Effective Microorganism Substance (EM-S) Reduces Development and Aggravation of Atopic Dermatitis-like Skin Lesions in NC/Nga Mice

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In a previous study, our group showed that the effective microorganism substance (EM-S) produced by fermentation of medicinal plants with effective microorganisms has an anti-inflammatory effect on atopic dermatitis-like lesions in NC/Nga mice. However, the possible anti-inflammatory effect and skin barrier function of EM-S for inflammatory cell infiltration, Interleukin-4 (IL-4) production, C-C chemokine receptor 10 (CCR10), and filaggrin (*FLG*) expression were not reported. Therefore, effects of EM-S on the development of atopic dermatitis-like skin lesions in NC/Nga mice were evaluated. Efficacy of EM-S was judged by measurement of scratching behavior, T-cell subset infiltration, cytokine production, and *FLG* expression. Topical application of EM-S significantly reduced scratching behavior in NC/Nga mice caused by house dust mite antigen (*Dermatophagoides farinse* extract, *DfE*) sensitization. IL-4 production and $CD4^+$ and $CD45^+$ cell infiltrations were significantly reduced by EM-S. CCR10 expression was also significantly inhibited by EM-S. EM-S treatment also increased the level of *FLG* reduced by *DfE* sensitization. These results demonstrate EM-S, when applied topically, may be an effective substance for management of atopic dermatitis patients.

Key words: anti-inflammatory effects, atopic dermatitis, effective microorganism substance, filaggrin, NC/Nga mouse

Atopic dermatitis (AD) is a chronically relapsing pruritic inflammatory skin disease with a wide range of severity, and is usually the first manifestation of atopic disease. Manifestation of AD results from a complex interaction of environmental factors, skin barrier defects, and immune disorders [Dubrac *et al*., 2010]. It is one of the most common skin disorders in developed countries, affecting approximately 20% of children and 1-3% of adults [Simpson, 2010]. AD is generally considered to be a model of typical type 2 helper T cell (Th2)-mediated disorders [Nakazato *et al*., 2008]. It has been consistently shown that skin-infiltrating Th2 chemokines producing cells play a pivotal role in initiation and amplification of atopic skin inflammation, and that infiltration of inflammatory cells in the skin is regulated by Th2 chemokines [Homey *et al*., 2006].

In human AD patients and AD-like murine models, it is well recognized that the cytokines such as interleukin-4 (IL-4), IL-5, and IL-13 produced by Th2 cells are responsible for increased serum IgE levels and the presence of blood eosinophilia [Nomura *et al*., 2003]. In particular, enhanced IL-4 is a hallmark of acute AD [Boguniewicz, 2004] inhibiting ceramide synthesis, and exogenous applications of IL-4 impede recovery of the permeability barrier after acute perturbations [Kurahashi *et al*., 2008].

Cutaneous T cell-attracting chemokine (CTACK/CCL27) is a skin-specific CC chemokine, which is produced constitutively by keratinocytes and is highly upregulated in inflammatory skin conditions such as AD [Morales *et*

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al., 1999; Homey *et al*., 2006]. CTACK/CCL27 binds to the chemokine receptor CCR10 [Homey *et al*., 2000], which is expressed almost exclusively on cutaneous lymphocyte associated antigen (CLA)-positive lymphocytes [Morales *et al*., 1999], which are the lymphocytes bound to the skin, specifically on a small $CD4^+$ $CD45RO^+$ $CCR7^-$ $CD29^-$ memory/effector subset [Soler *et al*., 2003]. Expression of CTACK/CCL27 has only been reported in the skin, and not in other organs, and is especially expressed constitutively in epidermal keratinocytes [Morales *et al*., 1999].

Filaggrin (*FLG*) is a key protein that plays an important role in formation of the cornified cell envelope (CCE), which is critical for an effective skin barrier [Marenholz *et al*., 2006]. *FLG* gene defects have recently been identified as a major risk factor for development of atopic eczema [Marenholz *et al*., 2006; Palmer *et al*., 2006]. These skin barrier defects increase the risk of early onset of severe and persistent forms of atopic eczema.

Effective microorganism fermented extract (EM-X) is a refreshment drink commonly found in East Asia. EM-X contains over 40 minerals, α-tocopherol, lycopene, ubiquinone, saponin, and flavonoids, such as quercetin, quercetin-3-*O*-glucopyranoside, and quercetin-3-*O*-rhamnopyranoside [Deiana *et al*., 2002; Ke *et al*., 2009]. EM-X inhibits *in vitro* growth and reduces the regenerative potential of cancer cells [Chui *et al*., 2006]. EM-X also provides *in vivo* protection of the liver and kidneys from oxidative stress-dependent damage, and demonstrates anti-inflammatory activity [Aruoma *et al*., 2002; Deiana *et al*., 2002].

In a previous study, we have shown that the effective microorganism substance (EM-S) produced by fermentation of medicinal plants with effective microorganisms has an anti-inflammatory effect on atopic dermatitis-like lesions in NC/Nga mice [Mok *et al*., 2010]. However, the possible anti-inflammatory effect and skin barrier function of EM-S for inflammatory cell infiltration, IL-4 production, CCR10, and *FLG* expression were not reported. The purpose of the present study was to determine the effect of EM-S on *Dermatophagoides farinse* extract (*DfE*)-sensitized itching behavior, T-cell subsets, cytokine production, *FLG*, and CCR10 expression in NC/Nga mice.

Materials and Methods

Reagents. *D. farina* extract (*DfE*) was purchased from the Arthropods of Medical Importance Resource Bank (Yonsei University, Seoul, Korea).

Enzyme-linked immunosorbent assay (ELISA) kits for IL-4 and interferon-γ (IFN-γ) were obtained from R&D Systems (Minneapolis, MN). Anti-mouse CD4 and CD45, and anti-CCR10 conjugated with FITC were obtained from BD Biosciences (San Jose, CA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tacrolimus ointment (0.1%) was purchased from Astellas (Tokyo, Japan). Other chemicals for reagent grade were purchased from Sigma (St. Louis, MO).

Plant materials and preparation of methanol (MeOH) extraction. Plant materials (Scutellariae Radix, Coptidis Rhizoma, Phellodendri Cortex, Lonicerae Flos, Chaenomelis Fructus, Green Tea, and Broccoli) used were purchased in October 2009 from Kyungdongmart (Seoul, Korea), which is a distributor center of Oriental medicine materials. The plant was identified and authenticated by Prof. Hong Jun Kim at the College of Oriental Medicine, Woosuk University. A voucher specimen has been deposited in the authors laboratory (S. I. Jang). The composition of the dried plant materials is shown in Table 1. For preparation of extracts, the dried materials were powdered; the powdered leaves (2,000 g) were then extracted with MeOH (5 L) for one week at room temperature and filtered through a nylon mesh (pore size 40 μm). Following centrifugation at 5,000 rpm for 20 min at 4°C, the supernatant of the MeOH extract was evaporated under vacuum to give the MeOH extract (85.8 g).

Preparation of EM-S. Strains of effective microorganism used in the experiment were obtained from EverMiracle (Jeonju, Korea), the Korean branch of the EM Research Organization (Okinawa, Japan). EM-S was kindly supplied by EverMiracle. Briefly, cells, including those of photosynthetic bacteria (*Rhodopseudomonas palustris*), lactic acid bacteria (*Lactobacillus rhamnosus*), and yeast (*Pichia deserticola*) were pre-cultured with equal cell numbers (2×10^9 CFU for each microorganism) in medium containing 1% MeOH-extract from plant materials (Table 1), and EMs were further cultured in 3% molasses media at 38°C under anaerobic conditions for 21 days. The fermented supernatant was acidified to pH 3.5 and filtered through a membrane filter (pore size 0.2 μm), freeze-dried (yield, 40.5 g), and kept at −20°C. The stored material (EM-S) was dissolved immediately before use in vehicle (acetone/olive oil=1:1).

Animals. Male NC/Nga 6-week-old mice were purchased from Central Lab. Inc. (Seoul, Korea), the Korean branch of Charles River Japan (Kanagawa, Japan). Mice were maintained in an environmentally controlled rearing system and used for experiments after 2 weeks. All experiments were performed in accordance with Jeonju University Institutional Animal Care and Use Committee guidelines.

Induction of AD-like skin lesions and administration of EM-S. To determine the inhibitory effects of EM-S on the development of AD, AD-like skin lesions were induced in NC/Nga mice. *DfE* (1%) was used as an allergen, as previously reported [Kang *et al*., 2006; Mok *et al*., 2010]. Briefly, 10 mg *DfE* was dissolved in 1 mL phosphate buffered saline (PBS, pH 7.4) that contained 0.5% Tween 20. Both surfaces of NC/Nga mouse ear lobes were stripped three times using surgical tape. One hour after tape stripping, 10 μL of 10 mg/mL mite antigen solution was painted onto each surface of both ear lobes. Tape stripping and mite antigen painting were repeated five times at 7-day intervals. One group was further treated with 10 μL of 3.0% (w/v) EM-S solution (EM-S group), second group was treated with 10 μL of vehicle only (acetone/olive oil=1:1) (the *DfE* alone group), and the third group was sham-treated (the vehicle group) daily for 4 weeks. The active control group was treated with 0.1% (w/v) Tacrolimus ointment (TO) along with *DfE* (TO group), and the last group was treated with the 2.5% composition of medicinal plant (CMP) (CMP group). Each experimental group consisted of five mice and initial treatments of drugs/vehicles were started 2 h after the first *DfE* treatment. Ears for histological observation and study of immunological phenomena were separated 24 h after the fifth antigen application. Auricular lymph nodes for cytokines were also isolated 24 h after final antigen sensitization.

Scratching behavior. Scratching behavior was assessed according to the method of Mihara *et al*. [2004]. Before the experiments, five mice were placed in five compartments for 30 min of acclimatization. Their behaviors were then recorded using a digital video recording micro-camera (ONCCTV, Seoul, Korea) for 30 min. Videotapes were played back, and the cumulative time spent scratching over the 30 min period was noted. A series of scratching movements by the hind paw was defined as a scratching episode.

Preparation of lymph node cells. Lymph node cells were taken from the auricular region. Freshly isolated lymph node cells were made into single-cell suspensions, and contaminated erythrocytes were lysed by hypotonic shock with sterile distilled water. Cells were stimulated with soluble anti-CD3 (1 μg/mL) plus anti-CD28 (1 μg/mL) antibodies (Pharmingen, San Diego, CA) in RPMI1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B (Sigma), and 5×10^{-5} M 2-mercaptoethanol (2ME).

Cytokine measurement by ELISA. Cytokines levels in the culture supernatants were determined in appropriate dilutions by sandwich ELISA using antibody pairs against murine IL4 and IFN-γ from R&D Systems (Minneapolis, MN), following the manufacturer's instructions.

Western blotting analysis. Auricular lymph node cells were cultured, harvested, and lysed in 1×SDS sample buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 2 mM β-mercaptoethanol, 1 mM DTT, 0.1% BPB]. Cell lysates were electrophoresed on a 10 or 12% SDS polyacrylamide gel, and proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 300 mA for 1 h. After a brief rinse with PBST (PBS containing 0.1% Tween 20), the blots were blocked for 1 h at room temperature in a blocking buffer (PBST containing 4% non-fat dried milk). Primary antibodies against anti-murine IL-4 or anti-murine IFN-γ diluted in the blocking buffer (1:1,000) were added to the blots, and the blots were incubated for 1 h at room temperature or overnight in a refrigerator. After washing three times with PBST, the blots were incubated with HRP-conjugated secondary antibodies (1:10,000 dilutions in blocking buffer) for 1 h at room temperature. The blots were washed three times in PBST and developed with super signal enhanced chemiluminescence (ECL) substrate solution (Pierce, Rockford, IL) for an appropriate time, according to the manufacturer's instructions. Signals were then visualized using X-ray film.

Reverse transcription-polymerase chain reaction (RT-PCR). For analysis of CCR10 and *FLG* mRNA levels from ear tissues, total RNAs were isolated by the guanidine isothiocyanate method using Trizol Reagent (Sigma), and quantified. Total RNAs (1 μg) were denatured and 1 mM each dNTPs, 2 U RNasin, 0.5 mg of random hexamer, 0.5 μg of oligo (dT)12-18, and 20 U MMLV reverse transcriptase (Promega, Madison, WI) were added to make a reaction volume of 20 mL. The reaction mixtures were incubated at 42°C for 45 min and heat inactivated at 85°C for 5 min. Five microliters of the reaction mixture (cDNA) were used for each PCR reaction: 50 μL of the reaction solution contained 1× PCR buffer, 1.5 mM $MgCl_2$, 250 mM dNTPs, and 5 pmol of each forward and reverse PCR primer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an internal control. The amplification conditions were denaturation at 94°C for 1 min (5 min for the first cycle), annealing at 55°C for 1 min, and extension at 72°C for 1 min (10 min for the last cycle) for 40 cycles for all chemokines, and 30 cycles for GAPDH. Amplified products (10 μL each) were separated by electrophoresis on a 1% agarose gel containing 0.5 μg/mL ethidium bromides and visualized over the UV transilluminator. The primer sets used were 5'-GGG GAT GAA GAG

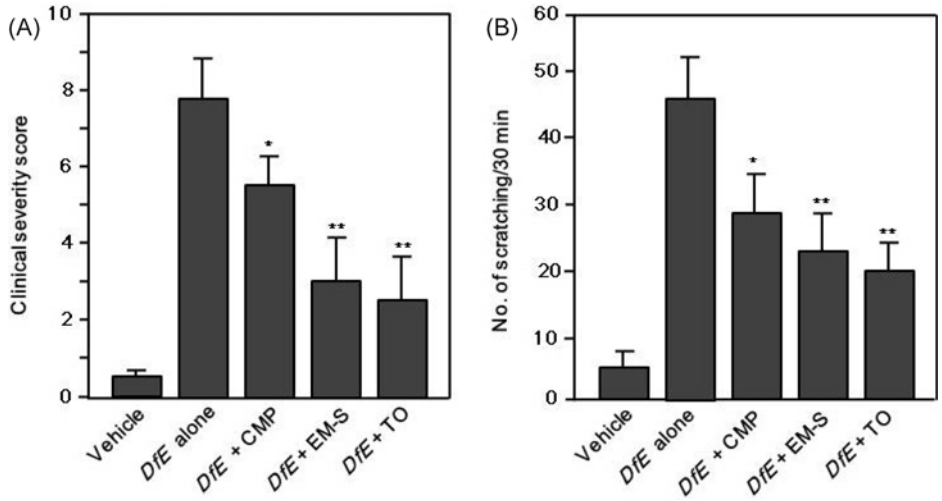

Fig. 1. Changes of total clinical severity scores (A) and number of scratches (B) for 30 min by topical EM-S treatment. *DfE* application on the ears of NC/Nga mice for 4 weeks induced AD-like skin lesions. The total clinical severity score was defined as the sum of scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five aspects: itch, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. Clinical scores and scratching counts were determined 28 days after completion of treatment. Data are shown as means± SE of changes in the total clinical severity scores on the face, ears, nose, and neck of five mice in each group, and number of scratches for 30 min. *p<0.05 and **p<0.01 compared with the *DfE* alone group.

GAC GCA TAC T-3' (forward) and 5'-CCT GGA CAT CGG CCT TGT-3' (reverse) for CCR10; 5'-GAC CAG CAC TGG AGG AAG AC (forward) and 5'-ATG GGA ACC TGA GTG TCC AG-3' (reverse) for FLG; 5'-GCC AAG GTC ATC CAT GAC AAC TTT GG-3' (forward) and 5'-GCC TGC TTC ACC ACC TTC TTG ATG TC-3' (reverse) for GAPDH.

Immunohistochemical analysis. Ear tissues were fixed with 4% paraformaldehyde, embedded in paraffin; 5-μm thin sections were then placed on silane-coated micro slides (Muto-glass, Tokyo, Japan). De-paraffinized skin sections were stained with anti-CD4, CD45, CCR10, and *FLG*. Sections for immunohistochemistry were de-paraffinized with xylene, rinsed with distilled water, and then rinsed with PBS. Nonspecific antigen-antibody reactions were inhibited by one-hour treatment with 10% normal goat serum. The sections were reacted overnight at 4°C in diluted solutions of each of the primary antibodies, including purified rat monoclonal antibodies against anti-mouse CD4, CD45, CCR10, and *FLG* antibodies. The sections were rinsed with distilled water and PBS, and were then covered with cover-slips. They were examined by fluorescence microscopy (Olympus, Tokyo, Japan) for assessment of molecular expression and cell infiltration.

Statistical analysis. Differences in data among the groups were analyzed by one-way ANOVA, and all values were expressed as means±SD. Differences between groups were considered significant at p<0.05.

Results

Effect of EM-S on clinical severity and scratching behavior. To evaluate the effect of EM-S on AD-like skin lesions, clinical signs and symptoms on the face, ears, nose, and neck were scored according to criteria for five major clinical signs and symptoms of AD: itch, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness [Matsuda *et al*., 1997]. Application of *DfE* antigen on the ears of NC/Nga mice for 4 weeks induced AD-like skin lesions. The total clinical severity score was defined as the sum of scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five aspects: itch, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. Clinical severity scores and scratching counts were determined 28 days after completion of treatment. Total clinical severity scores were significantly increased at day 28 of treatment and reached a clinical score of more than 7 points in the *DfE* alone group (p<0.01), compared to those of the vehicle group (Fig. 1A). However, topical application of the CMP tended to be lower than those for the *Df*E alone group (p<0.05). Topical application of EM-S or Tacrolimus was significantly lower than that for the *DfE* alone group (p<0.01). On the other hand, to evaluate effect of EM-S on the scratching behavior of NC/Nga mice sensitized by *DfE* antigen, the number of scratches was scored using a digital video recording micro-camera for 30 min. Scratching frequencies for topical application in the *DfE* alone group after 4

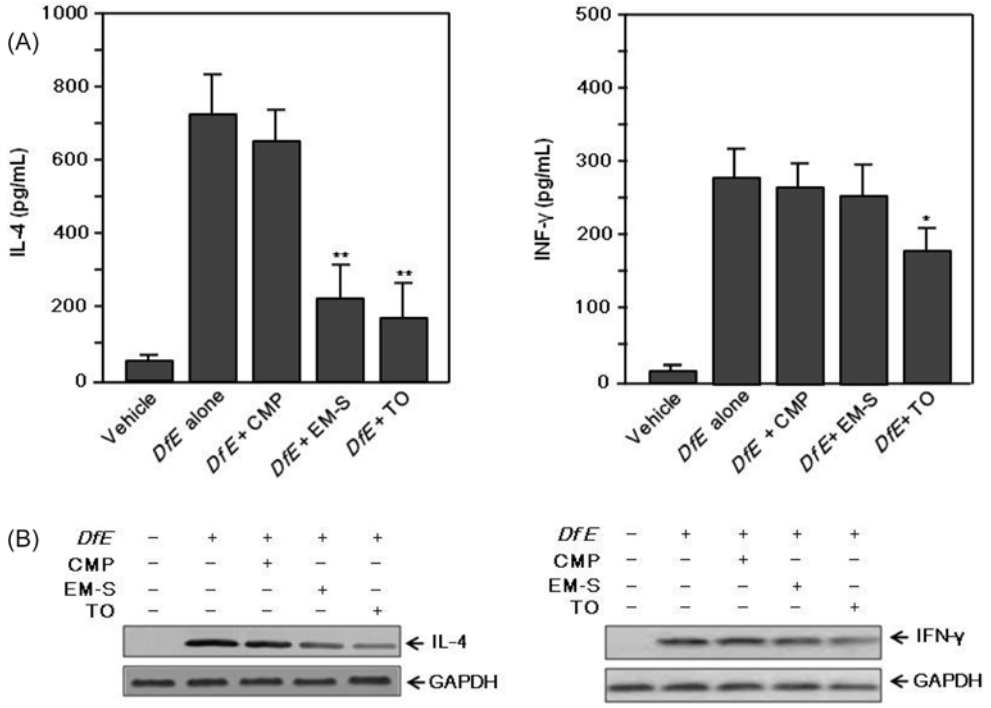

Fig. 2. Effects of EM-S on cytokine production (A) and expression (B). *DfE* application on the ears of NC/Nga mice for 4 weeks induced AD-like skin lesions. One day after the final *DfE* antigen application, auricular lymph node cells were isolated and stimulated with anti-CD3 plus anti-CD28 antibodies for 2 days, and their *ex vivo* cytokine production and expression were examined by ELISA and Western blot. Data are shown as the cumulative means±SE of 5 individual mice. *$p<0.05$ and **$p<0.01$ compared with the *Df*E alone group.

weeks were significantly higher than those for the *DfE*-untreated vehicle group (Fig. 1B). However, scratching frequencies for topical application of the CMP tended to be lower than those for the *DfE* alone group ($p<0.05$). Furthermore, scratching frequencies for topical application of EM-S were significantly lower than those for the *DfE* alone group ($p<0.01$). The reducing effect of EM-S on scratching behavior was similar to that of TO.

Effect of EM-S on IL-4 and IFN-γ expression in auricular lymph nodes. To investigate the effect of EM-S treatment on inflammatory or regulatory cytokines related to AD, cytokine levels of IL-4 and IFN-γ were measured by ELISA and Western blot. Production (Fig. 2A) and expression (Fig. 2B) of IL-4 and IFN-γ were significantly increased in the *DfE* alone group, compared to that of the *DfE*-untreated vehicle group. In contrast, protein production and expression of IL-4 were significantly down-regulated in the EM-S or Tarcrorimus group, compared to that of the *DfE* alone group ($p<0.01$), whereas the CMP- and EM-S treated groups, but not TO, showed no change in the level of IFN-γ. These results demonstrate that EM-S effectively suppressed inflammatory cytokines in AD-like skin lesion NC/Nga mice.

Effect of EM-S on immune cell infiltration in ear tissues. For histological determination of the inhibitory effect of EM-S on $CD4^+$ or $CD45^+$ infiltration, immunohistochemical staning was performed on ears of the specimens of NC/Nga mice. Sections of the ears in the *DfE* alone group revealed hyperplastic epidermis and edematous dermis, with a large number of infiltrated $CD4^+$ or $CD45^+$ cells (Fig. 3A and B). In contrast, sections of the ears in the CMP group showed significantly diminished cellular infiltration into the dermis ($p<0.05$). Furthermore, the EM-S ($p<0.01$) and TO ($p<0.01$) groups showed markedly diminished cellular infiltration into the dermis.

Effect of EM-S on CCR10 expression in ear tissues. To investigate the inhibitory effect of EM-S on CCR10 expression, immunohistochemical staining and RT-PCR were performed on specimens of the ears of NC/Nga mice. CCR10 expression in ear specimens of NC/Nga mice was increased in the *DfE* alone group (Fig. 3A). In contrast, the expression was markedly decreased with topical application in the EM-S and TO groups. The effect of EM-S on CCR10 mRNA expression was also analyzed in specimens of the ears of NC/Nga mice. In parallel with ear tissue expression of CCR10 molecule, topical application of CMP, EM-S or TO markedly suppressed the *DfE*-induced CCR10 mRNA expression in ear tissues (Fig. 4 B and C). These results demonstrate

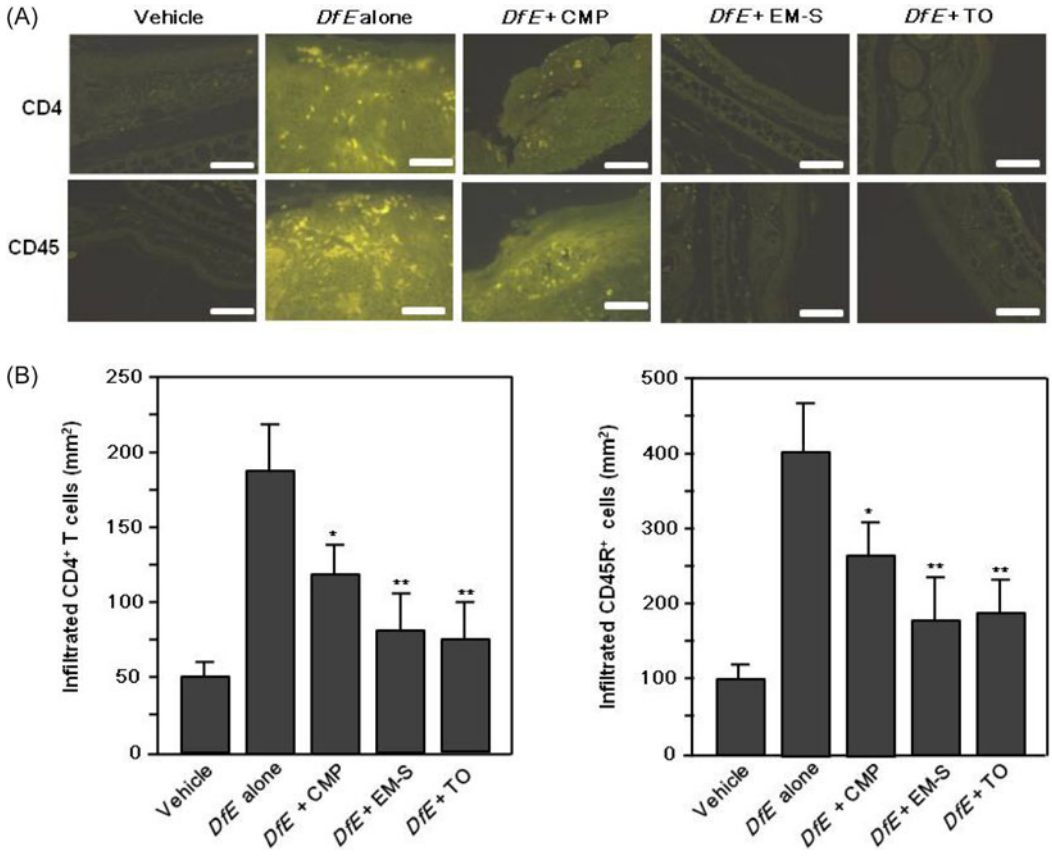

Fig. 3. Effect of EM-S against infiltration of CD4$^+$ and CD45$^+$ cells on *DfE*-induced AD-like ear tissues of NC/Nga mice. Application of *DfE* antigen on the ears of NC/Nga mice for 4 weeks induced AD-like skin lesions. One day after the final *DfE* antigen application, tissue samples were prepared from ears, and infiltrations of CD4$^+$ and CD45$^+$ in ear tissues were stained by immunochemistry with anti-mouse CD4 and CD45 antibodies (A). A relative score for infiltrated CD4$^+$ and CD45$^+$ cells was compared from both groups (B). Data are shown as means±SE of changes in infiltration of CD4 and CD45 from five mice in each group. Scale bar=150 μm. *p<0.05 and **p<0.01 compared with the *DfE* alone group.

that EM-S suppressed *DfE*-induced CCR10 mRNA expression in NC/Nga mice sensitized with *DfE* antigen.

Effect of EM-S on *FLG* expression in ear tissues. To further investigate the inhibitory effect of EM-S on *FLG* expression, immunohistochemical staining and RT-PCR were performed on specimens of the ears of NC/Nga mice. *FLG* expression in ear specimens of NC/Nga mice increased in the *DfE* alone group (Fig. 3A), whereas was markedly suppressed with topical application in the EM-S or TO group. The effect of EM-S on *FLG* mRNA expression in specimens of the ears of NC/Nga mice was also analyzed. In parallel with ear tissue expression of *FLG* molecule, topical application of EM-S or TO markedly suppressed *DfE*-induced *FLG* mRNA expression in ear tissues (Fig. 4B and C). These results demonstrate that EM-S suppressed *DfE*-induced *FLG* mRNA expression in NC/Nga mice sensitized with *DfE* antigen.

Discussion

EM-X is a refreshment drink developed in Japan, and it is produced by fermentation of unpolished rice, papaya, and seaweed with effective microorganisms, including photosynthetic bacteria, lactic acid bacteria, and yeast [Deiana *et al*., 2002]. The potential of EM-X for clinical application has been validated by recent reports showing an *in vivo* protective effect on the liver and kidney, as well as anti-inflammatory [Deiana *et al*., 2002; Do *et al*., 2007] and neuroprotective effects [Datla *et al*., 2004]. Although the accumulated evidence shows that the antioxidant properties displayed by flavonoids, saponins, ubiquinones, lycopene, and vitamin E are mostly associated with the activities of EM-X; however, its actions cannot be fully explained due to the very low level of antioxidant content of EM-X (below 1 μg/mL).

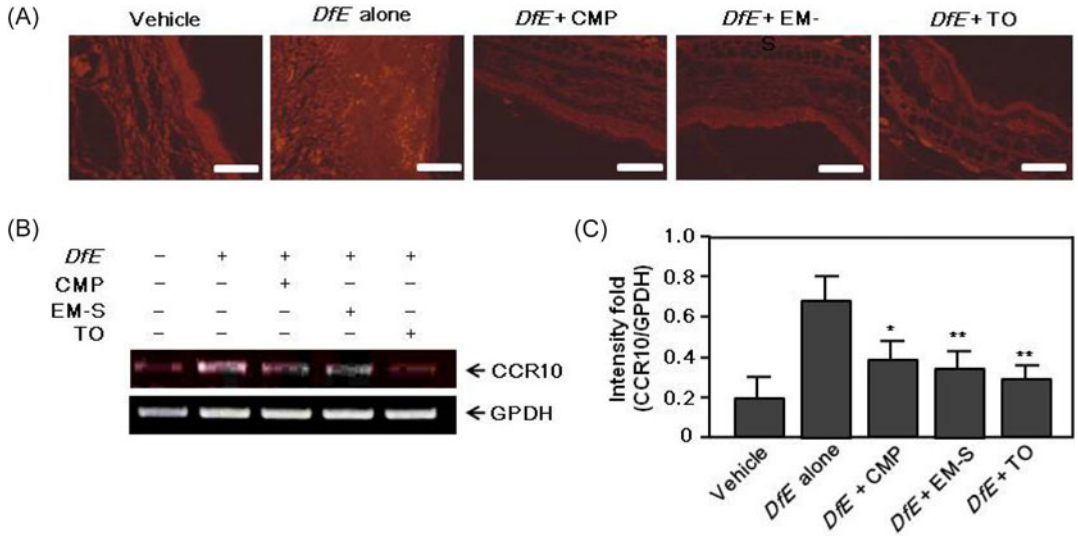

Fig. 4. Effects of EM-S on protein (A) and mRNA (B, C) expression of CCR10 on *DfE*- induced AD-like lesion of NC/Nga mice. Application of *DfE* on the ears of NC/Nga mice for 4 weeks induced AD-like skin lesions. One day after the final *DfE* application, tissue samples were prepared from ears, and expressed CCR10 were determined by immunochemical staining with anti-mouse CCR10 antibody. For analysis of mRNA expression, total RNA was extracted from ear tissues. CCR 10 and GAPDH mRNA were analyzed by RT-PCR using specific primers. Data are shown as means±SE of CCR10 mRNA expression intensity rates against GAPDH from five mice in each group. Scale bar=150 μm. *p<0.05 and **p<0.01 compared with the *DfE* alone group.

Therefore, new EM products, which contain high antioxidant level above 20 μg/mL, was developed by addition of fermented medicinal plants (Table 1) for regulation of skin inflammatory diseases such as AD and contact dermatitis, followed by evaluation of the effects of EM-S on mouse models of acute and chronic inflammatory responses.

In our previous study, the anti-inflammatory effects of EM-S on *DfE*-induced AD-like lesions in NC/Nga mice were investigated [Mok *et al*., 2010]. However, the possible anti-inflammatory effects and skin barrier functions of EM-S on the development and aggravation of AD-like skin lesions in NC/Nga mice still remained unclear. Therefore, in the present study, the reducing effects of EM-S on development and aggravation of the clinical severity and itching behavior of *DfE*-induced AD-like NC/Nga mice were first analyzed.

Table 1. CMP for EM culture medium additives

Crude drug name	Scientific name	Rate (%, w/v)
Scutellariae Radix	*Scutellaria baicalensis*	10
Coptidis Rhizoma	*Coptis chinensis*	5
Phellodendri Cortex	*Phelloedndron amurense*	5
Lonicerae Flos	*Lonicera japonica*	5
Chaenomelis Fructus	*Chaenomeles sinensis*	30
Green Tea	*Camellia sinensis*	15
Broccoli	*Brassica oleracea* var. *italica*	30

Although the therapeutic effects of EM-S were less than those of TO, an active control, topical application of EM-S significantly reduced scratching behavior as well as significantly lessened the clinical signs provoked by mite antigens, such as erythema, edema, scaling, and excoriation (Figs. 1 and 2). Histologically, topical EM-S treatment markedly decreased epidermal hyperplasia, and infiltration of mononuclear cell and eosinophil into the dermis of AD-like skin lesions, showing results similar to those of previous data [Mok *et al*., 2010].

Thus, attempt was made to determine whether EM-S exerts an anti-inflammatory effect by inhibition of chemokine receptor and other cytokines related to immune dysregulation in AD. Quantitative ELISA, Western blot, and RT-PCR results revealed that topical application of EM-S significantly down-regulated over-production and/or expressions of IL-4, $CD4^+$, $CD45^+$, and CCR10 on AD-like skin lesions. Immunohistochemical analysis of skin lesions also showed that topical application of EM-S markedly diminished the over-expressions of $CD4^+$, $CD45^+$, and $CCR10^+$ in AD-like skin lesions. EM-S was also found to effectively enhance the recovery of *FLG* reduced by *DfE* sensitization in NC/Nga mice (Fig. 5). These results demonstrated that topical application of EM-S can suppress *DfE*-sensitized skin pathological lesions, Th2-related cytokine/chemokine, infiltrations, and CCR10, but enhances the recovery of

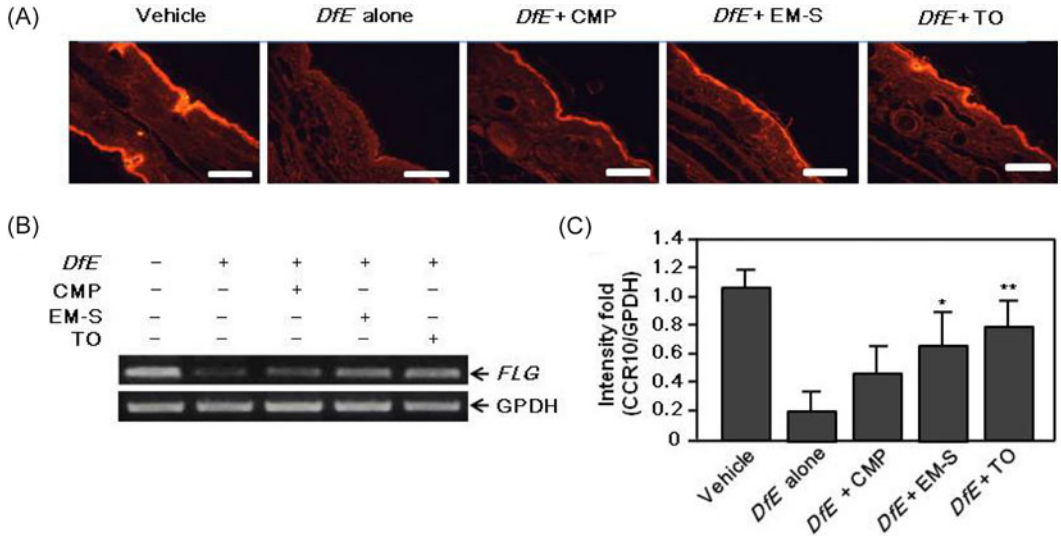

Fig. 5. Effects of EM-S on protein (A) and mRNA (B, C) expression of filaggrin (*FLG*) on *DfE*-induced AD-like lesion of NC/Nga mice. Application of *DfE* on the ears of NC/Nga mice for 4 weeks induced AD-like skin lesions. One day after the final *DfE* application, tissue samples were prepared from ears, and expressed *FLG* were determined by immunochemical staining with anti-mouse *FLG* antibody. For analysis of mRNA expression, total RNA was extracted from ear tissues. *FLG* and GAPDH mRNA were analyzed by RT-PCR using specific primers. Data are shown as means±SE of *FLG* mRNA expression intensity rates against GAPDH from five mice in each group. Scale bar=150 μm. *$p<0.05$ and **$p<0.01$ compared with the *DfE* alone group.

FLG reduced by *DfE* sensitization.

AD is a skin inflammatory disease, in which the presence of mediators, such as IL-4, CCL27, and IgE, the cellular infiltrate ($CD4^+$ or $CD45^+$ T cells, eosinophils, and mast cells), and the association with allergic disorders, all point to a Th2-associated disease [Homey *et al*., 2006]. Repeated *DfE* application caused an increase in IL-4 and INF-γ expressions in auricular lymph nodes of NC/Nga mice [Noh *et al*., 2008]. In contrast, protein production and expression of IL-4 significantly suppressed over-production of IL-4, but not IFN-γ (Fig. 2). These results demonstrate that EM-S effectively suppressed the inflammatory cytokine in AD-like skin lesions in NC/Nga mice.

$CD4^+$ and CLA^+ are lymphocytes associated with invasion of the skin in inflammatory skin diseases. They are attracted by chemokines, particularly CTACK/CCL27, which binds to CCR10, and thus is of interest in this context [Homey *et al*., 2000; 2002]. CTACK/CCL27 is continuously expressed by keratinocytes, but highly upregulated in inflammatory skin diseases such as AD [Homey *et al*., 2006]. $CCR10^+$ lymphocytes invading the skin in AD have Th2 profiles. Therefore, CCR10 expression was examined by immunohistochemical staining and RT-PCR in ear tissues of NC/Nga mice. Results demonstrated that topical application of EM-S significantly suppressed *DfE*-induced expression of $CCR10^+$ lymphocytes *via* inhibition of CCR10 mRNA expression in NC/Nga mice.

On the other hand, *FLG* is a crucial component of the CCE in the outer layer of the epidermis [Leung, 2009]. The significance of *FLG* in AD development has also been suggested in the cases of AD lacking specific mutations in *FLG*. Skin inflammation in AD is typically associated with increased expression of cytokines, such as IL-4 and IL-13, both of which reduce *FLG* gene and protein expression in keratinocytes [Howell *et al*., 2007]. Thus, although it appears that approximately 50% of all AD cases can be explained by the presence of at least one *FLG* null allele, an acquired defect in *FLG* might also be present in a subset of individuals whose AD is due to other inherited or acquired abnormalities [Howell *et al*., 2007]. Fallon *et al*. [2009] showed that the flaky-tail (*ft*/*ft*) mouse, which lacks processed *FLG* due to a homozygous frameshift mutation in pro-*FLG* that is analogous to the human null mutations in *FLG*, has the potential to address some of the remaining questions with regard to the relative importance of *FLG* compared with other factors involved in the pathobiology of AD. Moreover, when exposed repeatedly to topical haptens at doses that produced no inflammation in control mice, ft/ft mice had a severe AD-like dermatosis with a further deterioration in barrier function and features of Th2 immune response [Leung, 2009]. Therefore, the purpose of the present study was to determine the effect of EM-S on *FLG* reduced by *DfE* sensitization in NC/Nga mice. EM-S was

found to effectively enhanced the recovery of *FLG* reduced by *DfE* sensitization (Fig. 5). However, further studies are required to fully understand the recovery effect of EM-S on *FLG* reduced by *DfE* sensitization. Additionally, studies on the pharmacological actions of EM-S in relation to skin specific cytokines and chemokines, as well as interactions between EM-S and the AD system are needed.

In summary, the present study provided evidence that topical application of EM-S could improve AD-like skin lesions in NC/Nga mice by suppression of clinical severity and Th2 chemokines. Taken together, topical application of EM-S may be a beneficial approach to the treatment of AD.

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