IL-4 and IL-13 expression in CD4+ T cells (20), arazyme was not observed to be effective at reducing the serum IgE levels in AD-like BALB/c mice. In contrast to the effects of arazyme on IgE expression in BALB/c mice, arazyme downregulated the serum levels of IgE and inhibited histological inflammation in Nc/Nga mice.

To elucidate this difference, future studies are required in order to determine how arazyme inhibits the pathophysiological mechanisms of AD. Arazyme induces anti-oxidant signaling in addition to the inhibition of Th2 cytokine release (9,14). Additionally, arazyme induces expression of filaggrin and involucrin included in skin barrier proteins (9). As arazyme is a metalloprotease, it may exert its function via a protease-activated receptor. Further studies are required to examine these complex inhibitory mechanisms in greater detail.

In conclusion, arazyme alleviated the clinical features in BALB/c and Nc/Nga models of AD and diminished the synthesis of Th2 cytokines, including IL-4 and IL-13, in addition to the serum IgE levels. These observations indicated that arazyme may be valuable for the treatment of allergic diseases such as AD.

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References

1. Bieber T: Atopic dermatitis. *N Engl J Med* 358: 1483-1494, 2008.
2. Barnes KC: An update on the genetics of atopic dermatitis: scratching the surface in 2009. *J Allergy Clin Immunol* 125: 16-29, 2010.
3. Habu Y, Seki S, Takayama E, Ohkawa T, Koike Y, Ami K, Majima T and Hiraide H: The mechanism of a defective IFN-gamma response to bacterial toxins in an atopic dermatitis model, NC/Nga mice, and the therapeutic effect of IFN-gamma, IL-12, or IL-18 on dermatitis. *J Immunol* 166: 5439-5447, 2001.
4. Jin H, He R, Oyoshi M and Geha RS: Animal models of atopic dermatitis. *J Invest Dermatol* 129: 31-40, 2009.
5. Shiohara T, Hayakawa J and Mizukawa Y: Animal models for atopic dermatitis: are they relevant to human disease? *J Dermatol Sci* 36: 1-9, 2004.
6. Berke R, Singh A and Guralnick M: Atopic dermatitis: an overview. *Am Fam Physician* 86: 35-42, 2012.
7. Bersanetti PA, Park HY, Bae KS, Son KH, Shin DH, Hirata IY, Juliano MA, Carmona AK and Juliano L: Characterization of arazyme, an exocellular metalloprotease isolated from *Serratia proteamaculans* culture medium. *Enzyme Microb Technol* 37: 574-581, 2005.
8. Kwak J, Lee K, Shin DH, Maeng JS, Park DS, Oh HW, Son KH, Bae KS and Park HY: Biochemical and genetic characterization of arazyme, an extracellular metalloprotease produced from *Serratia proteamaculans* HY-3. *J Microbiol Biotechnol* 17: 761-768, 2007.
9. Kim IS, Kim MJ, Shin DH, Son KH, Park HY and Lee JS: Arazyme inhibits cytokine expression and upregulates skin barrier protein expression. *Mol Med Rep* 8: 551-556, 2013.
10. Kim IS, Yang EJ, Shin DH, Son KH, Park HY and Lee JS: Effect of arazyme on the lipopolysaccharide-induced inflammatory response in human endothelial cells. *Mol Med Rep* 10: 1025-1029, 2014.
11. Lee JS, Kim IS, Ryu JS, Kim JH, Kim JS, Kim DH and Yun CY: The inhibitory effect of *Duchesnea chrysanthi* extract on the development of atopic dermatitis-like lesions by regulating IgE and cytokine production in Nc/Nga mice. *Phytother Res* 26: 284-290, 2012.
12. Kim IS, Kim DH, Yun CY and Lee JS: A (S)-(+)-decursin derivative, (S)-(+)-3-(3,4-dihydroxy-phenyl)-acrylic acid 2,2-dimethyl-8-oxo-3,4-dihydro-2H,8H-pyrano[3,2-g]-chromen-3-yl-ester, attenuates the development of atopic dermatitis-like lesions in NC/Nga mice. *Mol Biol Rep* 40: 2541-2548, 2013.
13. Witter RF and Grubbs LM: An evaluation of the Reitman-Frankel method for the determination of serum glutamic oxalacetic transaminase. *Clin Chim Acta* 13: 524-527, 1966.
14. Park JK, Jeong DH, Park HY, Son KH, Shin DH, Do SH, Yang HJ, Yuan DW, Hong IH, Goo MJ, Lee HR, Ki MR, Ishigami A and Jeong KS: Hepatoprotective effect of Arazyme on CCl4-induced acute hepatic injury in SMP30 knock-out mice. *Toxicology* 246: 132-142, 2008.
15. Tanaka A, Amagai Y, Oida K and Matsuda H: Recent findings in mouse models for human atopic dermatitis. *Exp Anim* 61: 77-84, 2012.
16. Romagnani S: Lymphokine production by human T cells in disease states. *Annu Rev Immunol* 12: 227-257, 1994.
17. Suto H, Matsuda H, Mitsuishi K, Hira K, Uchida T, Unno T, Ogawa H and Ra C: NC/Nga mice: a mouse model for atopic dermatitis. *Int Arch Allergy Immunol* 120 (Suppl 1): 70-75, 1999.
18. Yang EJ, Lee JS, Yun CY, Kim JH, Kim JS, Kim DH and Kim IS: Inhibitory effects of *Duchesnea chrysanthi* extract on ovalbumin-induced lung inflammation in a mouse model of asthma. *J Ethnopharmacol* 118: 102-107, 2008.
19. Luzina IG, Keegan AD, Heller NM, Rook GA, Shea-Donohue T and Atamas SP: Regulation of inflammation by interleukin-4: a review of "alternatives". *J Leukoc Biol* 92: 753-764, 2012.
20. Yamanaka K and Mizutani H: The role of cytokines/chemokines in the pathogenesis of atopic dermatitis. *Curr Probl Dermatol* 41: 80-92, 2011.