한방안이비인후피부과학회지 제18권 제1호(2005년 4월)
The Journal of Korean Oriental Medical Ophthalmology & Otolaryngology & Dermatology 2005;18(1):27-49

Effects of Tang-gwi-eum-za-gagambang along with External Spray Therapy on the Spontaneously Occurring Atopic Dermatitis Development in NC/Nga Mouse

Sung-Hun Kim · Jung-Hwa Choi · Jong-Han Kim · Su-Yeon Park

Dept. of Ophthalmology & Otorhinolaryngology & Dermatology, Oriental Medical College, Dongshin University

當歸飮子加減方과 外治方 병용이 NC/Nga 아토피 생쥐에 미치는 영향

김성훈 · 최정화 · 김종한 · 박수연

當歸飮子加減方과 外治方 병용의 아토피 치료 기전을 규명하고자 NC/Nga 생쥐의 동물 병태 모델을 이용하여 다양한 면역 반응을 관찰하였던 바, 다음과 같은 결론을 얻었다.

1. NC/Nga 생쥐의 피부손상 정도는16주와 20부에 대조군에 비해 36.0%, 37.8% 감소하였다.
2. NC/Nga 생쥐의 血中 IgE, IL-4, IL-5, IL-6, IgM, IgG1 및 IgG2a 수준은 대조군에 비하여 유의성 있게 감소하였고, IL-13 수준은 대조군에 비하여 감소하였으나 유의성을 나타내지 않았다. 반면, IFN-γ 수준은 유의성 있게 증가하였다.
3. NC/Nga 생쥐의 비장 무게는 대조군에 비하여 유의성있게 감소하였다.
4. NC/Nga 생쥐의 lymph node에서 B/f ratio는 증가된 대조군에 비하여 감소하였으며, $CD4^+$와 $CD8^+$ 세포 발현은 대조군에 비하여 증가하였고, $CD4^+$는 유의성있는 감소를, $CD8^+$는 유의성 없는 약간의 증가를 나타내었다. $CD69^+$, CD11a 세포 발현은 대조군에 비하여 유의성있게 감소하였다.
5. NC/Nga 생쥐의 피부조직배양에서 IL-4, IL-5, CCR3 유전자 발현은 대조군에 비하여 현저히 감소하였고, IL-6, IL-13, $CD69^+/CD3\varepsilon^+$, $CD19^+/CD44^+$ 발현량은 유의성있게 감소하였으며, IFN-γ의 유전자 발현은 대조군에 비하여 증가하였다.
6. NC/Nga 생쥐 귀, 목의 피부 조직 변화에서는 표피와 진피의 염증 정도와 침윤된 염증 면역세포 등이 대조군에 비하여 현저하게 감소되었다.
7. Lymphokine assay에서 IL-4 발현량은 대조군에 비하여 유의성 있게 減少하였고, IFN-γ의 발현량은 유의성 있게 증가하였다.

주제어 : 當歸飮子加減方, 아토피피부염

교신저자: 최정화. 동신대학교부속광주한방병원
안이비인후피부과학교실
(Tel. 062-350-7217. E-mail: mkyu0@hanmail.net)

Introduction

Atopic dermatitis (AD) is one of the most common skin diseases during infancy and childhood with a family history of atopy[1,2] and is frequently associated with elevated IgE levels in the serum against many kinds of inhaled allergens[3,4]. AD is characterized by itching and the patients having family history of atopic diseases are known to higher epidemiological incidence[5,6], suggesting that the onset of AD is influenced by genetic as well as environmental factor(s).

Although pathogenesis underlying AD is largely unknown, several lines of studies strongly implicate the involvement of abnormal regulation of immune system. Mast cells in the skin lesions are involved in the pathogenesis of AD[3,7-11]. IgE-mediated mast cell activation leads to release of various kinds of chemical mediators, which results in infiltration of inflammatory cells into the skin lesion, such as eosinophils and lymphocytes. IL-4 is able to exert switching signals on B cells for IgE synthesis and the IL-4-dependent IgE synthesis is promoted by IL-5[12]. Both mast cells[13-15] and Th2 helper T cells[16,17] can generate and secrete these cytokines. On the other hand, Th1 helper T cells are known as effector cells in contact sensitivity[18] produce IFN-γ that has a potent activity to suppress IgE synthesis by B cells[19] and proliferation of Th2 helper T cells[20]. In patients with AD, decreased production of IFN-γ is considered to be associated with IgE hypersynthesis and Th2 immune responses[21].

Considering increasing cases of atopy patients in a broad range of age group including newborn babies, it would be critical to understand the precise cause of AD and thus develop drugs. In the present study, an attempt examining the effects of the oriental medicinal drugs Tang-gwi-eum-za-gagambang (TG) and Oe-chi-bang (ET) on atopy rash has been made using a genetic AD model mouse called NC/Nga. TG, which was used as an internal administration is Tang-gwi-eum-za augmented with Angelicae dahuricae radix (白芷), Angelicae pubescentis radix (獨活), and Cyperi rhizoma (香附子). In the oriental medicine, Tang-gwi-eum-za is known to relieve fever, complement the blood and body fluid, and thus has been used for the cure of diverse skin diseases. Combined prescription of Fagopyrum esculentum Moench (蕎麥), Oleum Terebinthinae (松津), Gypsum Fibrosum (石膏) and Bamboo salt (竹鹽) as an ET is known to be effective for fever relief, detoxification, antiseptic function and also complementation of blood and body fluid. Thus, by applying TG and ET at the same time, clinical effects and related mechanism were aimed to examine in an NC/Nga mouse model.

By administration of TG and ET at the same time into the NC/Nga mice, we found that the levels of major chemical mediators for AD development can be controlled. These findings further imply potential therapeutic application of the present experimental approach.

Material and Methods

1. Materials

1) Reagents and apparatus

(1) Reagents

The reagents used in the present study are as follows; diethyl pyrocarbonate (DEPC), 3-4,5- dimethyl-thiazol-2,5-carboxymethoxy phenyl-2,4-sulfophenyl- 2H-

tetrazolium (MTS), 2,7,-dichl-orodihydro-fluorescein diacetate (DCFH-DA), NH_4Cl, $KHCO_3$, Na_2 EDTA, collagenase IV, complete adjuvant, chloroform, RPMI-1640 medium, isopropanol, RBC lysis solution, ethidium bromide, dulbecco's phosphate buffered saline (D-PBS), dulbecco's minimum essential medium (DMEM), formaldehyde, formamide, and magnesium chloride ($MgCl_2$) were all purchased from Sigma (USA). Fetal bovine serum (FBS) was from Hyclone (Logan, Co., U.S.A), agarose (FMC, U.S.A), anti-$CD28^+$ and anti-$CD3\varepsilon^+$ was from Serotec (Kidlington, UK), $RNAzol^B$ from Tel-test (Friendswood, USA), RNase *inhibitor* and *Taq* polymerase from Takara (Shimogyo-ku, Japan), random primer, dNTP, Moloney murine leukemia virus reverse transcriptase were all purchased from promega(M-MLV RT, Madison, USA). anti-$CD3\varepsilon^+$-PE (phycoerythrin), anti-$CD4^+$-FITC (fluorescein isothiocyanate), anti-Gr1-PE, anti-$CD8^+$-FITC, anti-$CD25^+$-PE, anti-$CD19^+$-FITC, anti-$CD11b^+$-FITC, anti-$CD44^+$-PE, anti-$CD69^+$-FITC, propidium iodide (PI) and RNase from Pharmingen (Torreyana, Co, U.S.A), IL-6 and TNF-α, and the ELISA kit from R&D system (Minneapolis, U.S.A). Other chemicals were used with the highest quality available

(2) Apparatus

The instruments used in this study are as follows; heat extractor (Daewoong, Korea), rotary vacuum evaporator (Büchi B-480, Switzerland), freeze dryer (EYELA FDU-540, Japan), CO_2 incubator (Forma scientific Co., U.S.A), clean bench (Vision scientific Co., Korea), autoclave (Sanyo, Japan), micro-pipet (Gilson, France), water bath (Vision scientific Co., Korea), vortex mixer (Vision scientific Co., Korea), spectrophotometer (Shimazue, Co., Japan), centrifuge (Sigma, U.S.A), deep-freezer (Sanyo, Co., Japan), thermocycler system (MWG Biotech Co., Germany), ice-maker (Vision scientific Co., Korea), homogenizer (OMNI, U.S.A), plate shaker (Lab-Line, Co., U.S.A), flow cytometer (Becton Dickinson, U.S.A), ELISA reader (Molecular Devices, Co., U.S.A) Mab-Based Mouse Ig isotyping kit (PharMingen, San Diego, Calif) 24-well Costar plate (Corning Inc, Cambridge, Mass) and Primus 96 Legal PCR system (MWG Biotech Co., Germany).

2) Experimental animals

C57BL/6, Balb/C female mice (6 weeks old) were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB) and NC/Nga atopic dermatitis mice (AD, 6 weeks old male) were obtained from SLC (Shizuoka, Japan). Animals were housed in the room where the temperature was maintained at 22±2℃ and the humidity was 55±15 % at 12 hr of day (light intensity: 200-300 Lux) and dark cycle. Animals were adjusted to the environment for 2 weeks before the experiment. The composition of food pellets (Samyang Co., no antibiotics added) contains crude protein (higher than 22.1 %), crude fat (less than 8.0 %), crude fibers (less than 5.0 %), crude minerals (less than 8.0 %), calcium (less than 0.6 %), phosphorus (higher than 0.4 %). The food and water were supplied with no limitation.

3) Drugs

The drugs Tang-gwi-eum-za-gagambang (TG) and oe-chi-bang (ET) used in this study were obtained from Daejeon University Oriental medicine hospital, and used after purification. The composition of a seal of the TG & ET prescription used in the present study is as follows (Table 1, Table 2).

Table 1. Prescription of Tang-gwi-eum-za-gagambang (TG)

Scientific name	Amount (g)
Atractylodis Rhizoma	12
Coicis Semen	12
Angelicae gigantis Radix	6
Rehmanniae Radix Preparat	6
Citri Pericarpium	6
Paeonia Radix Alba	4
Polygoni multiflori Radix	4
Cinnamomi Ramulus	4
Schizonepetae Herba	4
Angelicae dahuricae Radix	4
Angelicae pubescentis Radix	4
Tribuli Fructus	4
Cyperi Rhzoma	4
Glycyrrhizae Radix	2
Total Amount	76

Table 2. Prescription of external spray treatment (ET)

Scientific name	Amount (g)
Fagopyrum esculentum Moench	17.0
Oleum Terebinthinae	22.5
Gypsum Fibrosum	15.0
Bamboo salt	25.0
Total Amount	79.5

2. Procedure

1) Drug preparation

The TG (76 g) was suspended in 2000 ml of distilled water, heat extracted for 3 hr, and filtered. The filtrate was concentrated by vacuum evaporation using the rotary evaporator and freezing drying with the freeze dryer. The product obtained was kept at -84 ℃, and used after appropriate dilution. The ET used as external therapy by spraying was prepared as follows. A mixture of Fagopyrum esculentum Moench (17 g), Oleum Terebinthinae(pine tree resin) (22.5 g), gypsum (15 g), and salt baked in the inside of bamboo tree stem (25 g) in 1200 ml of H_2O was boiled for 3 hr and further heated for 24 hr until complete evaporation of water. Then, 2.7 liters of the extract of the Japanese apricot (Hanmong Mall Co., Korea; composed of 50 % of Japanese apricot, 45 % of sugar, and 5 % of oligosaccharide) were added to it and it is filtered the filtrate was used for spraying treatment.

2) Drug administration into the NC/Nga atopy dermatitis mice

NC/Nga mice were maintained under the conventional non-sterile environment to provide the favorable condition for the induction of AD as describe previously[22]. TG (385 mg/kg) was given to NC/Nga atopy dermitis mice (8 weeks old) by oral administration TG and ET spray treatment. ET vapor was consistently sprayed at 11 am everyday on the neck on a daily basis. In human, regular and persistent stimulation induces an intense inflammation and dermatitis atopy. Thus, similar pathological outcomes were generated by rubbing on the skin with a sandpaper three times a week (Mondays, Wednesdays, and Fridays) for several weeks.

3) Clinical skin severity[22]

The clinical severity of atopic dermatitis disorders were defined by classifying into 4 steps; 0 for none, 1 for mild, 2 for moderate, and 3 severe by evaluating the following 5 signals and symptoms including itch, erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness. The symptoms were evaluated by observing skin dryness, eruption and injury on the body parts such as the ears, face, head and back.

4) Cytokine assay in the serum

About 100 $\mu\ell$ of blood was taken from the eyes using the capillaries from 8, 12, 16, and 20 week old mice, and IgE amount in the serum was determined. Similarly, blood was taken directly at the heart from 15

weeks old NC/Nga mice after anesthetized with ethyl ether, and IL-4, 5, 6, 13 and INF-γ levels in the serum were determined by ELISA. IgM, IgG1, and IgG2a amount in the serum was measured by using monoclonal antibody-based Mouse Ig isotyping kit.

5) Fluorescence Activated Cell Sorting analysis (FACS analysis)

Spleen and lymph node tissues were removed from 15 weeks old NC/Nga mice, and cells were separated using 100 mesh. Cells were washed twice after 5 mins centrifugation at 1700 rpm with D-PBS and dissociated cells were selected by passing through the cell strainer (Falcon) to remove cell debris and impure materials. Cells were treated with buffered ammonium chloride (ACK) solution containing 8.3 g of NH_4Cl and 1 g $KHCO_3$, in 1 liter of 0.1 ml EDTA solution at room temperature for 5 mins to lyse erythrocytes, washed with D-PBS twice, and stained with 0.04 % tryphan blue to count cells. Number of splenocytes and lymph node cells were adjusted to 2×10^5 and used for immunofluorescence staining at 4 ℃. Antibody reaction was performed with phycoerythrin (PE)-anti-mouse CCR3, phycoerythrin (PE)-anti-mouse $CD8^+$, phycoerythrin (PE)-anti-mouse $CD44^+$, anti-$CD^3\varepsilon^+$-FITC, anti-$CD4^+$-FITC, and fluorescein isothiocyanate (FITC)-anti-mouse $CD19^+$ antibodies for 30 mins on ice. After the reaction, cell were washed with PBS more than three times and stained splenocytes and lymph node cells were analyzed by the flow cytometer and determined expression of $CD3\varepsilon^+$, $CD4^+$, $CD8^+$, $CD11a^+$, $CD19^+$ $CD44^+$, $CD69^+$. The ratios of $CD69^+/CD3\varepsilon^+$, $CD19^+/CD44^+$ were obtained by the Cell Quest Program (Becton Dikinson, USA)

6) Reverse Transcription Polymerase Chain Reaction(RT-PCR)

(1) Total RNA extraction from the skin tissue

NC/Nga skin tissues (0.1 g) were fragmented and mixed with 500 μℓ of RNAzol[B] for cell solubilization and 50 μℓ of chloroform ($CHCl_3$) was added to the supernatant for 15 sec. Then the sample was incubated on ice for 15 min and centrifugated at 13,000 rpm to collect the supernatant. 200 μℓ of supernatant was mixed with an equal volume of 2-propanol and then incubated on ice for 15 min. The phase was separated again by centrifugation at 13,000 rpm and the pellet was washed with 80 % of ethanol and vacuum dried for 3 mins. Extracted total RNA was resuspended in 20 μℓ of DEPC water and used for reverse transcription-polymerase chain reaction (RT-PCR).

(2) RT-PCR

Total RNA (3 μg) was denatured for 5 mins at 75 ℃. The denatured RNA was mixed with 2.5 μℓ 10 mM dNPTs mix, 1 μℓ random sequence hexanucleotides (25 pmole/25 μℓ), 1 μℓ RNasin (20 U/μℓ), 1 μℓ 100 mM DTT, 4.5 μℓ 5× RT buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 μℓ M-MLV RT (200 U/μℓ), and DEPC water to bring up the reaction to 20 μℓ. The sample was mixed well and incubated for 60 mins for the synthesis of first stranded cDNA, and the reaction was stopped by placing for 5 mins at 95 ℃.

(3) cDNA PCR

Synthesized cDNA was used for PCR by using Primus 96 Legal PCR system. 3 μℓ of cDNA template, 1.0 μℓ of sense and antisense primers (20 pmole/μℓ each) for β-actin, IL-4, IL-5, IL-6, IL-13, CCR-3, and IFN-γ, 3 μℓ of 2.5 mM dNTPs, 3μℓ of 10× PCR buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), and 0.18 μℓ of Taq. polymerase (5 U/μℓ) were added to the reaction and the reaction volume was adjusted to 30 μℓ. The condition for PCR was pre-denaturation at 95 ℃ for 5 mins, annealing at

55 ℃ for 1 min, elongation at 72 ℃ for 1 min, and denaturation at 95 ℃ for 5 min. After 25 cycles of the reaction, the sample was post-elongated at 72 ℃ for 3 mins. 20 ㎕ of individual PCR products were analyzed by running the agarose gel for 20 mins at 120 volts on 1.2 % agarose gel electrophoresis.

■ The oligonucleotides used as primers were as follows;

Gene	Primer	Sequence
IL-4	sense	5'-CCGTCGATAGTGGCATCCATGAAAC-3'
	antisense	5'-GGACCAATACCTGCTATAGGG-3'
IL-5	sense	5'-AACCCTTACTGAACTCAGATTGTTAG-3'
	antisense	5'-TAAGTCAGTTTAAATGCTTAGGG-3'
CCR3	sense	5'-TTCAAATGAGATTGTGGGAAAAT-3'
	antisense	5'-ACCGATACAGTACAGTACAGTA-3'
IFN-γ	sense	5'-CCGATATTTAGATACGTTAAAC-3'
	antisense	5'-ATGGCCTAGTCAGTCTCTAAAT-3'
β-actin	sense	5'-TGGAATCCTGTGGTCCATGAAAC-3'
	antisense	5'-GTCACAGTCAGCTGTATAGGG-3'

7) IL-6 and IL-13 quantitation in the skin tissues and cell surface molecule analysis by FACS

Facial and skin tissues (1 g each) were removed and washed with DMEM. After chopping into small pieces, the cells were cultivated in 10% FBS-DMEM medium for 7 days, and then the supernatant was removed and fresh 10 % FBS-DMEM medium was added. After culture for 7 days, culture supernatant was used for the analysis of IL-6 and Il-13 by ELISA. Cultured cells were used for immunofluorescence staining of cells. Cell concentration was adjusted to $2\text{-}5\times10^5$ and washed once with PBS (pH 7.4) containing 1 % fetal bovine serum (FBS) and 0.01 % NaN_3 and reacted for 30 min at 4 ℃ with FITC or PE conjugated antibodies such as anti-$CD3\varepsilon^+$-PE, anti-$CD69^+$-FITC, anti-$CD19^+$-FITC, anti-$CD44^+$-PE. After antibody reaction, $CD3\varepsilon^+$, $CD69^+$, $CD19^+$, $CD44^+$ cells were washed twice with buffer and the labeled cells were analyzed by flow cytometer.

8) Histopathological examination

The ear and neck skin tissues were removed from the animal and fixed in 10 % paraformaldehyde solution for 24 hrs. The tissues were embedded into the paraffin and 5 μm thickness of blocks were prepared. The sections were stained with hematoxylin and eosin (H&E) to differentiate inflammation and edema in epidermis, dermis, keratinocytes, neutrophils and eosinophils.

9) Lymphokine assay

Spleen cells (2×10^6/㎖) were plated on 24 well plates and immunoreacted with anti-$CD28^+$-PE (1 ㎍/㎖) antibody (Pharmingen) and anti-$CD3^+$-PE antibody (1 ㎍/㎖). Cells were cultured in the presence of TG and ET (100 ㎍/㎖) for 48 hr. Then, IL-4 and IFN-γ levels were measured by ELISA kit.

10) Statistical analysis

The number data were represented as mean±standard error, and a criterion for the statistical significance was determined by Student's t-test[23].

Results

I. Determination of AD severity in NC/Nga mice after drug administration

NC/Nga mice were maintained under the conventional non-sterile condition for several weeks beginning at the age of 8 weeks old as describe in the Materials and Methods. Fig 1A shows that AD-like skin lesions were developed in NC/Nga mice on the

face, neck, ears and dorsal skin in 12 weeks old animals (Fig 1A).

NC/Nga mice showed increases in severity of dermitis as increases in the exposure periods to the conventional environment. When the animals were treated with TG plus ET, the severity of dermitis was significantly decreased compared to control animals in 8, 12, 16 and 20 weeks old mice (Fig 1B). The skin severity values in non-treated NC/Nga mice were 7.33 ± 2.59 and 11.3 ± 3.04 at 16 and 20 weeks old of age respectively whereas those in the treated NC/Nga mice were 2.04 ± 0.65 and 4.26 ± 1.53 at the corresponding age.

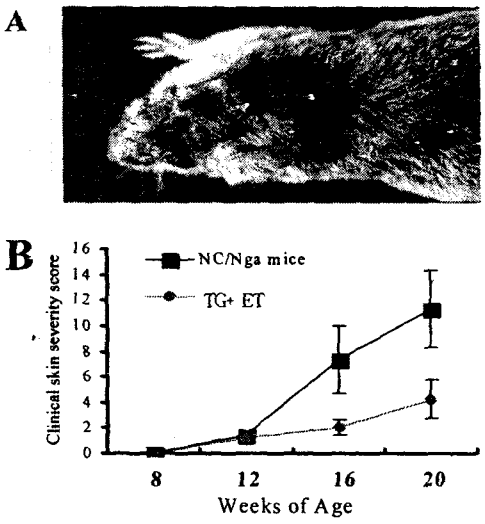

Fig. 1. Clinical skin features and severity of dermatitis in NC/Nga mice

2. Effects of TG and ET on Serum IgE and IL-6 levels in AD mice

As shown in Fig 2, IgE and IL-6 in the serum of non-treated NC/Nga mice were increased as animal's age was increased. IgE levels in μg/ml were 4.6 ± 1.3, 51.2 ± 12.6, 216 ± 59.1, and 365 ± 84 at 8, 12, 16, and 20 weeks old of age respectively, and IL-6 levels in pg/ml were 87 ± 21.2, 305 ± 76.5, 722 ± 89.9, and 798 ± 168.4 at 8, 12, 16, and 20 weeks old of age respectively. But the IgE and IL-6 protein levels in the serum were not increased at the corresponding age groups of the Balb/C mice. Total IgE levels for Blab/c were 0.5 - 1.1 μg/ml between 8 - 20 weeks old of age and IL-6 protein levels were 46 ± 5.5, 50 ± 12, 87 ± 30.2, and 82 ± 21.2 at 8, 12, 16, and 20 weeks old of age respectively, When the NC/Nga mice were treated with TG and ET, IgE and IL-6 levels in the serum were lower than the corresponding NC/Nga mice with no drug treatment. For this group animal, total IgE levels in μg/ml were 2.5 ± 0.3, 22.3 ± 5.6, 101 ± 16.5, and 221 ± 76.2 at 8, 12, 16, and 20 weeks old of age respectively, and IL-6 levels in pg/ml were 68 ± 12.7, 186.5 ± 54.3, 278.5 ± 41, and 323 ± 48.7 at 8, 12, 16, and 20 weeks old of age respectively.

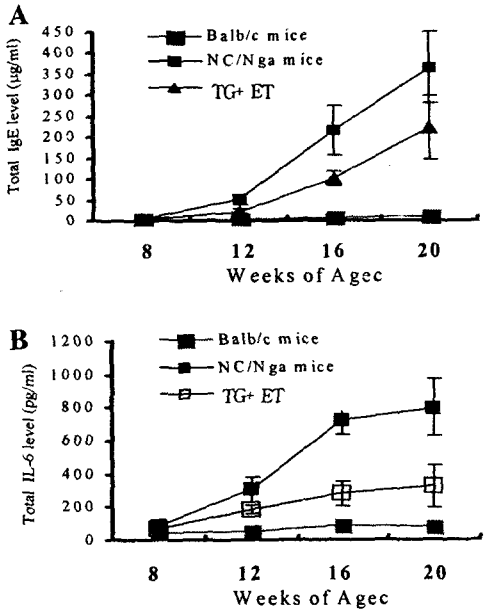

Fig. 2. Serum IgE and IL-6 elevation and development of dermatitis in NC/Nga atopy dermatitis model mice
Statistically significant values compared with NC/Nga mice group data by T test (*p<0.05, **p<0.01, ***p<0.001).

3. Effects of TG and ET on Serum IL-4 and IL-5 levels in NC/Nga mice

As shown in Fig 3, IL-4 and IL-5 levels in the serum of NC/Ng mice were increased compared to Balb/C control mice. IL-4 levels in pg/ml were 17.5 ± 5.6 for Balb/c vs 78.3 ± 7.9 for NC/Nga, and IL-5 levels for 48 ± 12.1 for Balb/c vs 250 ± 5.6 for NC/Nga. When the NC/Ng mice were treated with TG and ET, IL-4 and IL-5 levels in the serum were significantly decreased compared to untreated NC/Nga mice (31 ± 9.6 for IL-4 and 126 ± 25 for IL-5; unit, pg/ml) (Fig 3).

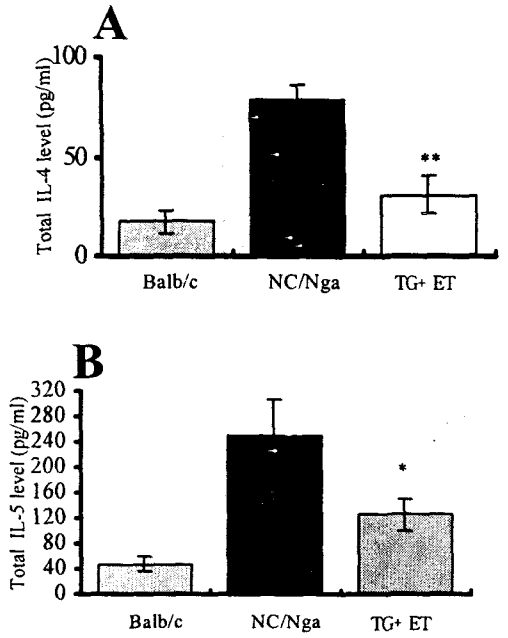

Fig. 3. Serum IL-4 and IL-5 elevation and development of dermatitis in NC/Nga atopy dermatitis model mice

4. Effects of TG and ET on Serum IgE and IL-13 and IFN-γ levels in NC/Nga mice

As shown in Fig 4, IL-13 and IFN-γ in the serum of Blab/c mice were barely detected. But, IL-13 and IFN-γ were greatly induced in the serum of NC/Nga mice (2105 ± 365 for IL-13 and 962 ± 363 for IFN-γ; unit pg/ml). When the NC/Nga mice were treated with TG and ET, IL-13 in the serum were decreased compared to untreated NC/Nga mice though the decrease was not statistically significant (1480 ± 254 for IL-13 and 1654 ± 296 for IFN-γ; unit pg/ml). Treatment with TG and ET in NC/Nga mice induced further increased in serum IFN-γ levels compared to untreated NC/Nga mice.

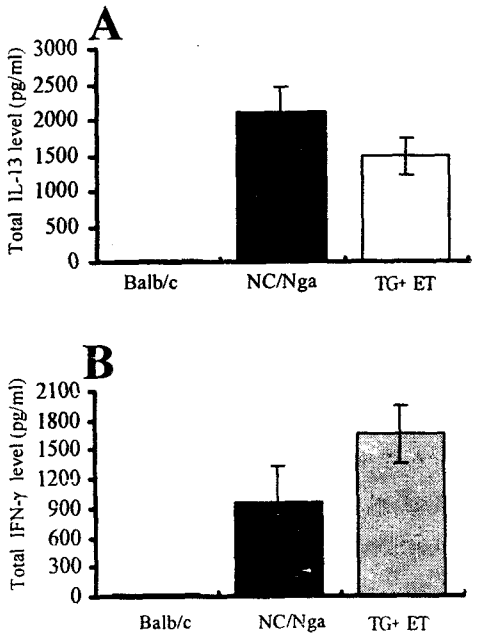

Fig. 4. Serum IL-13 and IFN-g elevation and development of dermatitis in NC/Nga atopy dermatitis model mice

5. Effects of TG and ET on spleen weight of NC/Nga mice

Spleen weight was measured in Balb/c, untreated and treated NC/Nga mice. As shown in Fig 5, spleen weights were similar between Balb/c and NC/Nga mice. Then, the treatment of TG and ET in NC/Nga mice significantly decreased spleen weight in 8 weeks old

NC/Nga mice for 12 weeks. The spleen weight in grams were 0.142 ± 0.009 for Balb/c, 0.152 ± 0.004 for NC/Nga, and 0.116 ± 0.011 for TG plus ET treated NC/Nga mice.

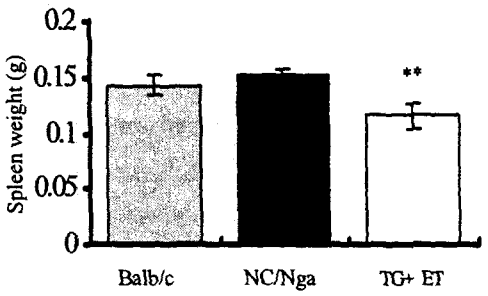

Fig. 5. Spleen weight in NC/Nga atopy dermatitis model mice

6. Effect of TG and ET on serum IgM in NC/Nga mice

As shown in Fig 6, IgM in the plasma of NC/Nga mice were significantly increased when compared to Balb/c control mice. When NC/Nga mice were treated with TG and ET, IgM in the serum was significantly decreased compared to untreated NC/Nga mice. Total IgM levels in μg/ml were 59 ± 12.3 for Balb/c, 683 ± 163 for non-treated NC/Nga, and 294 ± 104 for TG plus ET-treated NC/Nga mice.

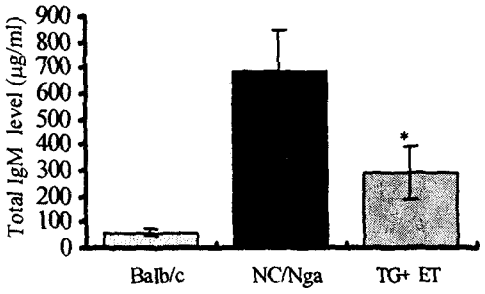

Fig. 6. IgM in NC/Nga atopy dermatitis model mice

7. Effects of TG and ET on serum IgG1 and IgG2a in NC/Nga mice

As shown in Fig 7, IgG1 and IgG2a in the serum of NC/Nga mice were greatly increased compared to Balb/c mice. When the NC/Nga mice were treated with TG and ET, IgG1 and IgG2a levels in the serum were significantly decreased compared to untreated NC/Nga mice. Total IgG1 levels in μg/ml were 575 ± 39 for Balb/c, 3024 ± 120 for NC/Nga, and 1690 ± 440 for TG plus ET-treated mice.

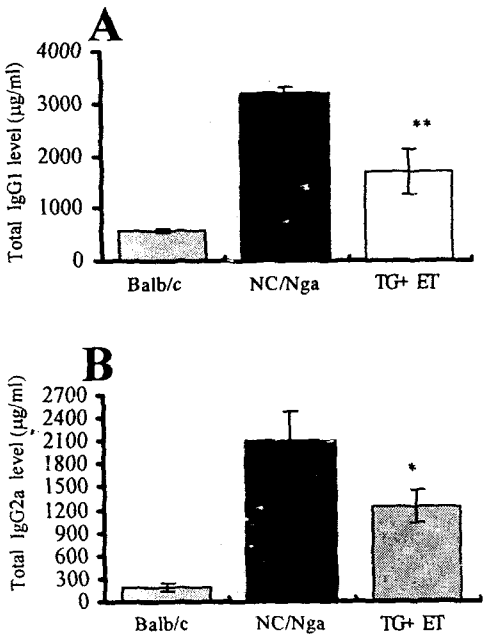

Fig. 7. Immunoglobulin G1 and G2a in NC/Nga atopy dermatitis model mice

8. FACS analysis in the lymph node of NC/Nga mice

To determine the contents of T cells and B cells in the lymph node, $CD3\varepsilon^+$ T cells and $CD19^+$ B cells were measured from the isolated lymph node of Balb/c mice and NC/Nga mice with or without TG and ET

administration. CD3ε$^+$ T cells were decreased in NC/Nga mice compared to Balb/c mice (Fig 8A). Then, the treatment of TG and ET significantly increased T cells levels in the Balb/c mice. In contrast, pattern of CD19$^+$ B cells was changed in the opposite manner. CD19$^+$ B cells were increased in NC/Nga mice. Treatment with TG plus ET significantly decreased B cell population in Blab/c mice.

To determine relative changes between T and B cells, the ratio of B cells to T cells was analyzed. As presented in Fig 8B, the ratio was greatly increased in NC/Nga mice compared to Balb/c group. Then, the treatment of TG and ET decreased the ratio of B cells to T cells (Table 3, Fig 8).

The amount of CD4$^+$ and CD8$^+$ cells was determined in the lymph node by flow cytometer. CD4$^+$ and CD8$^+$ positive cells were measured from the isolated lymph node of Balb/c mice and NC/Nga mice with or without TG and ET administration. CD4$^+$ cells were decreased in NC/Nga mice compared to Balb/c mice (Fig 9A). Then, the treatment of TG and ET significantly increased T cells levels similar to the Balb/c mice. Similar pattern of CD8$^+$ cell population was observed. CD8$^+$ B cells were decreased in NC/Nga mice. The treatment with TG plus ET recovered B cell population similar to that in Blab/c mice (Table 3, Fig 9B).

The relative changes of CD69$^+$ positive cells to CD3 ε$^+$ cells or CD11a+ to CD19$^+$ cells were determined in the lymph node cell population by flow cytometer. The ratio of CD69$^+$/CD3ε$^+$ cells was measured from the isolated lymph node of Balb/c mice and NC/Nga mice with or without TG and ET treatment. CD69$^+$/CD3ε$^+$ ratio was increased in NC/Nga mice compared to Balb/c mice (Fig 10A). Then, the treatment of TG plus ET significantly decreased T cells levels in the Balb/c mice. Similar pattern in the ratio of CD11a$^+$ to CD19$^+$ cells was observed. The ratio of CD11a$^+$ to CD19$^+$ cells was increased in NC/Nga mice compared to Balb/c mice. Treatment with TG and ET significantly recovered the ratio of CD11a$^+$ to CD19$^+$ cells in Balb/c mice (Table 3, Fig 10B).

Table 3. Cell Content in Lymph Node of NC/Nga Atopy Dermatitis Model Mice

Leucocytes type	Organ	Normal Balb/c	NC/Nga atopy dermatitis mice(scratching/drug)	
			Control	TG+ET
CD3ε$^+$(T)	LN*	71.3±2.55	38.5±4.21	70.6±3.66***
CD19$^+$(B)	LN	27.4±1.49	60.6±3.75	28.4±3.59***
CD69$^+$/CD3ε$^+$	LN	5.3±0.26	9.7±1.22	5.2±0.75**
CD4$^+$	LN	48.7±3.62	29.8±2.35	41.3±1.74**
CD8$^+$	LN	27.7±3.68	13.9±1.89	18.9±3.72
CD11a$^+$/CD19$^+$	LN	21.4±2.45	47.5±3.87	31.1±1.61**
*LN : Lymph node	B/T Rate	0.38	1.57	0.40

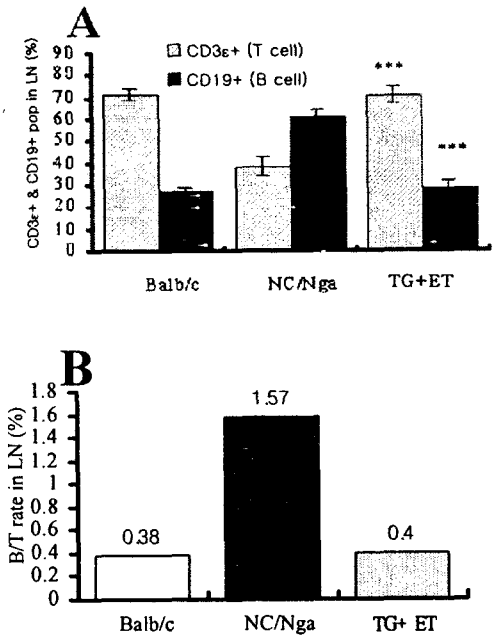

Fig. 8. Effect of TG and ET cotreatment on CD3ε$^+$ and CD19$^+$ (%) population in NC/Nga atopy dermatitis mouse lymph node cells.

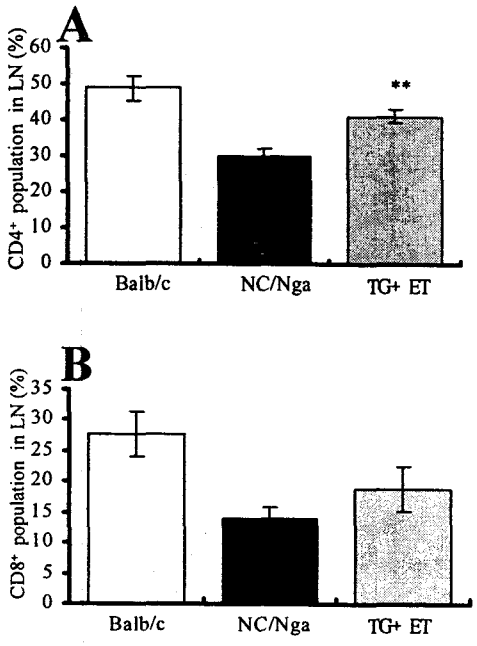

Fig. 9. Effect of TG and ET cotreatment on $CD4^+$ and $CD8^+$ (%) population in NC/Nga atopy dermatitis mice lymph node cells.

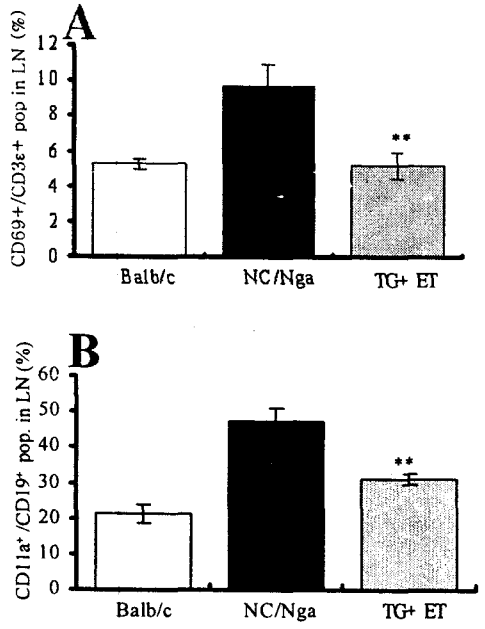

Fig. 10. Effect of TG and ET cotreatment on $CD3\varepsilon^+$/$CD69^+$ and $CD11a^+$/$CD19^+$ (%) population in NC/Nga atopy dermatitis mice lymph node cells.

9. FACS analysis in the spleen

The amounts of $CD3\varepsilon^+$ and $CD19^+$ were determined in the spleen tissues by flow cytometer. $CD3\varepsilon^+$ and $CD19^+$ cells were measured from the isolated spleen tissues of Balb/c mice and NC/Nga mice with or without TG and ET treatments. $CD3\varepsilon^+$ cells was decreased in NC/Nga mice compared to Balb/c mice (Fig. 11A). The treatment of TG and ET significantly increased T cells levels. $CD19^+$ cells were decreased in NC/Nga mice compared to Balb/c mice. Treatment with TG and ET decreased $CD19^+$ cells, which was even higher than that in the Balb/c mice.

The ratio of B cells to T cells was determined in the spleen tissues by flow cytometer. The ratio was increased in NC/Nga mice compared to Balb/c mice (Table 4, Fig. 11B). NC/Nga mouse group with TG and ET treatment decreased B/T cells levels compared with NC/Nga with no treatment.

The amounts of $CD4^+$ and $CD8^+$ cells were determined in the spleen by flow cytometer. $CD4^+$ and $CD8^+$ cells were measured from the isolated spleen cells of Balb/c mice and NC/Nga mice with or without TG and ET treatment. $CD4^+$ cells were increased in NC/Nga mice compared to Balb/c mice (Fig 12A). The treatment of TG and ET significantly decreased $CD4^+$ cell levels compared to untreated NC/Nga mice. $CD8^+$ cells were increased in NC/Nga mice compared to Balb/c mice. Treatment with TG plus ET further increased than untreated NC/Nga mice (Table 4, Fig. 12B).

The ratio of $CD3\varepsilon^+$ to $CD69^+$ cells or $CD11a^+$ to $CD19^+$ cells were determined in the spleen cells by flow cytometer. The ratios was measured from the isolated spleen cells of Balb/c mice and NC/Nga mice with or without TG and ET treatment. The ratio of

$CD3\varepsilon^+$ to $CD69^+$ cells was increased in NC/Nga mice compared to Balb/c mice (Fig. 13A). The treatment of TG and ET significantly decreased the ratio of $CD3\varepsilon^+$ to $CD69^+$ cells similar to that in Balb/c mice. The ratio of $CD11a^+/CD19^+$ cells was increased in NC/Nga mice compared to Balb/c mice. Then, the treatment with TG plus ET significantly decreased the ratio (Table 4, Fig. 13B).

Table 4. Cell Content in Spleen of NC/Nga Atopy Dermatitis Model Mice

Leucocytes type	Organ	Normal Balb/c	NC/Nga atopy dermatitis mice(scratching/drug)	
			Control	TG+ET
$CD3\varepsilon^+$(T)	SP*	49.8±3.64	20.6±3.44	28.8±2.17*
$CD19^+$(B)	SP	38.3±2.02	74.4±5.64	68.0±1.61
$CD69^+/CD3\varepsilon^+$	SP	0.87±0.04	3.40±0.26	1.56±0.02*
$CD4^+$	SP	5.48±0.74	12.5±0.68	10.4±0.25**
$CD8^+$	SP	4.97±0.53	7.40±1.15	8.55±0.31
$CD11a^+/CD19^+$	SP	55.3±4.58	76.8±3.80	66.7±1.73*
*SP : spleen	B/T Rate	0.77	3.61	2.36

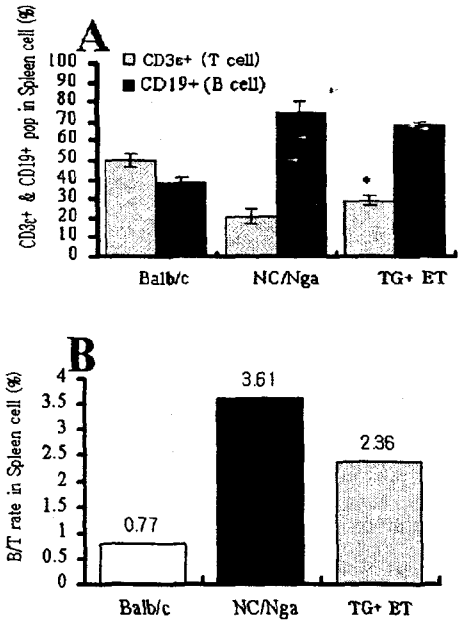

Fig. 11. Effect of TG and ET cotreatment on $CD3\varepsilon^+$ and $CD19^+$ (%) population in NC/Nga atopy dermatitis mice spleen cells.

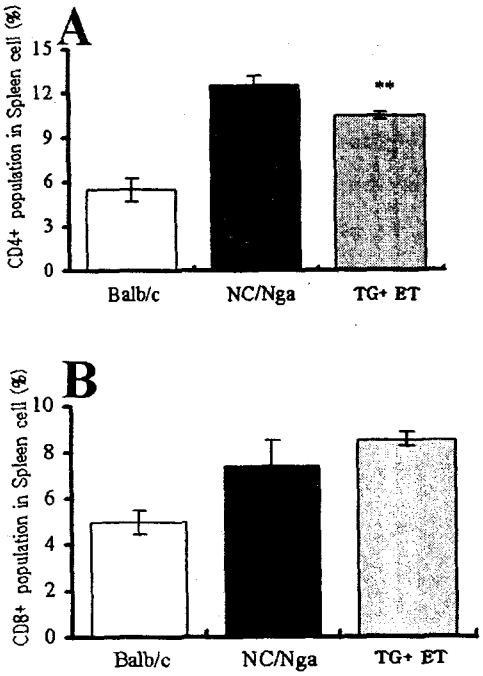

Fig. 12. Effect of TG and ET cotreatment on $CD4^+$ and $CD8^+$ (%) population in NC/Nga atopy dermatitis mice spleen cells.

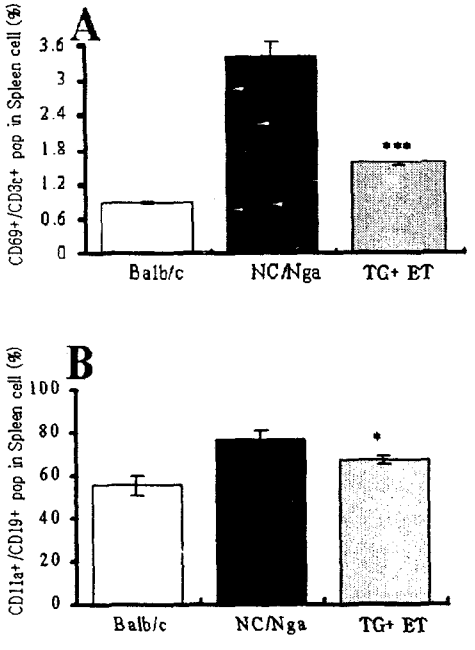

Fig. 13. Effect of TG and ET cotreatment on $CD3\varepsilon^+/CD69^+$ and $CD11a^+/CD19^+$ (%) population in NC/Nga atopy dermatitis mice spleen cells.

10. RT-PCR isolated from the facial skin tissues

1) Effects of TG and ET on IL-4 and IFN-γ mRNA expression

As shown in Fig 14, IL-4 mRNA levels were increased in the skin tissues isolated from the dorsal part of the neck of NC/Nga mice compared to Balb/c mice. Treatment of TG and ET deceased IL-4 levels of mRNA, suggesting possible inhibitory effects of TG and ET on the induction of inflammatory cytokines in NC/Nga mice. IFN-γ mRNA levels were not changed in the skin tissues isolated from the dorsal part of the neck in NC/Nga mice compared to Blab/c mice. Treatment of TG and ET in NC/Nga mice increased IFN-γ mRNA levels.

2) Effects of TG and ET on IL-5 and CCR3 mRNA expression

As shown in Fig. 15, IL-5 mRNA levels were increased in the skin tissues isolated from the dorsal part of the neck of NC/Nga mice compared to Balb/c mice. Treatment of TG and ET deceased IL-5 levels of mRNA, suggesting possible inhibitory effects of TG and ET on the induction of inflammatory cytokines in NC/Nga mice. CCR3 mRNA levels were remarkably increased in the skin tissues isolated from the dorsal part of the neck in NC/Nga mice compared to Blab/c mice. Then the treatment of TG and ET in NC/Nga mice decreased IFN-γ levels of mRNA.

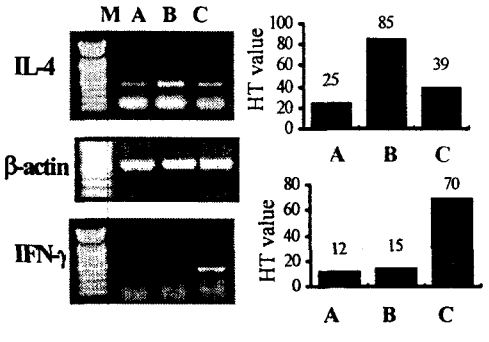

Fig. 14. RT-PCR analysis for IL-4 and IFN-γ mRNA
Normal Balb/c mouse skin (A), NC/Nga mouse skin control (B), and TG and ET (C). The numbers above each bar denotes the Ht value for the treatment. M: Molecular weight size marker.

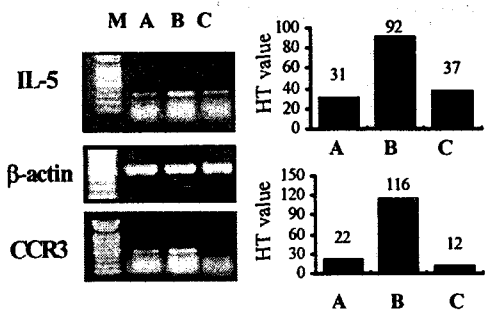

Fig. 15. RT-PCR for IL-5 and CCR3 mRNA
Normal Balb/c mouse skin (A), NC/Nga mouse skin control (B), and TG and ET (C). The numbers above each bar denotes the Ht value for the treatment. M: Molecular weight size marker.

11. Effects of TG and ET on IL-6 and IL-13 mRNA expression

As shown in Fig 16A, IL-6 mRNA levels were increased in the skin tissues isolated from the dorsal part of the neck of NC/Nga mice compared to Balb/c mice. Treatment of TG and ET significantly decreased IL-6 levels of mRNA although its level was higher than that from Blab/c mice, suggesting possible inhibitory effects of TG and ET on the induction of inflammatory cytokines in NC/Nga mice. IL-13 mRNA levels were remarkably increased in the skin tissues isolated from the dorsal part of the neck in NC/Nga mice compared to Blab/c mice. Then the treatment of TG and ET in NC/Nga mice significantly decreased IL-13 levels of mRNA (Fig. 16B). Total IL-5 levels in pg/ml were 7.5 ± 1.2 for Balb/c, 64.3 ± 5.6 for NC/Nga, and 37.6 ± 6.8 for TG plus ET-treated

NC/Nga mice. Total IL-13 levels in pg/ml were 22 ± 4.3 for Balb/c, 305 ± 36.4 for NC/Nga, and 133 ± 28 for TG plus ET-treated NC/Nga mice.

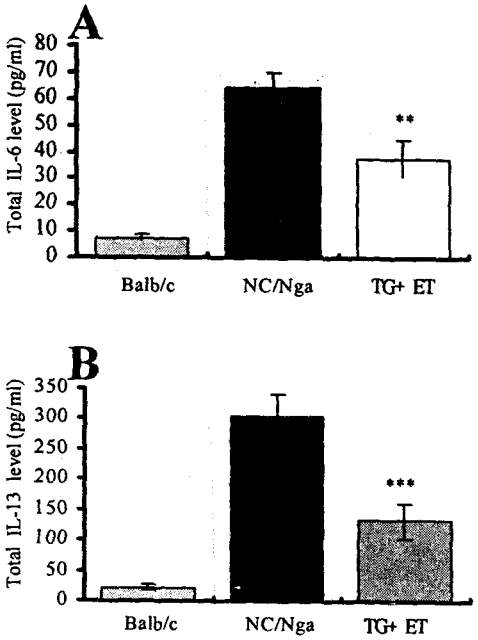

Fig. 16. Culture supernatant IL-6 and IL-13 production in facial skin biopsy of atopic dermatitis-like skin lesions in NC/Nga mice

12. Determination of ratios of $CD69^+/CD3\varepsilon^+$ and $CD19^+/CD44^+$ by FACS analysis

The ratios of $CD69^+/CD3\varepsilon^+$ and $CD19^+/CD44^+$ in cells isolated from facial and neck skins were measured by FACS analysis. As shown in Fig 17, $CD3\varepsilon^+$ cells relative to $CD69^+$ cells in NC/Nga mice were increased after the treatment of TG and ET. Comparison of $CD19^+$ cells to $CD44^+$ cells showed a decrease in cell population positive to both $CD19^+$ and $CD44^+$ cells.

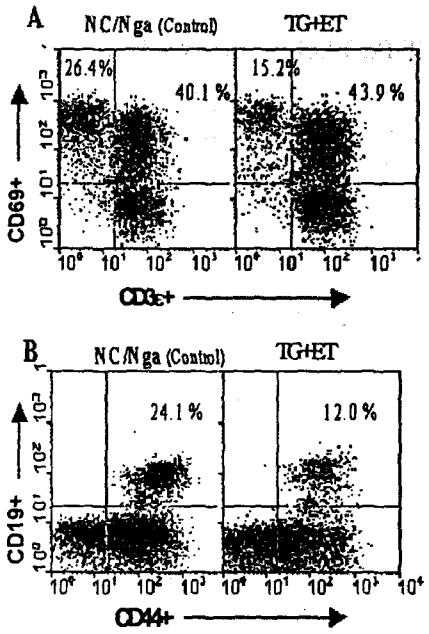

Fig. 17. FACS analysis of cultured facial & neck skin cells in dermatitis-like skin lesions in NC/Nga mice

13. Histopathological analysis of lesioned ear skin tissues

To investigate whether the treatment of TG and ET affected the severity of skin lesions in NC/Nga mice, ear skin tissues was histologically analyzed. After H&E staining, the morphological features of ear skins were compared among groups with different treatment. Histogical analysis of epidermis or dermis in the Balb/c mice showed clear morphological features of epidermis, dermis, and bone tissues (Fig 18A and B). In contrast, NC/Nga control mouse tissue showed the extended shape of epidermis and dermis tissues due to edema and also leukocyte infiltration (Fig. 18C and D). Then, in experimental NC/Nga mice which were treated with TG and ET, infiltrated leukocytes were much less in numbers compared to untreated NC/Nga mice, and the cell morphologies in the dermis and epidermis layers were similar to Balb/c mice (Fig. 19E and F).

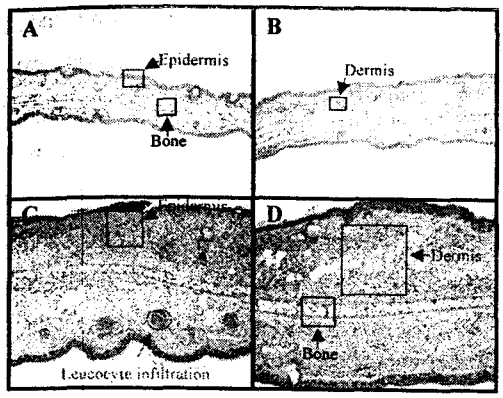

Fig. 18. Histologic features of ear lesion in NC/Nga mouse
(A, B): ear tissues from Balb/c mouse. (C or D): ear tissues from NC/Nga mouse with no treatment.

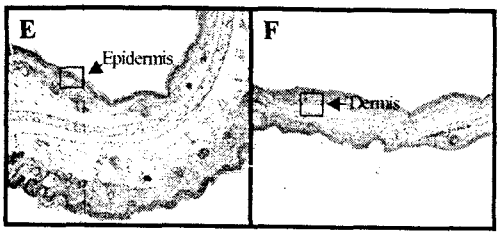

Fig. 19. Histologic features of ear lesion in NC/Nga mouse
(E or F): ear tissues from NC/Nga mouse with TG plus ET treatment.

14. Histopathological analysis of lesioned skin tissues

The skin tissues of dorsal part of the neck among mice with different treatments were histologically examined. H&E staining of Balb/c mice tissues showed normal morphological features in epidermis, dermis, and baseline at 100X magnification (Fig. 20A and B). But, the corresponding epidermis tissue of NC/Nga mice was migrated toward the dermis layer, and sever skin inflammation and eruption were observed (Fig. 20C and D). In contrast, in the tissues treated with TG and ET, infiltrated leukocytes were much less in numbers compared to untreated NC/Nga mice, and the cell thickness in the dermis and epidermis layers were similar to Balb/c mice (Fig. 21E and F).

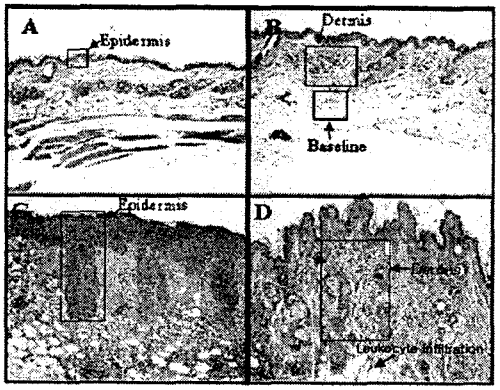

Fig. 20. Histologic features of lesioned skin tissue in NC/Nga mouse
(A, B): skin tissues from Balb/c mouse. (C or D): skin tissues from NC/Nga mouse with no treatment.

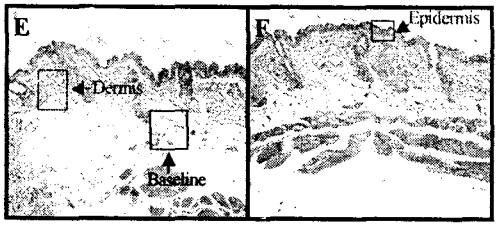

Fig. 21. Histologic features of lesioned skin in NC/Nga mouse
(E or F): skin tissues from NC/Nga mouse with TG plus ET treatment.

15. Lymphokine assay in the splenocytes

As shown in Fig. 22, IL-4 mRNA levels were highly increased in the splenocytes of C57BL/6 mice in the presence of anti-CD3ε^+ plus anti-CD28$^+$ antibodies (labeled CT in the Fig. 22), but not in the presence of anti-CD3ε^+ plus anti-CD28$^+$ antibodies (labeled 'CsA' for cyclosporin A in the Fig. 22). Treatment of TG and ET decreased IL-4 levels of mRNA, suggesting possible inhibitory effects of TG

and ET on the induction of inflammatory cytokines in activated splenocytes. In the similar procedure, changes of IFN-γ were investigated. IFN-γ levels were strongly increased in cells cultured on anti-CD3ε$^+$ plus anti-CD28$^+$ antibody-coated plates or in the presence of anti-CD3ε$^+$ plus anti-CD28$^+$ antibodies. Induction of IFN-γ was further significantly elevated by TG plus ET treatments. Thus, TG and ET have a positive effect on IFN-γ production in activated mouse splenocyets.

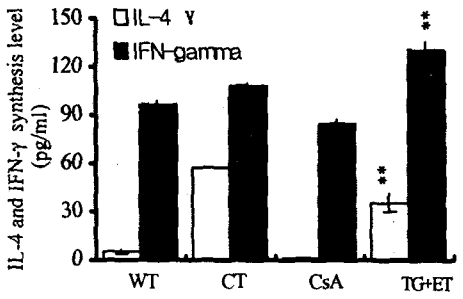

Fig. 22. IL-4, IFN-γ synthesis in splenocytes from treated with TG and ET in C57BL/6 mice.

WT : Normal C57BL/6 mice

CT : anti-CD3ε$^+$ (1 ㎍/㎖) plus anti-CD28$^+$ (1 ㎍/㎖) antibody coated plate.

CsA : anti-CD3ε$^+$ plus anti-CD28$^+$ plus cyclosporin A (: CsA, 10 ㎍/㎖)

TG+ET : anti-CD3ε$^+$ plus anti-CD28$^+$ plus TG+ET.

Discussion

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with cutaneous hyperreactivity to environmental triggers that are innocuous to normal non-atopic individuals[24]. Although written descriptions of AD date back to the early 1800s, an objective laboratory test does not exist for AD. AD usually presents during early infancy and childhood, but it can persist into or start in adulthood[25]. The lifetime prevalence of AD is 10 - 20 % in children and 1 - 3 % in adults. Its prevalence has increased two- to threefold during the past three decades in industrialized countries but remains much lower in countries with predominantly rural or agricultural areas. Wide variations in prevalence have been observed within countries inhabited by groups with similar genetic backgrounds, suggesting that environmental factors play a critical role in determining expression of AD.

Various studies indicate that AD has a complex etiology, with activation of multiple immunologic and inflammatory pathways[26]. The clinical phenotype of AD is the product of complex interactions among susceptible genes, the host's environment, defects in the skin barrier function, and systemic and local immunologic responses. In a viewpoint of atopy as a systemic disease, AD is the cutaneous manifestation of a systemic disorder that also gives rise to asthma, food allergy, and allergic rhinitis[25,27,28]. These conditions are all characterized by elevated serum IgE levels and peripheral eosinophilia. Although the precise immunologic pathways are fully understood, key molecular factors underlying the development of acute and chronic AD have been revealed29). It is well known that Th2 cells circulating in the peripheral blood of AD patients result in elevated serum IgE and eosinophils.

These T cells express the skin homing receptor, CLA, and recirculate through unaffected AD skin where they can engage allergen-triggered IgE$^+$ LCs and mast cells that contribute to Th2 cell development[30,31].

Skin injury by environmental allergens, scratching, or microbial toxins activates keratinocytes to release proinflammatory cytokines and chemokines that induce the expression of adhesion molecules on vascular endothelium and facilitate the extravasation of inflammatory cells into the skin[32]. Keratinocyte-derived

thymic stromal lymphopoietin (TSLP) and DC-derived IL-10 also enhance Th2 cell differentiation. AD inflammation is associated with increased Th2 cells in acute skin lesions, but chronic AD results in the infiltration of inflammatory IDECs, macrophages, and eosinophils. IL-12 production by these various cell types results in the switch to a Th1-type cytokine milieu associated with increased IFN-γ expression[33,34].

Because the clinical phenotype is very complex and, accordingly, medications may vary in their effectiveness for the treatment of different forms of AD[35], numerous research attempts have recently been made to exploit potential drugs for AD treatment. One of them was concerned about the fact that the onset of AD are associated with a high familial occurrence, which further implies that a certain genetic factors could be critical for the progression of AD. There has been particular focus on chromosome 5q31-33, as it contains a clustered family of Th2 cytokine genes, i.e., IL-3, IL-4, IL-5, IL-13, and GM-CSF[36]. Case-control comparisons have suggested a genotypic association between the T allele of the -590C/T polymorphism of the IL-4 gene promoter region and AD. The fact that this allele is associated with increased IL-4 gene promoter activity suggests that it may increase allergic responses in AD. Similarly, IL-13 coding region variants, a gain-of-function polymorphism in the a subunit of the IL-4 receptor (located on chromosome 16q12), and a functional mutation in the promoter region of RANTES (located on chromosome 17q11) have been reported in AD.

In order to examine the potential role of certain genetic factors, several experimental animal models including transgenic mouse overexpressing candidate gene (gain of function mutant), knockout mouse inactivating the target gene (loss of function mutants), and spontaneous mutants have been developed. Although transgenic mice and knockout mouse models are advantageous in that specific role of the candidate gene can be studied in relation to AD, in many cases, they are lethal and also generate unstable genotype.

As a spontaneous mutant for AD model, NC/Nga mice were established as an inbred strain in 1955[37,38]. The NC/Nga strain has been reported to have some biological characteristics: liver and kidney esterase like a DBA/2 strain, high susceptibility to X-irradiation, and high susceptibility to anaphylactic shock from ovalbumin[39,40]. Some Japanese researchers noticed development of spontaneous dermatitis just before or after weaning, but the cause and pathogenesis have been unclear. Matsuda[41] have reported that when NC/Nga mice were in an air-uncontrolled conventional room, in other words, when they were not maintained under specific pathogen-free (SPF) conditions, skin lesions which occurred in the atopy patient in human were observed. Furthermore, IgE levels in the serum and increases in $CD4^+$ cells expressing IL-4 were observed[41]. Other clinical symptoms include itching, hemorrhage, scaling, dryness, and alopecia at the age of 8 weeks and these skin lesions are typically observed on the face, nose, ears, neck and back[42-44]. The clinical severity of the dermatitis increases with age and reaches a maximum at around 17 weeks of age[41].

In this study, we have found that the administration of oriental medicinal prescriptions was effective in alleviating the development of atopic dermatitis in the mouse model NC/Nga. Measurement of chemical mediators possibly involved in atopic development showed significant changes such that in the serum of NC/Nga mouse, IgE, IL-6, IL-13, IgM, and IgG1 and G2a protein levels were significantly increased compared to wild type Balb/c mice. Moreover, some of these cytokines and immunoglobulin proteins were

regulated at gene expression levels. RT-PCR analysis further indicated that mRNA expression for IL-4, IL-5, IL-6, IL-13, and IgE were increased in the lesioned tissues, suggesting that increased production of these cytokines were regulated at gene expression levels. Also, mRNA encoding CCR3 was increased in NC/Nga lesioned tissues as CCR protein levels were previously reported to be elevated. CCR is the eotaxin receptor which plays a role in controling eosinophil migration[45]. Although our data suggest that increased production of inflammatory cytokines, IgE, and IgG are regulated at the gene expression levels, regulation at posttranslational level cannot be excluded completely. For instance, increased stability of synthesized protein can be maintained by decreasing protease activity or by posttranalational modification of the existing proteins. During type I hypersensitivity, Th2 $CD4^+$ cells are increased and secrete cytokines (e.g., IL-4, IL5 and IL-10) that antagonize Th1 effects and promote the synthesis of IgE. Thus, our data on the increased content of $CD4^+$ cells in the splenocytes would be related to increased synthesis of IgE (Table 1 and 2). Further studies using cultured cells would be helpful for clarifying the regulation mechanism of IgE and other cytokines such as IL-4, IL-5 and IL-13.

Then, the treatment of TG and ET changed levels of several chemical mediators related to atopic development. The Tang-gwi-eum-

za-gagambang (TG) used as an internal administration in this study is the prescription for the cure of diverse skin diseases due to both blood defect and chilliness in the clinical oriental medicine. TG is Tang-gwi-eum-za augmented with Angelicae dahuricae radix (白芷), Angelicae pubescentis radix (獨活), and Cyperi rhizoma (香附子), all of which have the relieving activity of both chilliness and fever, and thus is expected for the cure of atopy. The external therapy is a prescription of a mixture of Fagopyrum esculentum Moench (蕎麥), Oleum Terebinthinae (松津), Gypsum Fibrosum (石膏) and Bamboo salt (竹鹽) and is currently used for dermal problems in the civil society. Therefore, it was expected that the parallel administrations of TG and ET were effective for the cure of atopy dermatitis by regulating the complementation of blood and 'yin', removal of chilliness and fever relief and detoxification.

Treatment of TG and ET significantly decreased induced IL-4, IL-5 and IL-13 levels. Similar changes of these mediators were found to be regulated at mRNA expression levels.

IFN-γ was increased by the administration of TG and ET. FACS analysis showed that the ratio of B cells to T cells was significantly increased in NC/Nga mice, and then the treatment of TG and ET decreased the B/T ratio. Finally, histological analysis indicated that histopathological features observed in NC/Nga mice were significantly improved by TG and ET treatment.

Together, combined administration may be effective for the treatment of atopic dermatitis at least in the experimental animal and further implicate possible application of these prescriptions to human therapy.

Several previous studies have indicated that the treatment of herbal drugs attenuated the severity of AD in the numerous skin tissues of NC/Nga mice. Kotani[46] has shown that persimmon leaf extract and astragalin were effective in inhibiting the development of dermatitis.

The persimmon leaf is known to contain antiallergic substances that inhibit histamine release by human basophilic cell. Oral intake of this extract into NC/Nga mice significantly reduced not only infiltration of inflammatory cells, especially degranulated mast cells, thickening of the epidermis, and prominent hyperkeratosis. but also down-regulated the capacity of

spleen T cells to produce both IL-4 and IL-13, but not IFN-γ. Similar physiological effects such as skin symptoms and transepidermal water loss of persimmon leaf extract were reported[47,48].

Also, administration of royal jelly suppressed the development of AD-like skin lesions. In this study, the development of AD-like skin lesions in NC/Nga mice was induced by repeated application of picryl chloride (PiCl) under specific pathogen-free (SPF) conditions. Then, oral administration of royal jelly inhibited the development of AD-like skin lesions in these mice as exemplified by the significant decrease in the total skin severity scores and the decrease in hypertrophy, hyperkeratosis, and infiltration of the epidermis and corium by inflammatory cells. Besides the potential effects of natural herbal drugs mentioned above, steroid drugs such as dehydro-epiandosterone and 2,3,7,8-tetrachlorodibenzo-p-dioxin were also effective in attenuating atopic dermatitis responses in NC/Nga mouse. Together, these studies suggest the possibility that atopic dermatitis can be regulated by appropriate natural drugs as well as chemical drugs. In this aspect, our findings on the attenuating effects of combined treatments of TG and ET on AD provide broader insight into the use of natural herbal products for atopy therapy.

While the present results along with several previous studies implicate the possibility for the therapeutic application, it should be noted that there might be an intrinsic difference between animal model and human therapy. It was reported that about 50% of NC/Nga mouse displayed the incidence of AD-like lesions although histopathological characteristics of atopic dermatitis in NC/Nga mouse model are very similar as occurs in human atopy. Furthermore, the genetic factors which might be critical for the onset of AD in NC/Nga mouse have not been characterized yet, and there are a certain levels of variability for the onset of AD in aged mouse, which is 8-17 months. AD onset in NC/Nga mouse requires hapten administration and conventional maintenance condition. Therefore, while all these features which were characterized from NC/Nga mice can provide important clues for the development of effective drugs in the treatment of AD, there are certainly much more complexities and individual variations reflecting genetic predispositions and environmental influences in humans.

Therefore, it would be very important to keep in mind that the pathophysiological significance of our data which were obtained from NC/Nga mouse model can be strengthened by extending the realm of investigation, for example by investigating along with other animal models such as hapten-induced model as well as transgenic animal model whenever available. Hapten-induced mouse model that can be produced by repeated application of 2,4,6-trinitrochlrobenzen (TCNB) at 2 day intervals to the same skin site induces skin lesion similar to human AD. In this AD model, increased production of Th2 cytokines (IL-4 and IL-10) was observed[49], and importantly, this mouse model revealed 100 % incidence of AD-like lesions. Transgenic mouse model which overexpresses IL-18 also showed 100 % of incidence of AD-like lesions. Thus, by examining TG and ET in diverse animal model systems, its potential efficacy and possible application to human AD patients would be positively acted on.

In the future, it would be critical to analyze components of TG and ET at the molecular level. As mentioned above, some natural herbal drugs such as persimmon are known to contain the components which inhibit inflammatory cytokines and IgE in cultured cells. Chemical analysis is thus of great importance not only for understanding of AD mechanism but also for the development of drugs.

Conclusion

As a long-term goal of developing therapeutic drugs for human atopic dermatitis, oriental medicinal drugs TG and ET were administered into NC/Nga mouse, a genetic model of AD. Biochemical and histological examinations showed overall improvement of parameters related to AD by TG and ET co-treatment. The major finding are summarized as follows.

1. The skin severity of NC/Nga mice treated with TG and ET was decreased by 36.0% and 37.8% compared to the control group at 16 weeks and 20 weeks old respectively.
2. IgE, IL-4, IL-5, IL-6, IgM, IgG1 and IgG2a protein levels in NC/Nga mice treated with TG and ET were significantly decreased compared to non-treated control group and IL-13 protein levels were decreased in the experimental group, but statistically insignificant. IFN-γ levels in TG and ET treated NC/Nga mice were increased compared to the control group.
3. The spleen weight in TG and ET treated NC/Nga mice were significantly decreased compared to the control group.
4. The B/T ratio in the lymph node of TG and ET-treated group was decreased compared to the NC/Nga control group. $CD4^+$ and $CD8^+$ cells in the lymph node of NC/Nga animals were slightly increased compared to wild type mice, and TG and ET treatment of NC/Nga mice showed a significant increase in $CD4^+$ expression and an statistically insignificant increase in $CD8^+$ expression. $CD69^+$ and $CD11a^+$ were significantly decreased in TG and ET-treated NC/Nga mice compared to control group.
5. IL-4, IL-5, and CCR3 mRNA expression levels in the skin tissues of TG and ET treated NC/Nga mice showed a remarkable decrease, and IL-6, IL-13, $CD3\varepsilon^+/CD69^+$, $CD19^+/CD44^+$ expression was significantly decreased in TG and ET treated NC/Nga mouse group. IFN-γ mRNA was increased compared to control group.
6. The levels of inflammation and leukocyte infiltration were remarkably decreased in epidermis and dermis of the ears and neck tissues of NC/Nga mice teated with TG and ET compared to control group.
7. Lymphokine assay showed a significant decrease in IL-4 expression and a significant increase in IFN-γ expression levels in NC/Nga mice treated with TG and ET.

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