



Epigallocatechin-3-gallate improves *Dermatophagoides pteronissinus* extract-induced atopic dermatitis-like skin lesions in NC/Nga mice by suppressing macrophage migration inhibitory factor

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Abstract

Epigallocatechin-3-gallate (EGCG) has been shown to exert anti-inflammatory effects on the inflammatory skin conditions. However, little is known about its effect on atopic dermatitis (AD). We first attempted to assess the anti-inflammatory effect of topical application of EGCG *in vivo* AD model using NC/Nga mice and to determine whether EGCG exerts the anti-inflammatory effect by inhibiting macrophage migration inhibitory factor (MIF) and other cytokines that are related to immune dysregulation in the pathogenesis of AD. Murine AD-like skin lesions were made by painting *Dermatophagoides pteronissinus* extract (DPE). The effects of EGCG treatment were assessed by total clinical severity score and ear thickness, and by histological grading. In addition, the mRNA and protein expression of the cytokines including MIF were measured by real-time RT-PCR and immunohistochemistry. The serum levels of MIF and IgE were measured by ELISA. In the AD mouse model, EGCG significantly reduced the total clinical severity score and ear thickness ($p < 0.05$). The histological grading was also markedly improved. The mRNA expression of MIF, TNF- α , IFN- γ , IL-2 and IL-12 p40, but not of IL-4, IL-5 and IL-13 in the lesions was significantly reduced by EGCG ($p < 0.05$). On the immunohistochemistry, EGCG also markedly diminished the expression of MIF, TNF- α and IFN- γ . The serum MIF and IgE production was significantly reduced by EGCG ($p < 0.05$). These results demonstrate that topical application of EGCG may improve the AD-like skin lesions by suppressing MIF and T helper 1 cytokines. Taken together, it is suggested that EGCG may be a potential therapeutic modality for AD. © 2008 Elsevier B.V. All rights reserved.

Abbreviations: AD, atopic dermatitis; DPE, *Dermatophagoides pteronissinus* extract; EGCG, epigallocatechin-3-gallate; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; H₂O₂, hydrogen peroxide; IFN, interferon; IL, interleukin; MIF, macrophage migration inhibitory factor; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PFA, paraformaldehyde; RT-PCR, reverse transcription polymerase chain reaction; SPF, specific pathogen free; Th, T helper.

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1. Introduction

Green tea polyphenols referred to as catechins, are present in the leaves of the tea plant (*Camellia sinensis*). The four major polyphenols present in green tea leaves are (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG) and (–)-epigallocatechin-3-gallate (EGCG) (Fig. 1) [1]. Based on several *in vitro* [2,3] and *in vivo* [4–6] studies, it has become clear that a group of polyphenolic catechins, most notably its major constituent EGCG, exhibit multiple biological effects on the skin. Trompezinski et al. [2] have shown that EGCG inhibits, in a dose-dependent manner, the upregulation of both vascular endothelial growth factor and interleukin (IL)-8 in tumor necrosis factor (TNF)- α stimulated keratinocytes. Afaq et al. [4] have shown that topical application of green tea polyphenol to SKH-1 hairless mice before ultraviolet-B exposure resulted in a significant decrease in skin edema and infiltration of leukocytes via modulations in the mitogen-activated protein kinase and nuclear factor kappa B signaling pathways. These findings have suggested that EGCG may have utility as a novel therapy for various inflammatory skin diseases such as atopic dermatitis (AD).

AD is a chronic relapsing skin disorder with allergic inflammation [7]. The pathogenesis of AD has been known to be related with local and systemic immunologic dysfunction that leads to Th1/Th2 imbalance as well as being related to susceptibility genes and environmental factors [7–10]. It has been especially reported that there were biphasic T helper (Th) cell responses in the AD skin lesions according to local deviation, with a Th2-predominant acute phase and a Th1-predominant chronic phase. Two functionally distinct subsets of cells, Th1 and Th2 cells, are characterized by the different effector cytokines they secrete [11–13]. However, a more comprehensive understanding of the relative role of these factors in the pathogenesis of AD still remains to be elucidated. Shimizu et al. [14] have reported that macrophage migration inhibitory factor (MIF) is one of the most crucial immunoregulatory cytokines in the pathogenesis of AD. MIF has been recently re-evaluated as pro-inflammatory cytokine [15,16], and the over-expression of MIF stimulates Th1 [17,18] and thereby prolongs an exaggerated inflammatory response [19]. From these evidences, it is conceivable that MIF contributes to the dysregulation seen in the inflammatory and the immune responses in AD.

Although topical steroids are commonly used and they're still mainstay in the treatment of AD, chronic use is discouraged due to potential side effects such as skin atrophy, tachyphylaxis and symptomatic rebound following discontinuation of therapy. Moreover, evidence has been obtained for the upregulation of MIF by steroids. Calandra et al. [20] have shown that low concentrations of steroids induce rather than inhibit MIF production. Despite being induced by steroids, MIF is able to directly antagonize the effects of steroids [21]. These findings demonstrate that MIF counter-regulates the anti-inflammatory effects of steroids and so exacerbates systemic inflammatory diseases. In addition, Rosengren et al. [22] have identified MIF as a phenylpyruvate tautomerase with phenylpyruvate and *p*-hydroxyphenylpyruvate as its natural substrates; this suggests that MIF is involved in inflammation. Interestingly, it has been reported that various plant-derived polyphenol derivatives exert an inhibitory action on MIF as a tautomerase and so this results in the anti-inflammatory effect [23].

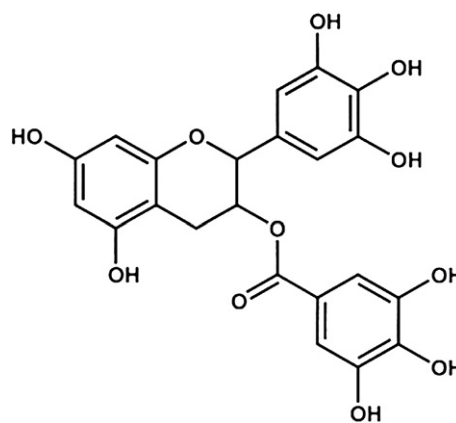


Figure 1 Chemical structure of epigallocatechin-3-gallate.

NC/Nga mice originated from Japanese fancy mice at Nagoya University (Japan) in 1957 and they were established as an inbred strain for creating an animal model of human AD. NC/Nga mice raised in air-uncontrolled conventional circumstances spontaneously suffer from AD-like skin lesions with a markedly elevated serum level of the immunoglobulin E (IgE), whereas NC/Nga mice maintained in specific pathogen-free (SPF) conditions do not show the clinical signs or IgE hyperproduction [24]. However, a variety of antigens can be involved in the development of AD-like skin lesions in NC/Nga mice under conventional conditions, which makes it difficult to analyze the pathogenesis of AD-like skin lesions [25]. A recent study demonstrated that *Dermatophagoides pteronissinus* extract (DPE), a representative mite allergen of human AD, could provoke dermatitis through the scratching behavior in NC/Nga mice under SPF conditions [26]. Those findings were valuable for originally contriving the pathogenesis of AD.

To date, there have been no experiments to assess the therapeutic effects of a topical application of EGCG on the mouse skin *in vivo* AD model. Therefore, the aim of this study was to assess the anti-inflammatory effects of EGCG by topically applying it to the AD-like skin lesions that were provoked by DPE in NC/Nga mice, and to determine whether EGCG exerts the anti-inflammatory effect by inhibiting MIF and other cytokines that are related to immune dysregulation in the pathogenesis of AD.

2. Materials and methods

2.1. NC/Nga Mice

Twenty five SPF NC/Nga female mice (14–22 g each and 5-weeks old) were purchased from Charles River Japan (Kanagawa, Japan). The animals were housed in a room that was under a 12-hour light–dark cycle and the mice were placed individually in separate cages with *ad libitum* access to food and water. The animal care, handling and experimental procedures were carried out in accordance with a protocol approved by the Animal Care and Use Committee of the Catholic University of Korea.

2.2. Induction of AD-like skin lesions

1% DPE (Woongbee Meditech, Inc., Seoul, Korea) was used as an allergen as previously reported [26]. 10 mg DPE was dissolved in 1 ml phosphate buffered saline (PBS) that contained 0.5% Tween 20.

Topical 2.5% EGCG application has relevance for evaluating the efficacy of an EGCG *in vivo* animal model, as was reported previously [4,27]. 50 mg EGCG (Sigma, St. Louis, MO, USA) was dissolved in 2 ml acetone/olive oil (1:1). EGCG is isolated by extracting the green tea leaves with hot water, back-extracted with ethyl acetate. The EGCG is chromatographically isolated from the organic phase. Purity (>95%) was monitored by high performance liquid chromatography (HPLC) analysis.

Induction of AD-like skin lesions using DPE was performed as described previously [26]. Briefly, 20 μ l of 1% DPE solution was painted onto the surface of each ear of NC/Nga mice every other day for 3 weeks. One group was further treated with 20 μ l of 2.5% EGCG solution (the EGCG group), another group was treated with 20 μ l of vehicle only (acetone/olive oil=1:1) (the vehicle group), and the other group was sham-treated (the sham group), daily except every Sunday for 4 weeks. Also, active control group was treated with 0.1% FK506 ointment (Astellas, Tokyo, Japan) along with DPE (the FK506 group) and the last group was treated with EGCG only (the EGCG only group). Each experimental group consisted of five mice.

2.3. Measurement of the total clinical severity score and the ear thickness

The severity of dermatitis was evaluated by scoring the clinical signs and symptoms as described previously [24]. The total clinical severity score was defined as the sum of scores grade as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for each of five aspects: itch, erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness. The clinical signs and symptoms were measured by taking pictures with a digital

Table 1 Primer sequences used for real-time reverse transcription polymerase chain reaction

Target (bp) [Accession no.]	Sequences (5'–3') (S: sense, A: antisense)
MIF (292) [NM_010798]	S: GTTCTGTGCGGAGCTCAC A: AGCGAAGGTGGAACCGTTCCA
TNF- α (175) [NM_013693]	S: CATCTTCTCAAAATTCGAGTGACAA A: TGGGAGTAGACAAGGTACAACCC
IFN- γ (199) [NM_008337]	S: CGGCACAGTCATTGAAAGCCTA A: GTTGCTGATGGCCTGATTGTC
IL-2 (141) [NM_008366]	S: CCTGAGCAGGATGGAGAATTACA A: TCCAGAACATGCCGAGAG
IL-4 (183) [NM_021283]	S: TCTCGAATGTACCAGGAGCCATATC A: AGCACCTTGGAGCCCTACAGA
IL-5 (117) [NM_010558]	S: AGCACAGTGGTGAAAGAGACCTT A: TCCAATGCATAGCTGGTGATT
IL-12 p40 (180) [NM_008352]	S: GGAAGCACGGCAGCAGAATA A: AACTTGAGGGAGAAGTAGGAATGG
IL-13 (150) [NM_008355]	S: CAATTGCAATGCCATCTACAGGAC A: CGAAACAGTTGCTTTGTGTAGCTGA
β -actin (349) [M12481]	S: TGGAACTCTGTGGCATCCATGAAAC A: TAAACGCAGCTCAGTAACAGTCCG

camera (Canon A95, Canon Inc., Tokyo, Japan) daily for 4 weeks. The ear thickness was also measured using a dial caliper (Kori Seiki MFG, Japan) every week.

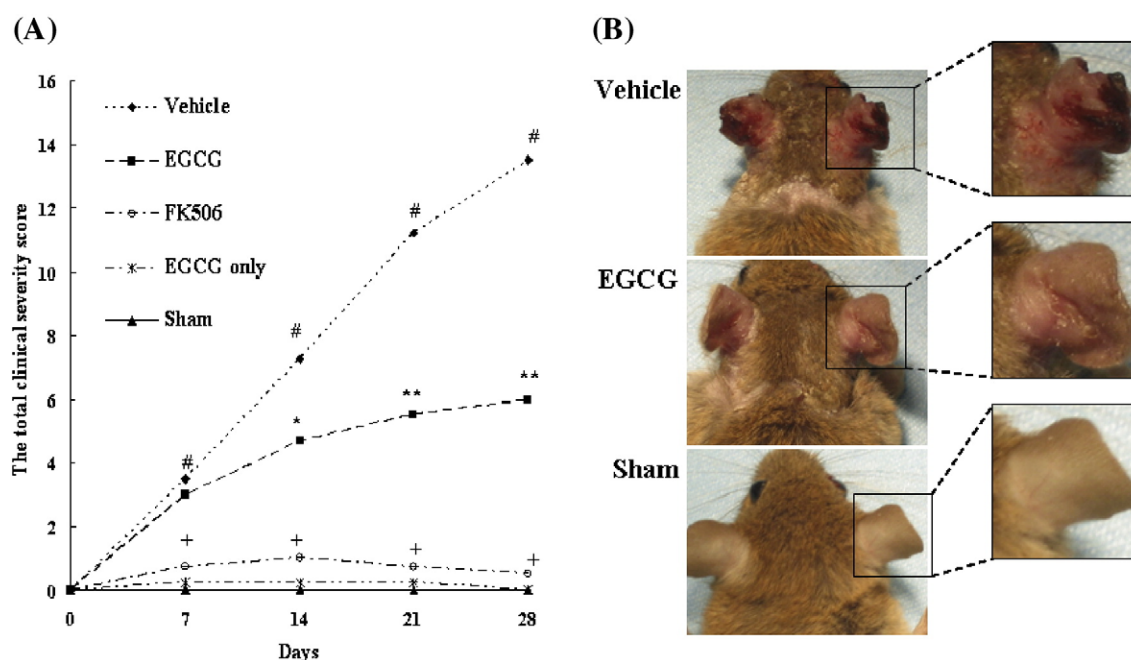


Figure 2 Changes of the total clinical severity score by topical EGCG treatment. DPE application on the ears of NC/Nga mice for 3 weeks induced the AD-like skin lesions. The total clinical severity score was defined as the sum of scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five aspects: itch, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. (A) Topical application of EGCG significantly lowered the total clinical severity scores provoked by DPE. (B) Photographs revealed that topical EGCG treatment significantly improved the clinical signs such as erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness on day 28. The squares indicate the enlarged ear features. Data are shown as mean $\pm$ S.D. of the changes in the total clinical severity scores on the face, ears, nose and neck of five mice of each group. # p <0.001 compared with the sham group. * p <0.005, ** p <0.001 compared with the vehicle group. + p <0.001 compared with the vehicle group. DPE = *Dermatophagoides pteronissinus* extract, EGCG = epigallocatechin-3-gallate.

2.4. Processing and preparation of samples

On the last day of experiment, blood was collected from the retro-orbital plexus via heparinized capillary tubes (Scientific Glass, Inc., Rockwood, USA), with the mouse under ether anesthesia. Serum samples were obtained by centrifugation and they were stored at -20°C until use. Enzyme-linked immunosorbent assay (ELISA) was performed for the measuring the serum levels for MIF and IgE. After blood collection, all of the ears were excised and used for the histological observations and the evaluation of the cytokine mRNA expression. One half of the ear was snap-frozen in liquid nitrogen for performing real-time reverse transcription polymerase chain reaction (RT-PCR) for Th1/Th2 cytokines such as MIF, TNF- α , interferon (IFN)- γ , IL-2, IL-4, IL-5, IL-12 p40 and IL-13, and the other half of the ear was embedded in OCT compound for immunohistochemistry for MIF, TNF- α , IFN- γ and IL-4. All the samples were stored at -70°C until further use. Some parts of the ears were fixed with 4% paraformaldehyde (PFA) and embedded in paraffin for hematoxylin and eosin (H&E) staining and immunohistochemistry for CD4+ T cell, and then these samples were stored at 4°C until further use.

2.5. Histological examination

The ear tissues were fixed with 4% PFA, embedded in paraffin and then 5- μm thin sections were made on silane coated micro slides (Muto-glass, Tokyo, Japan). The de-paraffinized skin sections were stained

with H&E. Some sections were rinsed with distilled water and endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H_2O_2) solution diluted with methanol. After rinsing with PBS, they were incubated with 0.01 M citrate buffer (pH 6) at 100°C for 15 min and then, at room temperature for 40 min. Then, nonspecific antigen-antibody reactions were inhibited by 1-hour treatment with 10% normal goat serum (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were reacted overnight at 4°C with purified rat monoclonal antibody against anti-mouse CD4 (1:100, BD PharMingen, San Diego, CA, USA). After rinsing with PBS, they were overlaid with biotinylated anti-rat IgG (1:500, Vector Laboratories, Inc., Burlingame, CA, USA) at room temperature for 1 h. After rinsing with PBS, they were reacted with avidin-biotin-peroxidase complex solution (ABC reagent, R.T.U. Vectorstain®, Vector Laboratories, Inc., Burlingame, CA, USA) at room temperature for 1 h and visualization of enzyme reaction was performed with the Vector® NovaRED™ substrate kit. After the chromogen reactions, the sections were rinsed and then counterstained with hematoxylin. After rinsing, the sections were dehydrated and covered with permount (Fisher Scientific, New Jersey, USA). They were examined by light microscopy to assess the histological changes.

2.6. RNA isolation and real-time RT-PCR

The expressions of mRNA transcripts of MIF, TNF- α , IFN- γ , IL-2, IL-4, IL-5, IL-12 p40, IL-13 and β -actin were evaluated by performing real-time RT-PCR [28]. The sequences of primers used are shown in Table 1 [28,29].

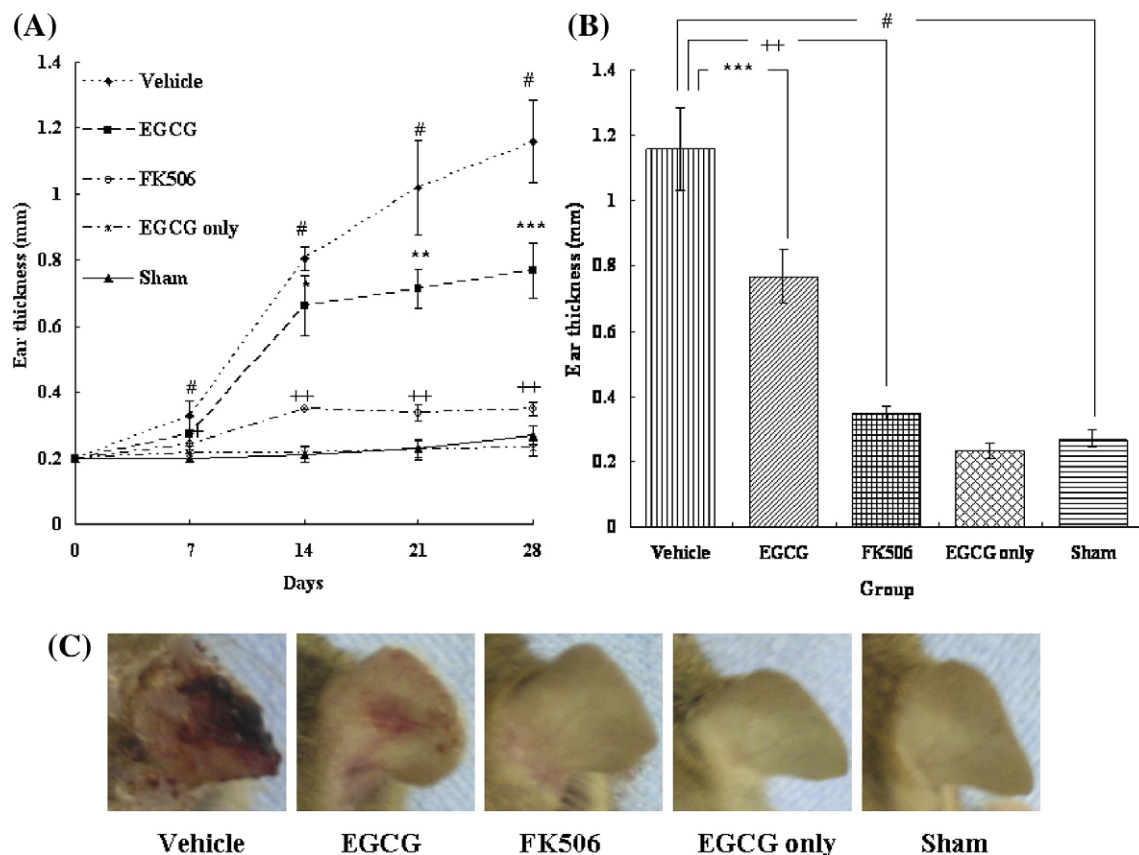


Figure 3 Changes of the ear thickness by topical EGCG treatment. DPE application on the ears of NC/Nga mice for 3 weeks induced the AD-like skin lesions. Ear thickness was measured using a dial caliper every week. (A) Topical EGCG treatment significantly reduced the ear thickness. (B) At the last day of experiment (day 28), the ear thickness of the EGCG group was significantly reduced by 41.6% (0.77 ± 0.08 mm), compared to that of the vehicle group (1.16 ± 0.13 mm). (C) Enlarged ear features of each group. Data are shown as mean $\pm$ S.D. of the changes in the thickness of both ears of five mice of each group. $\#p < 0.001$ compared with the sham group. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ compared with the vehicle group. $+p < 0.05$, $++p < 0.001$ compared with the vehicle group. DPE = *Dematophagoides pteronissinus* extract, EGCG = epigallocatechin-3-gallate.

The ears were ground up and suspended in TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The total RNA was extracted using TRIzol reagent according to the protocol provided by the manufacturer. Equal amounts of 10 µg total RNA was obtained from each sample, and these were reverse-transcribed into cDNA with oligo(dT)₁₅ primers using M-MLV reverse transcriptase (Promega, Madison, WI, USA) at 42 °C for 4 h. The reaction mixtures were amplified with a iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) in the presence of 1 µl of each of the sense and antisense primers for MIF, TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-12 p40 and IL-13, and with the primers for β-actin by using a iCycler iQ Real-Time PCR Detection system (Bio-Rad) with the following thermal cycling programs, cycled 30 times for: step 1 at 94 °C for 30 s; step 2 at 60 °C for 30 s; step 3 at 72 °C for 40 s. The fluorescence intensity was measured in real time by using the optical module and the melting curves were used to determine the product specificity. A relative expression level of the target gene, which was normalized to β-actin was given by $2^{-\Delta\Delta CT}$. All the experiments were performed in duplicate and they were repeated at least three times.

2.7. Immunohistochemical analysis

All the cryostat sections (5 µm) of the ear tissues were placed on silane coated micro slides and then fixed in acetone for immunohistochemical staining. The sections for immunohistochemistry were rinsed with distilled water and then reacted for 15 min with 0.3% H₂O₂ solution diluted with methanol to inactivate the endogenous peroxidase in the tissues. After rinsing with PBS, the nonspecific antigen–antibody reactions were inhibited by 1-hour treatment with 10% normal goat serum. The sections were reacted overnight at 4 °C in diluted solutions of each of the primary antibodies such as purified rat monoclonal antibodies against anti-mouse TNF-α, IFN-γ, IL-4 (1:200, BD PharMingen, San Diego, CA, USA) or rabbit polyclonal MIF antibody (1:50, Santa Cruz Biotechnology, Inc., Delaware, CA, USA) [14,30]. After rinsing with PBS, they were reacted with the secondary antibody solutions, that is, biotinylated anti-rat IgG (1:400) and anti-rabbit IgG (1:200, Vector Laboratories, Inc., Burlingame, CA, USA), at room temperature for 1 h. After rinsing with PBS, the sections were reacted with avidin–biotin–peroxidase complex solution at room temperature for 1 h. For the chromogen reactions, the Vector® NovaRED™ substrate kit was used

with reagent 1, 2 and 3 and H₂O₂ at room temperature for 2–4 min. After the chromogen reactions, the sections were rinsed with distilled water and PBS and then counterstained with hematoxylin. The rinsed sections were made transparent with xylene treatment via dehydration and then they were covered with permount. They were examined by light microscopy to assess the histological changes.

2.8. ELISA

The total IgE in the serum was measured using an enzyme-linked immunosorbent assay kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to the manufacturer's instructions [26]. Briefly, the capture antibodies were plated on a Nunc C bottom immunoplate that was contained in the kit and these plates were incubated 1 h at room temperature. After washing the wells with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0), 200 µl of blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) was added into each well. After 30-min incubation at room temperature, the well was washed; the serum samples (20-fold) and standards were diluted and plated in the wells, and then they were incubated for 1 h. After washing the wells, horseradish peroxidase-conjugated detection antibodies were diluted 5000-fold, transferred to each well and then incubated for 1 h. After washing, the enzyme reaction was initiated by adding substrate solution and the plate was kept for 30 min at room temperature in a dark place. The reaction was terminated by adding 2 M H₂SO₄ and the absorbance at 450 nm was measured with an ELISA reader (Spectra Max250, Molecular Devices, USA).

ELISA for MIF was performed as the direct method. For this assay, we used recombinant mouse MIF (R&D System, Inc., Minneapolis, MN, USA) to obtain the standard curve, for which good linearity was demonstrated between the MIF contents (0 to 250 ng/ml) and the absorbency [31]. Briefly, the serum samples (10-fold) and standards were diluted, plated in wells (Costar High Binding Type I plate, Corning Incorporated Corning, NY, USA). Anti-human MIF polyclonal antibodies (R&D System, Inc., Minneapolis, MN, USA) were diluted 100-fold and horseradish peroxidase-conjugated donkey anti-goat IgG secondary antibodies (R&D System, Inc., Minneapolis, MN, USA) were diluted 1000-fold. All the experiments were performed in duplicate and repeated at least three times.

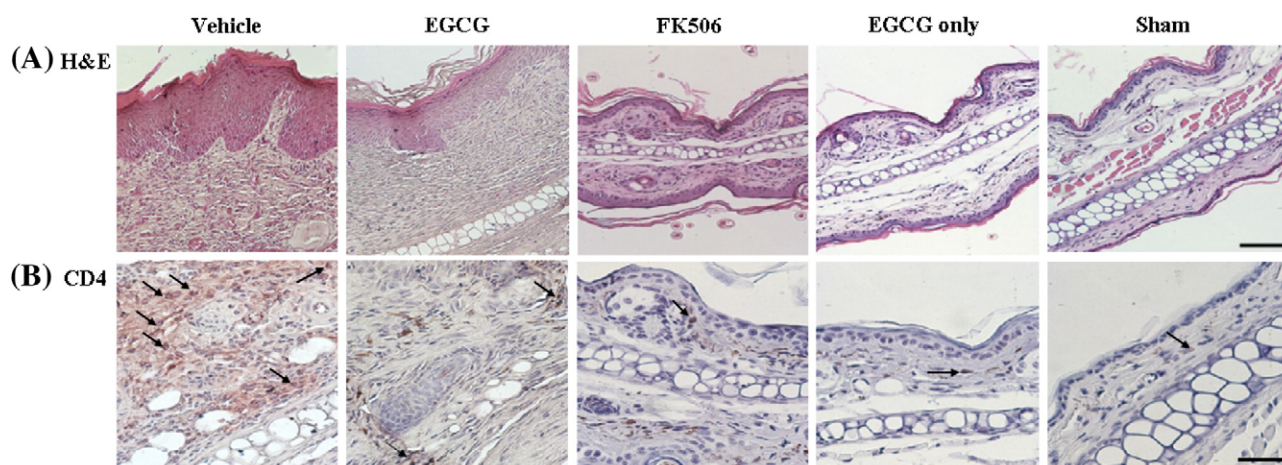


Figure 4 Histological features on the AD-like skin lesions topically treated with EGCG. DPE application on the ears in NC/Nga mice for 3 weeks induced the AD-like skin lesions. At the last day of experiments, the ear tissues were prepared for hematoxylin and eosin (H&E) staining and immunohistochemistry for CD4+ T cell. (A) Topical application of EGCG markedly reduced epidermal hyperplasia as well as mononuclear cell and eosinophil infiltration into the dermis provoked by DPE. Sections were observed at an original magnification of $\times 200$, scale bar = 60 µm. (B) Topical application of EGCG markedly reduced CD4+ T cell infiltration into the dermis provoked by DPE. Arrows (→) indicate CD4+ T cells. Sections were observed at an original magnification of $\times 400$, scale bar = 30 µm. A representative picture from three independent H&E and CD4 staining is shown. DPE = *Dermatophagoides pteronissinus* extract, EGCG = epigallocatechin-3-gallate.

2.9. Statistical analysis

The statistical analysis of the differences in the means for each experimental group of animals was carried out using the ANOVA test. SPSS v10.0 (SPSS Inc., Chicago, IL, USA) was used for all calculations. All the values are expressed as means and standard deviations. Differences were considered significant for $p < 0.05$.

3. Results

3.1. Topical application of EGCG significantly reduces the severity of AD-like skin lesions induced by DPE in NC/Nga mice

To evaluate the therapeutic effect of EGCG on the AD-like skin lesions, we scored the clinical signs and symptoms on the face, ears, nose and neck according to the criteria of five major clinical

signs and symptoms of AD; itch, erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness [24]. Immediately after topical application of DPE, the clinical signs and symptoms seen in the vehicle group began with itching, erythema and hemorrhage, followed by edema, superficial erosion, deep excoriation, scaling and dryness of the skin. The total clinical severity scores were significantly increased with time and reached a clinical score of more than 13 points in the vehicle group ($p < 0.001$), compared to those of the sham group. On the other hand, topical application of DPE and EGCG significantly lowered the total clinical severity scores ($p < 0.005$), compared to that of DPE and vehicle (Fig. 2A). At the last day of the experiment, the clinical signs such as erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness were rarely observed in the EGCG group (Fig. 2B). Topical application of DPE and FK506 ointment used as an active therapeutic control in this study also significantly lowered the total clinical severity scores ($p < 0.001$), compared to that of DPE and

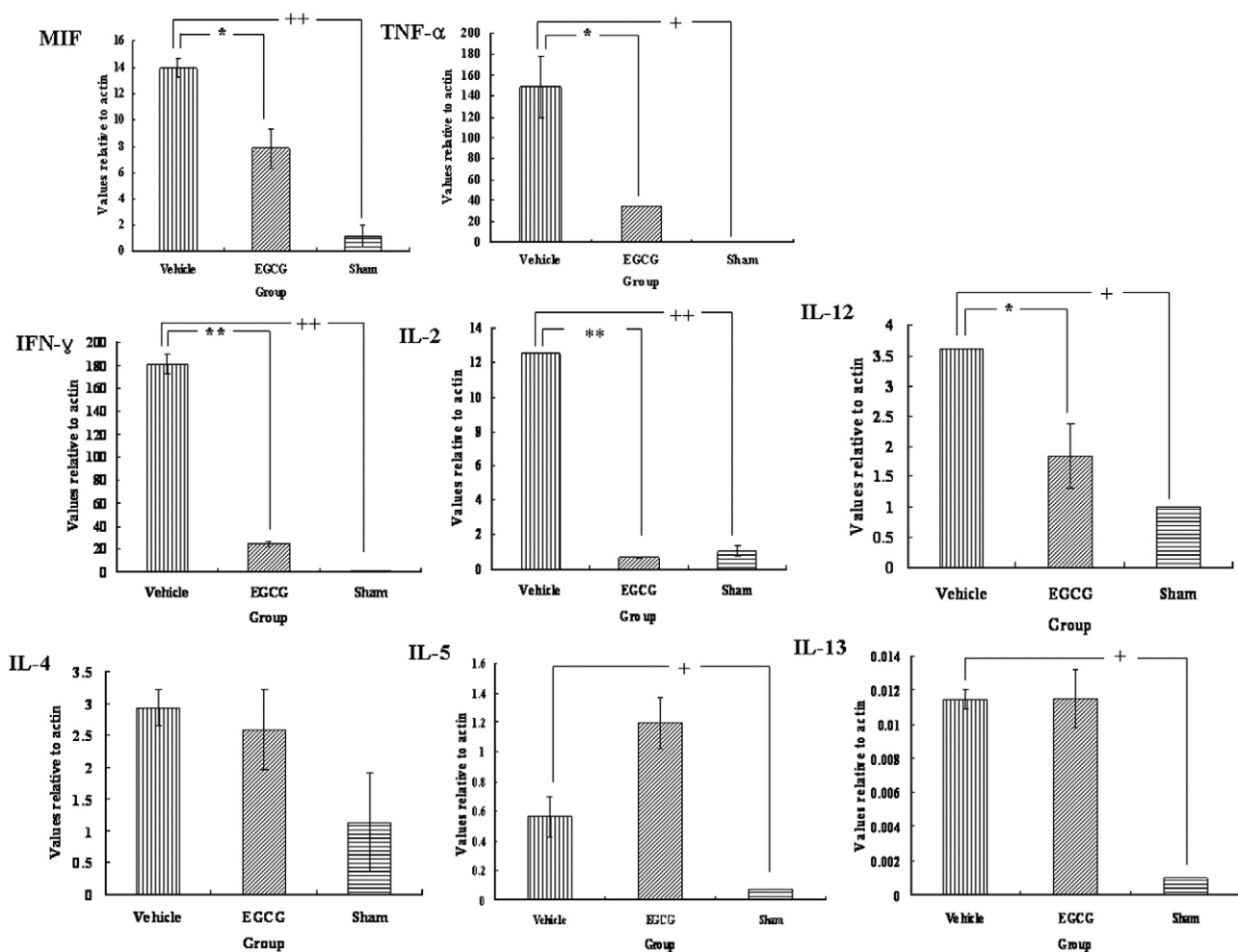


Figure 5 Effect of topical EGCG treatment on the mRNA expressions of MIF, TNF- α , IFN- γ , IL-2, IL-4, IL-5, IL-12 and IL-13 on the AD-like skin lesions. DPE application on the ears of NC/Nga mice for 3 weeks induced the AD-like skin lesions. 10 μ g of total RNA obtained from each ear sample was reverse-transcribed into cDNA. The real-time PCR analysis was performed with a SYBR Green assay system. The quantity of gene expression normalized to β -actin was shown by $2^{-\Delta\Delta CT}$. Topical application of EGCG significantly down-regulated the mRNA over-expressions of MIF, TNF- α , IFN- γ , IL-2 and IL-12, but not that of IL-4, IL-5 and IL-13 on the AD-like skin lesions. Each experimental group consisted of five mice. Quantitative RT-PCR was performed in duplicate to minimize experimental error on the value calculated. All columnar values were expressed as means and standard deviations. A pattern of results were analyzed by repeating at least three times. + $p < 0.05$, ++ $p < 0.005$ compared with the sham group. * $p < 0.05$, ** $p < 0.005$ compared with the vehicle group. DPE = *Dermatophagoides pteronissinus* extract., EGCG = epigallocatechin-3-gallate.

vehicle. The EGCG only group and the sham group commonly didn't show any changes of the total clinical severity scores until day 28.

3.2. Topical application of EGCG significantly reduces the ear swelling induced by DPE in NC/Nga mice

To further evaluate the therapeutic effect of EGCG on the AD-like skin lesions, we measured the ear thickness of NC/Nga mice that were treated with DPE along with EGCG or vehicle only. Thickening of the ear induced by the topical application of DPE and vehicle was first observed on day 7, it steeply increased on day 14, and then it steadily increased until day 28, compared to that of the sham-treated NC/Nga mice ($p < 0.001$). To the contrary, topical application of DPE and EGCG slowly decreased the ear thickness until day 14 ($p < 0.05$); afterward, topical application of DPE and EGCG significantly reduced the ear thickness on day 21 ($p < 0.005$) and 28 ($p < 0.001$), compared to that of DPE and vehicle (Fig. 3A). At the last day of the experiment, the ear thickness of the EGCG group was definitely reduced by 41.6% (0.77 ± 0.08 mm), compared to that of the vehicle group ($1.16 \pm$

0.13 mm) (Fig. 3B). Topical application of DPE and FK506 ointment used as an active therapeutic control in this study also significantly reduced the ear thickness until day 28 ($p < 0.001$), compared to that of DPE and vehicle. The EGCG only group and the sham group commonly didn't show any changes of the ear thickening until day 28.

3.3. Topical EGCG treatment also histologically suppresses the inflammation induced by DPE

To histologically evaluate the therapeutic effect of EGCG on the AD-like skin lesions, we performed H&E staining on specimens of the ears of the NC/Nga mice. As shown in Fig. 4A, sections of the ears in the vehicle group revealed a hyperplastic epidermis and edematous dermis, with a large number of infiltrated mononuclear cells together with some eosinophils. In contrast, sections of the ears in the EGCG group showed markedly diminished epidermal hyperplasia and cellular infiltration into the dermis.

We also conducted immunohistochemistry for CD4 to assess whether topical application of EGCG has an effect on the T cell

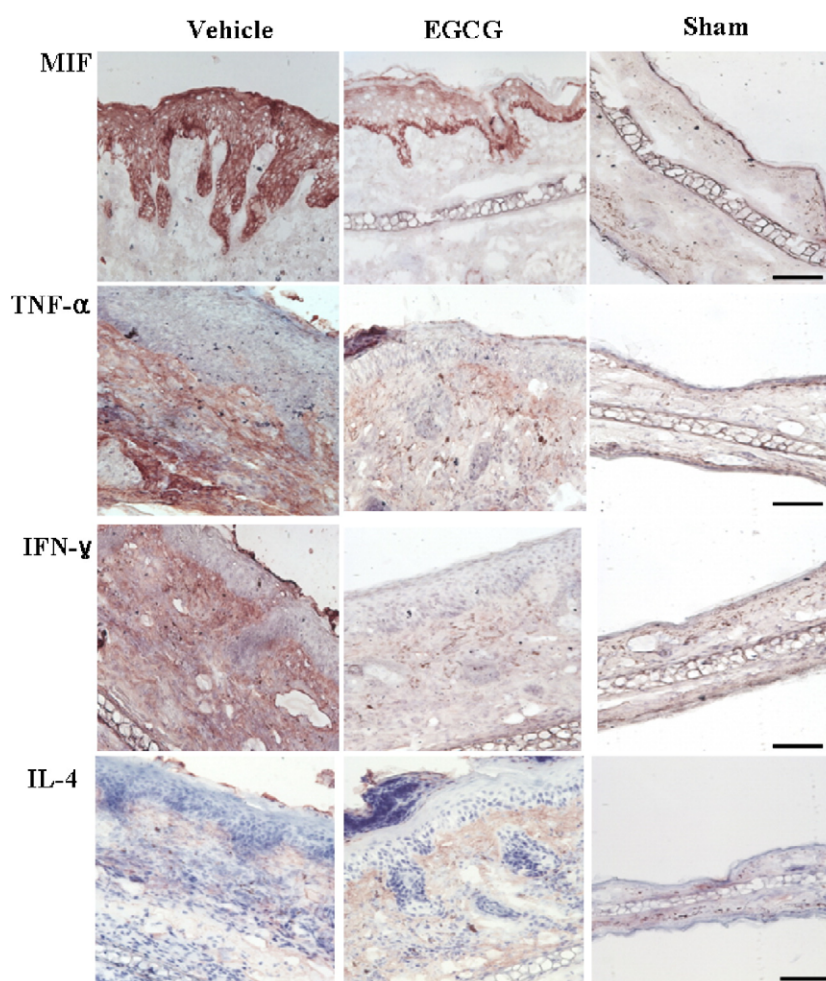


Figure 6 Effect of topical EGCG treatment on the expression of MIF, TNF- α , IFN- γ and IL-4 on the AD-like skin lesions. DPE application on the ears of NC/Nga mice for 3 weeks induced the AD-like skin lesions. At the last day of experiments, the ear tissues were prepared for immunohistochemistry. Topical application of EGCG markedly diminished the over-expression of MIF, TNF- α and IFN- γ on AD-like skin lesions. There was little difference in the IL-4 expression between the vehicle group and the EGCG group. Sections were observed at an original magnification of $\times 200$, scale bar = $60 \mu\text{m}$. A representative picture from three independent immunohistochemistry is shown. DPE = *Dermatophagoides pteronissinus* extract, EGCG = epigallocatechin-3-gallate.

population infiltrating into the AD-like skin lesions. As shown in Fig. 4B, CD4+ T cells infiltrating into the dermis were increased in the AD-like skin lesions in the vehicle group. In contrast, they were markedly decreased with topical application of EGCG. These results showed that topical application of EGCG histologically suppressed the inflammation induced by DPE.

Epidermal hyperplasia, inflammatory cell infiltration into the dermis or CD4+ T cells infiltration were rarely observed in the FK506 group. Also, the EGCG only group and the sham group didn't show any appearances relevant to the inflammation.

3.4. Topical EGCG treatment significantly down-regulates the mRNA expression of cytokines such as MIF, TNF- α , IFN- γ , IL-2 and IL-12 p40, but not that of IL-4, IL-5 and IL-13 on the AD-like skin lesions

To further investigate the effect of EGCG treatment on the inflammatory or regulatory cytokines related to AD, the cytokine mRNA levels for MIF, TNF- α , IFN- γ , IL-2, IL-4, IL-5, IL-12 p40 and IL-13 were quantitatively measured by real-time RT-PCR. As shown in Fig. 5, both the MIF ($p < 0.005$) and TNF- α mRNA expressions ($p < 0.05$) were significantly increased in the vehicle group, as compared to that of the sham group. The mRNA expressions of cytokines such as IFN- γ ($p < 0.005$), IL-2 ($p < 0.005$) and IL-12 p40 ($p < 0.05$) were also significantly up-regulated in the vehicle group, as compared to that of the sham group. In contrast, the mRNA expressions of MIF ($p < 0.05$), TNF- α ($p < 0.05$), IFN- γ ($p < 0.005$), IL-2 ($p < 0.005$) and IL-12 p40 ($p < 0.05$) were significantly down-regulated in the EGCG group, as compared to that of the vehicle group. On the other hand, there was no significant difference in the IL-4, IL-5 and IL-13 mRNA expression between the two groups. These results indicated that topical application of EGCG significantly decreased the mRNA expression of cytokines such as MIF, TNF- α , IFN- γ , IL-2 and IL-12 p40, but not that of IL-4, IL-5 and IL-13 on the AD-like skin lesions.

3.5. Topical application of EGCG markedly diminishes the over-expression of MIF, TNF- α and IFN- γ in the AD-like skin lesions

We performed immunohistochemistry for MIF, TNF- α , IFN- γ and IL-4 to assess the effect of EGCG treatment on the expression of the key regulatory cytokines in the AD-like skin lesions. As shown in Fig. 6, MIF was strongly expressed throughout the whole epidermal layer on the AD-like skin lesions in the vehicle group, as compared to the sham group, in which the MIF expression was mostly limited to the basal layer of the epidermis. On the other hand, the MIF expression in the EGCG group was weakly observed on the epidermis at large and it was relatively deeply expressed on the basal layer of the epidermis. These results showed that topical EGCG treatment decreased the expression of MIF on the AD-like skin lesions. In addition, we also investigated the effect of EGCG treatment on the expression of TNF- α and IFN- γ in the AD-like skin lesions. Both TNF- α and IFN- γ were strongly stained in the dermis of the AD-like skin lesions in the vehicle group. In contrast, the expressions of TNF- α and IFN- γ in the EGCG group were diminished in the dermis of the AD-like skin lesions. These results showed that topical EGCG treatment also decreased the expression of TNF- α and IFN- γ on the AD-like skin lesions. On the other hand, there was little difference in the expression of IL-4 between the vehicle group and the EGCG group.

3.6. Topical EGCG treatment significantly reduces the elevated MIF and IgE production in the serum induced by DPE in the NC/Nga mice

We performed ELISA to examine the effect of EGCG treatment on MIF and the IgE production in the serum induced by DPE in the NC/Nga mice. As shown in Fig. 7, the serum MIF ($p < 0.005$) and IgE ($p < 0.005$) levels of the vehicle group were significantly increased, as compared with those of the sham group. Interestingly, the serum MIF ($p < 0.005$) and IgE ($p < 0.005$) levels in the EGCG group were significantly reduced, compared to

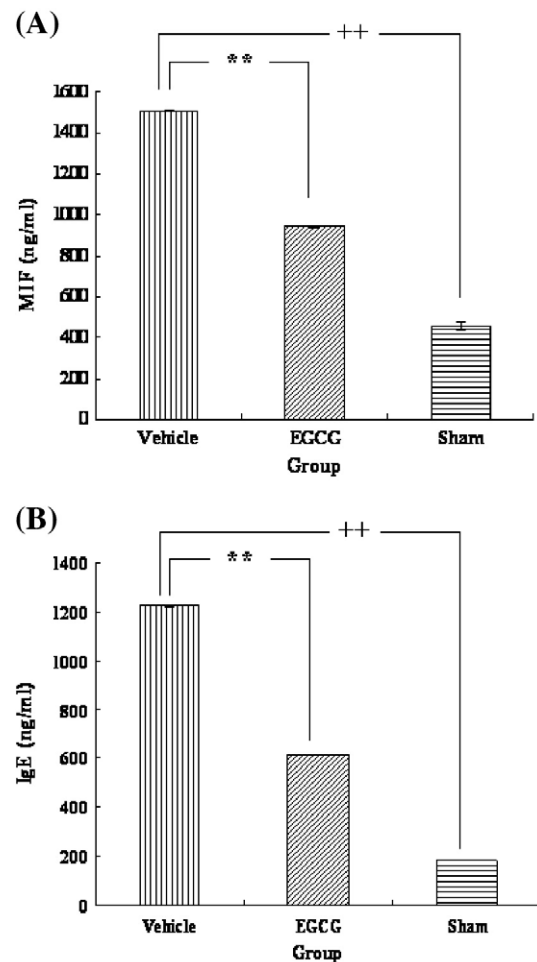


Figure 7 Effect of topical EGCG treatment on MIF and the IgE production in the serum induced by DPE in the NC/Nga mice. DPE application on the ears of NC/Nga mice for 3 weeks induced the AD-like skin lesions. At the last day of the experiments, each blood sample was collected from the retro-orbital plexus by heparinized capillary tubes and the serum MIF and IgE were quantified by ELISA. Topical application of EGCG significantly reduced the serum levels of MIF (A) and IgE (B), which were increased by DPE. Each experimental group consisted of five mice. ELISA was performed in duplicate to minimize experimental error on the value calculated. All columnar values were expressed as means and standard deviations. A pattern of results were analyzed by repeating at least three times. ++ $p < 0.005$ compared with the sham group. ** $p < 0.005$ compared with the vehicle group. DPE = *Dermatophagoides pteronissinus* extract, EGCG = epigallocatechin-3-gallate.

those of the vehicle group. These results indicated that even the topical application of EGCG also had a significant suppressive effect on the MIF and IgE production in the serum, which was increased by DPE in the NC/Nga mice.

4. Discussion

In this study, we first analyzed the therapeutic effect of EGCG by painting it directly on the AD-like skin lesions induced by DPE in the NC/Nga mice. Although the therapeutic effects of EGCG were less than those of FK506 used as an active control, topical application of EGCG significantly reduced the ear thickness and it also significantly lessened the clinical signs provoked by DPE, such as erythema, edema, scaling and excoriation. Histologically, topical EGCG treatment markedly decreased the epidermal hyperplasia, and the mononuclear cell and eosinophil infiltration into the dermis of the AD-like skin lesions. The number of CD4⁺ T cells was also markedly reduced with topical EGCG treatment. These results demonstrate that topical EGCG application has an anti-inflammatory effect on the AD-like skin lesions in NC/Nga mice.

Next, we attempted to determine whether EGCG exerts the anti-inflammatory effect by inhibiting MIF and the other cytokines related to immune dysregulation in AD. Quantitative RT-PCR results revealed that topical application of EGCG significantly down-regulated the mRNA over-expression of MIF, TNF- α , IFN- γ , IL-2 and IL-12 p40 but not that of IL-4, IL-5 and IL-13 on the AD-like skin lesion. Immunohistochemical analysis of skin lesions also showed that topical application of EGCG markedly diminished the over-expression of MIF, TNF- α and IFN- γ on the AD-like skin lesions, but not that of IL-4. These results indicated that topical application of EGCG can suppress MIF and other Th1-related cytokines on the AD-like skin lesions.

It is currently known that MIF is a pro-inflammatory cytokine that plays an essential role in the activation of T cells after antigenic stimulation [15,32]. The secreted MIF binds to its receptor CD74, which is expressed mainly by MHC II⁺ cells, and the antigen presenting cells such as macrophages, lymphocytes, dendritic cells and endothelial cells [33–35]. MIF thereby stimulates Th1 immune cells and induces inflammatory cytokines, resulting in an exaggerated inflammatory response [17–19]. Clinically, it has been reported that MIF is closely involved in the exacerbation of allergic disease [36–42]. Moreover, Bacher et al. [32] have shown that activated T cells produce MIF, neutralizing anti-MIF antibodies inhibit T cell proliferation, and IL-2 and IFN- γ production, and the expression of MIF mRNA is limited to the Th2 subset. Those researchers indicated that MIF released from the Th2 subset causes the Th1/Th2 imbalance.

From our results along with those of previous reports, it might be assumed that topical application of EGCG can down-regulate the over-expression of Th1 cytokines such as IL-2, IFN- γ and IL-12 p40 by suppressing the elevated MIF expression in the AD-like skin lesions provoked by DPE, resulting in restoring the immunological AD deviation by counterbalancing the Th1-predominant chronic inflammation on the AD-like skin lesions. Thus, MIF might be one of the principal cytokines that polarize immunological deviation in the AD-like skin lesions. Moreover, our results that EGCG can suppress the MIF expression on the DPE-induced AD-like skin lesions in the NC/Nga mouse model are interesting, and further investigation to determine whether EGCG can suppress the MIF-mediated Th1 reaction will be needed.

Although MIF was expressed throughout the entire epidermis of the AD-like skin lesions in this study, we could not exclude the possibility of the presence of other sources of MIF except for the keratinocytes. Shimizu et al. [31] have shown that peripheral blood mononuclear cells (PBMCs) could be an important source of increased serum levels of MIF in AD patients. Because MIF has the potential to induce local and systemic inflammatory and immune responses, it is conceivable that the MIF produced by PBMCs may affect the local and systemic pathologic features in AD. Furthermore, it was reported that MIF production by PBMCs obtained from 5 patients with AD decreased as clinical symptoms improved with steroid ointment treatment [31].

Likewise, our results showed that topical application of EGCG significantly decreased the levels of MIF and IgE in the serum. Based on these results, it might be assumed that topical application of EGCG has systemic effects as well as local effects on the AD-like skin lesions in NC/Nga mice. Moreover, the skin barrier of AD has been known to be defective and easy to penetrate. However, we have no convincing evidence that EGCG exerts systemic effects by direct absorption from skin lesions. While topical application of EGCG locally suppressed inflammation on the AD skin lesions, unknown another signal stimulated by EGCG may have systemically extended anti-inflammatory effect. Also, in the light of the previous reporting on the reduction of serum IgE on the MIF knockout mice [40], the down-regulation of serum MIF might improve the allergic inflammation of AD. In this study, although we could not determine the exact mechanism, our results that topical application of EGCG significantly decreased the levels of MIF and IgE in the serum are interesting.

For a long time, therapeutic strategies of AD have been dominated by the application of local or systemic steroids. Yet, the emerging evidence that steroids can induce MIF *in vitro* [20,43] and *in vivo* [44] limits their clinical usefulness. It has been also known that MIF possesses the unusual ability to tautomerize the phenylpyruvate, and this causes chronic inflammation, when phenylpyruvate accumulates in the tissues or in blood because of the abnormal metabolism of phenylalanine. Orita et al. [45] and Lubetsky et al. [46] have reported that the discovery of inhibitors of the tautomerase active site of MIF has potential for inducing novel anti-inflammatory action. Considering our results that EGCG suppressed the over-expression of MIF in the AD-like skin lesions in NC/Nga mice, EGCG might lead to a better exploitation of their putative steroid-sparing potency in AD therapy and EGCG might be one of the promising MIF antagonists that can bind to the catalytic site of MIF. However, further experiments will be necessary to analyze how EGCG overcomes the expression of MIF induced by steroids, and whether the tautomerase activity of MIF is linked to its physiological function.

In conclusion, our results demonstrated that topical application of EGCG could improve the AD-like skin lesions in NC/Nga mice by suppressing MIF and other Th1-related cytokines. Taken together, it is suggested that topical application of EGCG may be a novel approach to the treatment of AD.

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