



Quantitative evaluation of therapeutic effect of *Liriope platyphylla* on phthalic anhydride-induced atopic dermatitis in IL-4/Luc/CNS-1 Tg mice



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ABSTRACT

Ethnopharmacological relevance: A variety of previous pharmacological studies have suggested that *Liriope platyphylla* may exert beneficial biological effects on inflammation, diabetes, neurodegenerative disorder, obesity, and atopic dermatitis (AD).

Aim of the study: The therapeutic effect of aqueous extract of *Liriope platyphylla* (AetLP) on AD was quantified using the luciferase report system in IL-4/Luc/CNS-1 transgenic (Tg) mice.

Materials and methods: Alteration of the luciferase signal was quantified in IL-4/Luc/CNS-1 Tg mice co-treated with phthalic anhydride (PA) and AetLP for 2 weeks using the IVIS imaging system. Phenotypes of AD were assessed by ear thickness analysis, measurement of immune-related organ weights, ELISA, and histological and pathological analysis in Tg mice.

Results: A strong luciferase signal was detected in the abdominal region of IL-4/Luc/CNS-1 Tg mice treated with only PA. However, this signal was significantly reduced in IL-4/Luc/CNS-1 Tg mice co-treated with PA+AetLP in an AetLP concentration-dependent manner. Especially, three organs, the thymus, pancreas, and submandibular lymph node (SL), showed a high signal response to PA treatment. Furthermore, to verify whether or not alteration of the luciferase signal is associated with AD, these disease response phenotypes were measured in the same group of mice. Common allergenic responses including increases in ear thickness, lymph node weight, IgE concentration, and infiltrated mast cells were detected in IL-4/Luc/CNS-1 Tg mice treated with PA. However, these responses were dramatically decreased by AetLP treatment for 2 weeks.

Conclusion: These results indicate that the luciferase signal may successfully reflect the therapeutic effects of AetLP in IL-4/Luc/CNS-1 Tg mice. Further, we suggest additional evidence that *Liriope platyphylla* may be considered as an effective therapeutic drug for the treatment of AD.

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1. Introduction

AD is a common chronic inflammatory disorder that is caused by the excessive activation of certain white blood cells and basophils due to IgE production in response to environmental antigens (Hanifin and Rajka, 1980; Leung and Bieber, 2003). Further, AD is characterized by eczematous skin lesions, which give rise to asthma, allergic rhinitis, food allergies, and contact dermatitis (Kay, 2000, 2001). Risk factors of this disorder are

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regarded as four major environmental candidates, including infectious disease, environmental pollution, exposure to allergens, and dietary changes (Grammatikos, 2008). Until now, several traditional methods have been used in a clinical context to screen allergens among substances in the surrounding environment. Of these methods, the allergy skin prick test is a cheap, rapid, and accurate method, in which a few drops of isolated allergen are gently pricked onto the surface of the forearm skin in order to identify the causative allergens (Turner et al., 1980). Another method, the allergy-specific IgE antibody test, screens allergens by measuring amounts of IgE antibody for suspected allergens in blood samples (Johansson, 2004; Petersen et al., 2004). However, these methods are limited in that it is difficult to quantitatively evaluate the magnitude of the allergic response. To overcome these limitations, IL-4/Luc/CNS-1 Tg mice were recently produced

by microinjection with the luciferase gene under control of the human IL-4 promoter and the IL-4 enhancer (CNS). These mice were successfully applied to the evaluation of three types of allergens, including a respiratory sensitizer, vaccine additives, and crude extracts of natural allergens *in vivo* (Bae et al., 2011). However, no study has yet applied IL-4/Luc/CNS-1 Tg mice as a model to quantify the therapeutic effects of herbal medicines.

Liriope platyphylla has long been used for the treatment of asthma as well as bronchial and lung inflammation as a traditional oriental herbal medicine (Kim et al., 2002; Lee et al., 2005). Significant alteration of nerve growth factor (NGF) secretion and its related signaling pathway, including NGF receptor TrkA and p75^{NTR}, has been observed in PC12 cells treated with the extract or compounds from *Liriope platyphylla* (Hur et al., 2009; Choi et al., 2010). Furthermore, *Liriope platyphylla* has shown potential for the treatment of obesity and diabetes (Jeong et al., 2008; Choi et al., 2004). The homoisoflavone-enriched methanol fraction and LP9M80-H methanol fraction of *Liriope platyphylla* significantly induce insulin secretion and glucose transporter expression through mitogen-activated protein kinase (MAPK) signaling pathways (Choi et al., 2004; Lee et al., 2010; Kim et al., 2010b). Especially, *Liriope platyphylla* was shown to potently inhibit airway inflammation and hyperresponsiveness in a murine model of asthma by modulating the relationship between Th1/Th2 cytokine imbalance (Lee et al., 2005) as well as AD induced by phthalic anhydride treatment (Kim et al., 2010c). However, further studies are required to quantify the relieving effect of *Liriope platyphylla* on AD using simple and rapid methods.

As demonstrated in this study, the luciferase signal observed in IL-4/Luc/CNS-1 Tg mice effectively reflected the relieving effects of AEtLP on PA-induced AD phenotypes.

2. Materials and methods

2.1. Care and use of IL-4/Luc/CNS-1 Tg mice

All animal experimental procedures performed in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Pusan National University (PNU-2012-0061). The animals were handled in the Pusan National University-Laboratory Animal Resources Center accredited by the Korea FDA in accordance with USA NIH guidelines (Accredited Unit Number—00231). All mice were housed under specified pathogen-free (SPF) conditions and a strict light cycle (lights on at 06:00 h and off at 18:00 h) and were provided a standard irradiated chow diet (Purina Mills Inc., Missouri, USA) *ad libitum*. The 8-week-old IL-4/Luc/CNS-1 Tg and non-Tg mice used in this study were obtained from the National Institute of Food and Drug Safety Evaluation in Korea FDA (Osong, Korea).

2.2. Production and identification of IL-4/Luc/CNS-1 Tg mice

Large numbers of IL-4/Luc/CNS-1 Tg mice and non-Tg littermates were produced by breeding IL-4/Luc/CNS-1 Tg mice and HR1 mice. Founder mice, into which the IL-4/Luc/CNS-1 transgene was inserted, were identified by DNA-PCR of tail-derived genomic DNA. For DNA-PCR, 10 pmol each of sense (5'-GAA TGT ACC AGG AGC CAT ATC-3') and antisense (5'-CTC AGT ACT ACG AGT AAT CCA-3') primers were added into the genomic DNA template mixture, and the reaction mixtures were subjected to 25 cycles of amplification. Amplification was conducted in a thermal cycler (Perkin-Elmer, Waltham, MA, USA) under the following conditions: 30 s at 94°C; 30 s at 62°C, and 45 s at 72°C. The amplified PCR products were separated by 1% agarose gel electrophoresis, and band patterns were detected using a Kodak

Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, Rochester, NY, USA).

2.3. Preparation of AEtLP

Liriope platyphylla roots were collected from plantations in the Miryang area of Korea and dried with a hot-air drier (JSR Instruments, Uttaranchal, India) at 60 °C. The voucher specimens (WPC-11-010) were deposited at the Functional Materials Bank of PNU-Wellbeing RIS Center at Pusan National University. Six-hundred grams of dry roots was reduced to powder using an electric blender. Water extract was purified at 100 °C for 2 h using circulated extraction equipment (IKA Labortechnik, Staufen, Germany) after addition of 2 l of distilled water. Calories and composition of AEtLP were measured as described previously. Extract solutions were concentrated into dry pellets using a rotary evaporator (EYELA, Tokyo, Japan) and stored at -80°C until use.

2.4. Experimental design

Eight-week-old IL-4/Luc/CNS-1 Tg mice ($n=20$) were randomly divided into two groups. In the first group of Tg mice (AOO, $n=5$), 40 μ l of acetone-olive oil (AOO) was repeatedly spread on the dorsum of ears three times a week for 4 weeks. In the second group (PA, $n=15$), 40 μ l of 5% PA solution in AOO (4:1 acetone-olive oil, v/v:AOO) was repeatedly spread on the dorsum of ears three times a week for 4 weeks. Furthermore, the second group was further divided into three treatment groups: PA+Vehicle, PA+AEtLP25, and PA+AEtLP50. The PA+Vehicle group received a comparable volume of water daily via oral administration, whereas other two groups were received two different concentrations of AEtLP for 4 weeks. Age-matched non-Tg mice ($n=5$) were used as the control group.

2.5. Measurement of body weight, lymph node weight, and ear thickness

Alterations of body weight during the experimental procedure were measured with an electronic balance (Mettler Toledo, Greifensee, Switzerland) once a week for 4 weeks. Weights of lymph nodes collected from the sacrificed mice were also measured by the same method. Ear thickness was measured using a thickness gauge (Digimatic Indicator, Matusutoyo Co., Tokyo, Japan) to determine the degree of allergic skin inflammation induced by PA treatment.

2.6. Bioluminescence imaging and data analysis

In vivo imaging was conducted using an IVIS imaging system (Xenogen, CA, USA) as previously described. IL-4/Luc/CNS-1 Tg mice were anesthetized with Zoletil and injected i.p. with 150 mg/kg of D-luciferin (Sigma-Aldrich, MO, USA). Ten minutes after D-luciferin injection, images of mice were taken for 3 min using an IVIS imaging system. Photons emitted from specific regions were quantified using LivingImage software (Xenogen, CA, USA). *In vivo* luciferase activity was expressed in photons per second.

2.7. Enzyme-linked immunosorbent assay (ELISA) for detection of serum IgE concentration

The serum IgE concentration was measured using an ELISA kit (Shibayagi, Inc., Gunma, Japan) according to the manufacturer's instructions. Briefly, capture antibodies were plated in the Nunc C bottom immunoplate supplied in the kit. Next, wells were washed with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) three times. Then, serum samples and standards diluted

with buffer solution were added to the wells, and the plate was incubated for 2 h. The wells were washed again with washing solution, 50 μ l of Biotin-conjugated anti-IgE antibodies (1000-fold dilution) were added to each well and incubated further for 2 h to bind with captured IgE. The wells were washed again with washing solution, after which horseradish peroxidase-conjugated detection antibodies (2000-fold dilution) were added to each well and incubated for 1 h. An enzyme reaction was then initiated by adding tetramethylbenzidine (TMB) substrate solution (100 mM sodium acetate buffer pH 6.0, 0.006% H₂O₂) and incubating the plate at room temperature in the dark for 20 min. Finally, the reaction was terminated by adding acidic solution (reaction stopper, 1 M H₂SO₄), and absorbance of yellow product is measured spectrophotometrically at 450 nm. The final concentration of IgE was calculated using the standard curve.

2.8. Histological analysis

Ear skins were removed from mice, fixed with 10% formalin, embedded in paraffin wax, routinely processed, and then sectioned into 5 μ m thick slices. The skin sections were then stained with hematoxylin and eosin, followed by examination by light microscopy for the presence of edema and inflammatory cell accumulation. Further, thickness levels of the epidermis and dermis, and the number of lipid pores were measured using Leica Application Suite (Leica Microsystems, Wetzlar, Germany).

2.9. Toluidine blue staining

Mast cells were detected by staining with toluidine blue according to previously described methods (Kim et al., 2010a). After deparaffinization and dehydration, ear skin sections were stained with 0.25% solution of Toluidine blue (Sigma-Aldrich) and examined by light microscopy for the presence of mast cells. The number of cells per specific area was measured with Leica Application Suite (Leica Microsystems).

2.10. RT-PCR analysis of luciferase gene expression

RT-PCR was conducted to measure the relative quantities of mRNA for luciferase and IL-4 in lymph node from IL-4/Luc/CNS-1 Tg mice and non-Tg mice. For RT-PCR analysis, lymph node samples were frozen in liquid nitrogen. The frozen tissues were chopped with scissors and homogenized in RNAzol B solution (Tet-Test Inc., TX, USA). The isolated RNA was then measured by UV spectroscopy. Expression of the transgenes was assessed by RT-PCR with 5 μ g of the total RNA from each tissue. Next, 500 ng of the oligo-dT primer (Invitrogen, CA, USA) was annealed at 70 °C for 10 min. Complementary DNA, which was used as the template for further amplification, was synthesized by the addition of dATP, dCTP, dGTP, and dTTP with 200 units of reverse transcriptase. Ten picomoles each of the sense and antisense primers were then added, and the reaction mixture was subjected to 30 cycles of amplification. Amplification was conducted in a Perkin-Elmer Thermal Cycler as follows: 1 min at 94 °C, 1 min at 62 °C, and 1 min at 72 °C. In each case, minus-RT controls were included in order to differentiate between the DNA and RNA products. RT-PCR was also conducted using β -actin-specific primers in order to ensure RNA integrity. The primer sequences for luciferase expression were as follows: luciferase, 5'-CAG TCG ATG TAC ACG TTC GTC AC-3' and 5'-TAG CTG ATG TAG TCT CAG TGA GC-3'. The sequences of the β -actin sense and antisense primers were 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3', respectively. The experiment was repeated three times, and all samples were analyzed in triplicate. The final PCR

products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.11. Western blotting

Mesenteric lymph nodes from a subset of the groups ($n=5$ per group) was homogenized in a PRO-PREP™ Solution Kit (iNtRON Biotechnology, Sungnam, Korea) supplemented with 1/2 of a protein inhibitor cocktail tablet (Roche, Penzberg, Germany), followed by centrifugation at 10,000g for 10 min. The prepared proteins were electrophoresed through a 10% SDS-PAGE gel. Next, the proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences, Corston, UK) for 2 h at 45 V in transfer buffer (25 mM Trizma-base, 192 mM glycine, and 20% methanol). Efficiency of the transfer as well as equal protein loading were determined by staining the membrane with Amido Black Staining Solution (Sigma-Aldrich) and the gel with Coomassie Blue. Appropriate dilutions of primary antibodies, anti-IL-6 antibody (Santa Cruz Biotechnology, CA, USA), anti-VEGF antibody (Pepro Tech., NJ, USA), anti- β -actin (Sigma-Aldrich), were added to the membranes, which were allowed to hybridize overnight at 4 °C. After the antibodies were removed, the membrane was washed three times in a solution composed of 10 mM Trizma-base (pH 7.6), 150 mM NaCl, and 0.05% Tween-20 for 10 min. This was followed by incubation with horseradish peroxidase-conjugated anti-secondary antibody for 1 h at room temperature. The membrane was washed again as described above and developed using an enhanced chemiluminescence detection system (Amersham Bioscience). The results were quantitated using the Image Analyzer System (Estman Kodak 2000MM, NY, USA) and expressed as the fold-increase over control values. These results were confirmed by two independent researchers performing the experiments at least twice.

2.12. Statistical analysis

One-way ANOVA was used to determine whether or not significant differences existed between the PA-treated group and AOO-treated group (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL). Additionally, response differences between the AEtLP-treated group and vehicle-treated group in PA-treated group were evaluated by a *post hoc* test (SPSS for Windows, Release 10.10, Standard Version) of the variance and significance levels. All values were expressed as the mean $\pm$ SD. A p value of <0.05 was considered significant.

3. Results

3.1. Effects of PA or AEtLP treatment on body weight of mice

Previous studies have reported that PA is able to induce skin irritation in several strains of mice (Hayashi et al., 2001; Ban and Hettich, 2005; Bae et al., 2010). Therefore, to investigate the effects of PA and AEtLP treatment in mice, alteration of body weight in IL-4/Luc/CNS-1 Tg mice or non-Tg mice was measured using an electric balance during the experimental period. However, no significant difference in body weight was detected in IL-4/Luc/CNS-1 Tg mice or non-Tg mice after any of the treatments (Fig. 2A). Therefore, these results indicate that treatment with PA, AEtLP, or both did not induce any change in the body weight of mice.

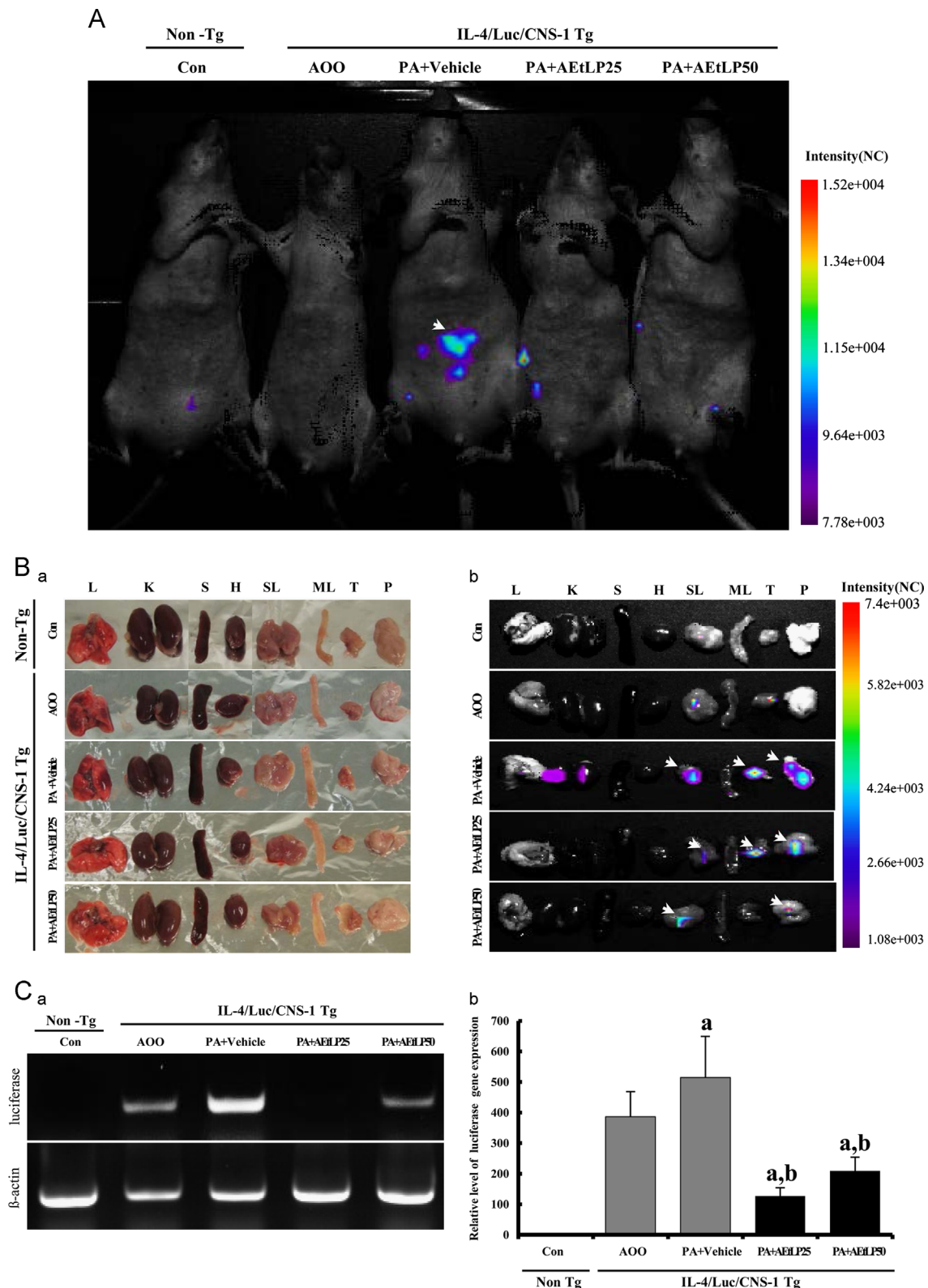


Fig. 1. Detection of luciferase signal in the whole body (A) and each organ (B) of IL-4/Luc/CNS-1 Tg mice. Mice were treated with AOO, PA+Vehicle, PA+AELP25, and PA+AELP50 for 4 weeks and imaged at 24 h after final treatment using Living Image software. Color overlay on the image represents the photons per second emitted from the organs, in accordance with the pseudocolor scale shown next to the image. In this image, red indicates the highest number of photons per second while blue indicates the lowest number of photons per second. L, Lung; K, kidney; S, spleen; H, heart; SL, Submandibular lymph node; ML, Mesenteric lymph node; T, Thymus; P, Pancreas. (C) Expression level of luciferase gene in SL was measured by RT-PCR using specific primer. Data shown are the mean $\pm$ SD ($n=5$). a, $p < 0.05$ is the significance level compared to the AOO-treated group. b, $p < 0.05$ is the significance level compared to the PA+Vehicle-treated group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Quantifying therapeutic effect of AEtLP treatment in IL-4/Luc/CNS-1 Tg mice treated with PA

Firstly, to quantify the therapeutic effect of AEtLP on the allergic response to PA treatment using the luciferase reporter system, luciferase signals from the whole body and eight organs were measured using LivingImage software in IL-4/Luc/CNS-1 Tg mice after individual PA+Vehicle or PA+AEtLP treatment. In the whole body image, the luciferase signal was highly detected in the abdominal region of IL-4/Luc/CNS-1 Tg mice treated with only PA+Vehicle, whereas the AOO-treated group showed no luciferase signal. However, this signal was greatly reduced in the groups treated with PA+AEtLP25 and AEtLP50 (Fig. 1A). In the organ image analysis, the highest luciferase signals were detected in the thymus, followed by the pancreas and SL, of IL-4/Luc/CNS-1 Tg mice treated with PA+Vehicle, whereas luciferase signals were maintained in all eight organs of the AOO-treated group. However, luciferase signals were dramatically reduced in these three organs in IL-4/Luc/CNS-1 Tg mice treated with PA+AEtLP25 or PA+AEtLP50. Especially, the luciferase signals in these three organs of IL-4/Luc/CNS-1 Tg mice treated with PA+AEtLP50 almost disappeared compared to the AOO-treated group (Fig. 1B). Furthermore, the mRNA level of luciferase gene was very similar with

the luciferase signal pattern in all groups, although the increase or decrease rate varied (Fig. 1C). Therefore, these results indicate that the relieving effect of AEtLP on the allergic response induced by PA treatment could be quantified using IL-4/Luc/CNS-1 Tg mice without sacrificing.

3.3. Comparison between luciferase signal and allergic response

Next, to determine whether or not the luciferase signal could corresponds with AD phenotypes, several indicators of the AD response, including ear thickness, lymph node weight, IgE concentration, and mast cell infiltration, were measured in IL-4/Luc/CNS-1 Tg mice.

3.3.1. Effects of AEtLP treatment on ear thickness and morphology

To investigate whether or not treatment with AEtLP can suppress the changes in ear phenotype induced by PA treatment, ear thickness and morphology were observed in IL-4/Luc/CNS-1 Tg mice for 4 weeks. Ear thickness rapidly increased in PA+Vehicle-treated mice compared to Con or AOO-treated mice. On the other hand, ear thickness in PA+AEtLP-treated mice slowly increased,

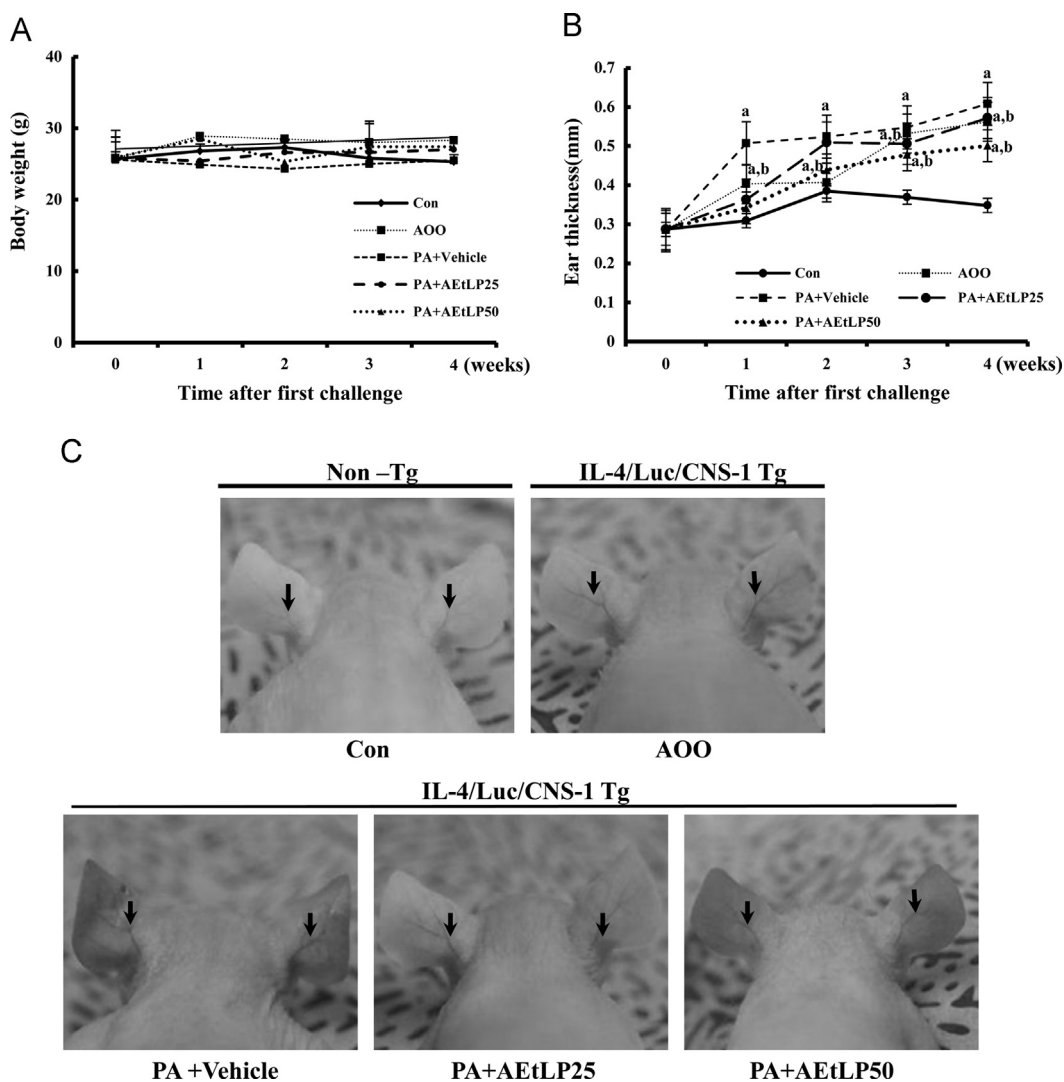


Fig. 2. Differences in body weight, ear thickness, and ear phenotypes. (A) Body weights of mice in the five groups were measured with a chemical balance. PA solution was repeatedly applied to the dorsum of ears of IL-4/Luc/CNS-1 Tg mice during oral gavage of AEtLP. After 4 weeks, ear thickness (B) and phenotypes (C) were observed by following the procedure described in Section 2. Arrowhead indicates the ear vein. Data shown are the mean $\pm$ SD ($n=5$). a, $p < 0.05$ is the significance level compared to the AOO-treated group. b, $p < 0.05$ is the significance level compared to the PA+Vehicle-treated group.

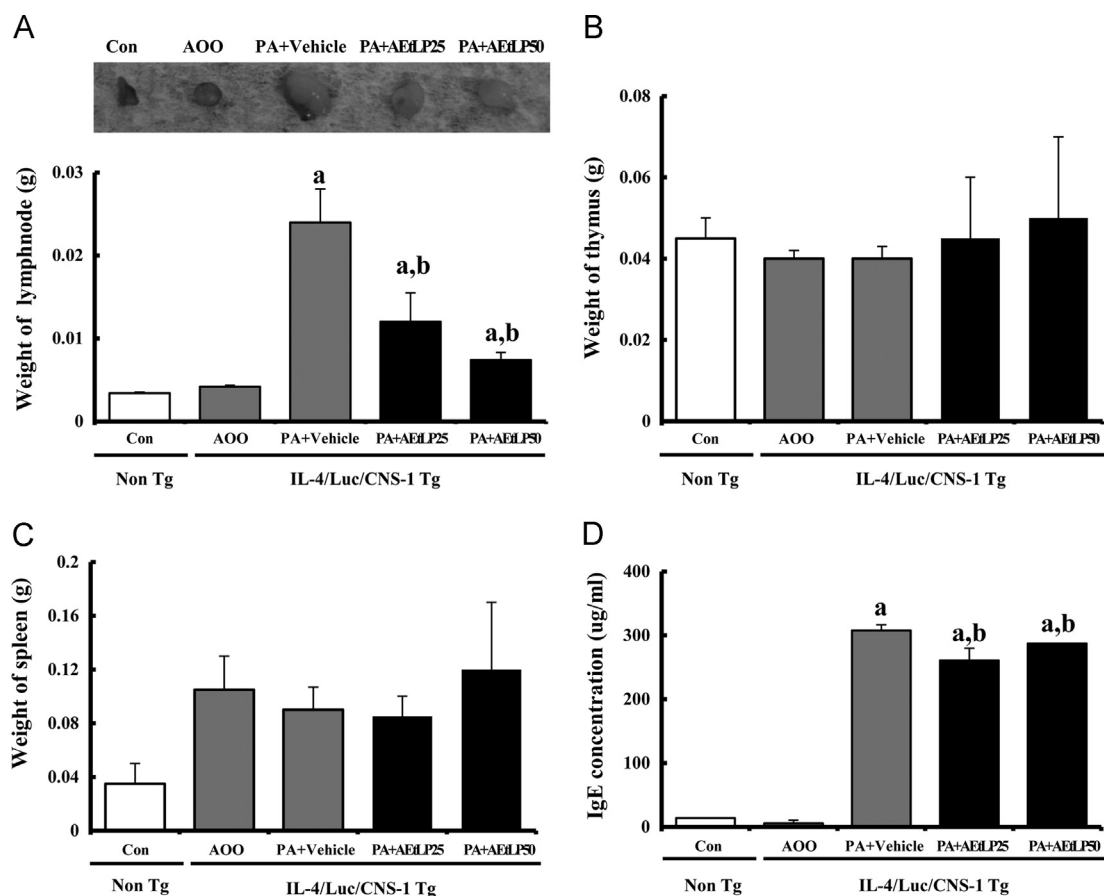


Fig. 3. Changes in auricular lymph node weight (A), thymus weight (B), spleen weight (C) and serum IgE concentration (D) in IL-4/Luc/CNS-1 Tg mice. After final treatment, mice from each group were sacrificed under anesthesia. The auricular lymph nodes were then harvested from the neck regions of the mice using a microscissor, after which they were weighed using a chemical balance. Serum used to measure the IgE concentration was prepared from blood samples collected from the abdominal veins of mice. Serum IgE concentration was quantified by ELISA. Data shown are the mean $\pm$ SD ($n=5$). a, $p < 0.05$ is the significance level compared to the AOO-treated group. b, $p < 0.05$ is the significance level compared to the PA+Vehicle-treated group.

although the PA+AETLP25-treated group showed a similar pattern as the PA+Vehicle-treated group from 2 to 4 weeks (Fig. 2B). Furthermore, the outline of the ear vein became distinct or thickened in the PA+Vehicle-treated group compared with the AOO-treated group, and ear color changed from flesh tint to dark brown. These alterations of ear morphology and ear thickness were dramatically reversed upon PA+AETLP cotreatment (Fig. 2C). Taken together, our results demonstrate that AETLP treatment may successfully recover the increases in ear and vein thickness induced by PA treatment. Further, these changes in ear thickness and phenotypes were in agreement with the luciferase signal observed in Fig. 1.

3.3.2. Effect of AETLP treatment on weight of lymph node and IgE concentration

Next, we investigated whether or not AETLP could suppress the increases in lymph node weight and IgE concentration. To accomplish this, we evaluated the auricular lymph node (AL) weight and serum IgE concentration in both non-Tg mice and IL-4/Luc/CNS-1 Tg mice. It is well known that the weight of local lymph nodes increases in response to topical application of agents that have allergenic or sensitizing potential (Stahlmann et al., 2006). As shown in Fig. 3A, PA treatment induced an increase in AL weight in IL-4/Luc/CNS-1 Tg mice compared with non-Tg mice. However, the weight of AL was significantly reduced in the PA+AETLP-treated group, although not to the level of the AOO-treated group. Furthermore, the weight of other organs including thymus

and spleen were maintained their level in PA+Vehicle, PA+AETLP25 or PA+AETLP50-treated group (Fig. 3B and C). It is well known that hyperproduction of IgE is one of the characteristic features of allergic hypersensitivity (Gao et al., 2004) as well as an indicator of the magnitude of the allergic immune response including AD (Dearman et al., 2003). Therefore, the serum IgE concentration was measured in the five groups of mice to determine whether or not AETLP contributes to the suppression of the allergic response induced by PA treatment. Repeated topical application of PA solution induced a significant increase in serum IgE concentration in IL-4/Luc/CNS-1 Tg mice. However, reduction of IgE concentration was observed in only the PA+AETLP-treated group (Fig. 3D). Taken together, these results suggest that AETLP treatment may contribute to the reduction of AL weight and IgE concentration in IL-4/Luc/CNS-1 Tg mice after PA treatment.

3.3.3. Effect of AETLP treatment on ear histology

To investigate the suppressive effect of AETLP treatment on ear histology, histological analysis of ear tissue from IL-4/Luc/CNS-1 Tg mice was performed. The dermis of the ear tissue was thicker in the PA+Vehicle-treated group than in the AOO-treated group, whereas the epidermis was thinner (Fig. 4A–C, G and H). However, the thickness of the dermis greatly decreased in the PA+AETLP-treated group, although there are no difference between PA+AETLP25 and PA+AETLP50 treatments (Fig. 4D, E, G and H). Especially, PA treatment induced large changes in the number of adipocytes in the dermis layer. Specifically, the PA+Vehicle-treated

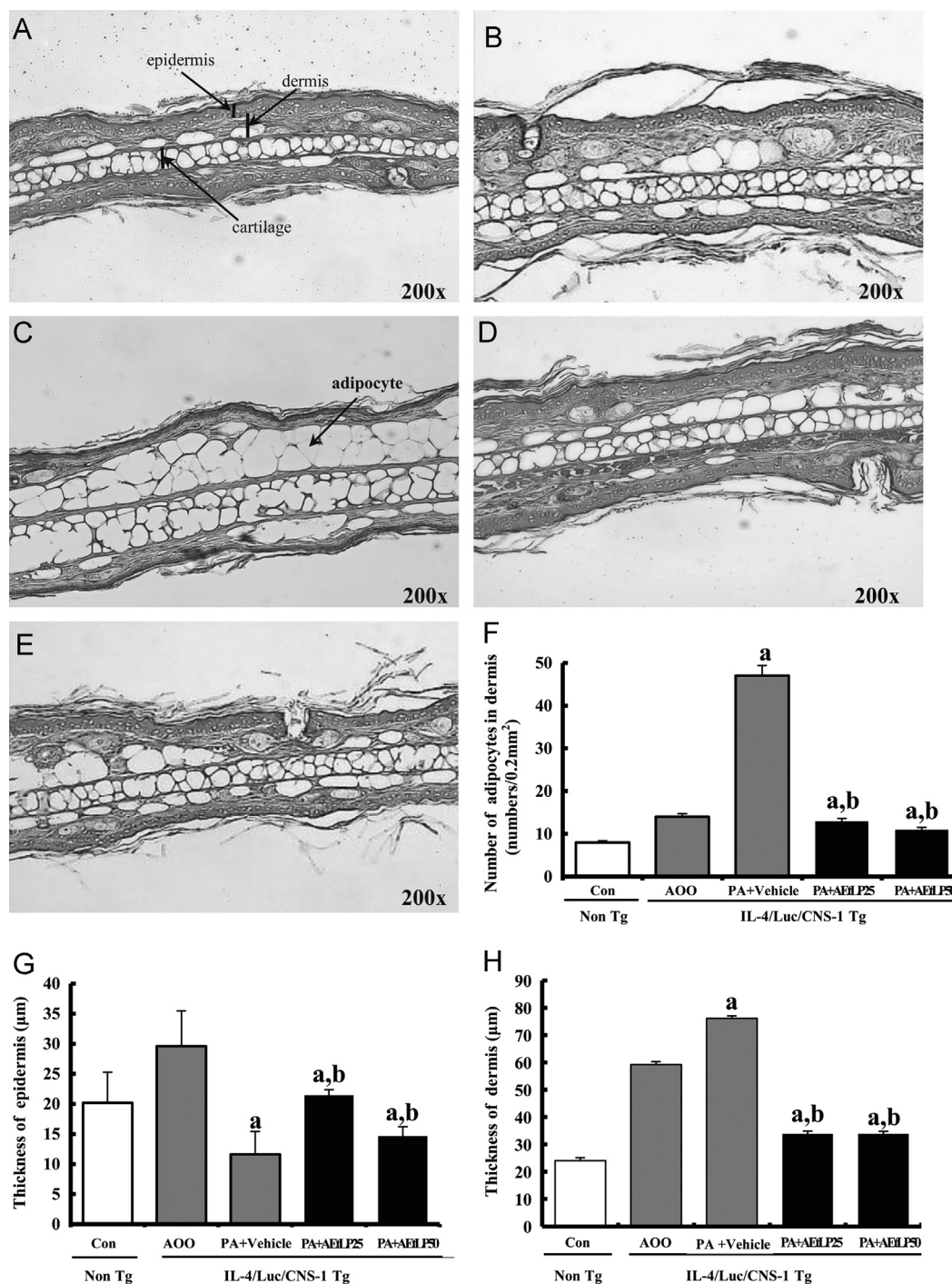


Fig. 4. Histopathology of ear skin in non-Tg (A) or IL-4/Luc/CNS-1 Tg mice treated with AOO (B), PA+Vehicle (C), PA+AELP25 (D) and PA+AELP50 (E). PA solution was repeatedly applied to the dorsum of ears of IL-4/Luc/CNS-1 Tg mice during oral gavage of AELTP. After 4 weeks, histological changes were observed as described in Section 2. Slide sections of ear tissue were stained with hematoxylin and eosin and observed at magnifications of 100 × or 200 ×. (A)–(E) Histological images and (F)–(H) numbers of adipocytes as well as thickness of the epidermis and dermis. Data shown are the mean ± SD ($n=5$). a, $p < 0.05$ is the significance level compared to the AOO-treated group. b, $p < 0.05$ is the significance level compared to the PA+Vehicle-treated group.

group showed 6-fold higher numbers of adipocytes compared to the AOO-treated group (Fig. 4B and C). However, AELTP treatment reversed this increase in adipocytes in a dose-dependent manner (Fig. 4D, E and F). These results show that AELTP improved the AD response induced by PA treatment.

3.3.4. Effect of AELTP treatment on infiltration of mast cells

Mast cells play important roles in asthma, eczema, itch, allergic rhinitis, and allergic conjunctivitis (Prussin and Metcalfe, 2003).

Therefore, in order to investigate the suppressive effects of AELTP on infiltration of mast cells, an ear skin section was stained with toluidine blue, and the alteration of mast cells were observed under a microscope. Mast cells stained with blue color were significantly greater in number in the PA+Vehicle-treated group (Fig. 5C) compared to the Con group, although it was lower than AOO-treated group (Fig. 5A and B). However, their numbers were significantly reduced upon PA+AELTP treatment according to their concentration (Fig. 5D–F). These data suggest that AELTP may contribute to the suppression of mast cell infiltration in the dermis of ear skin.

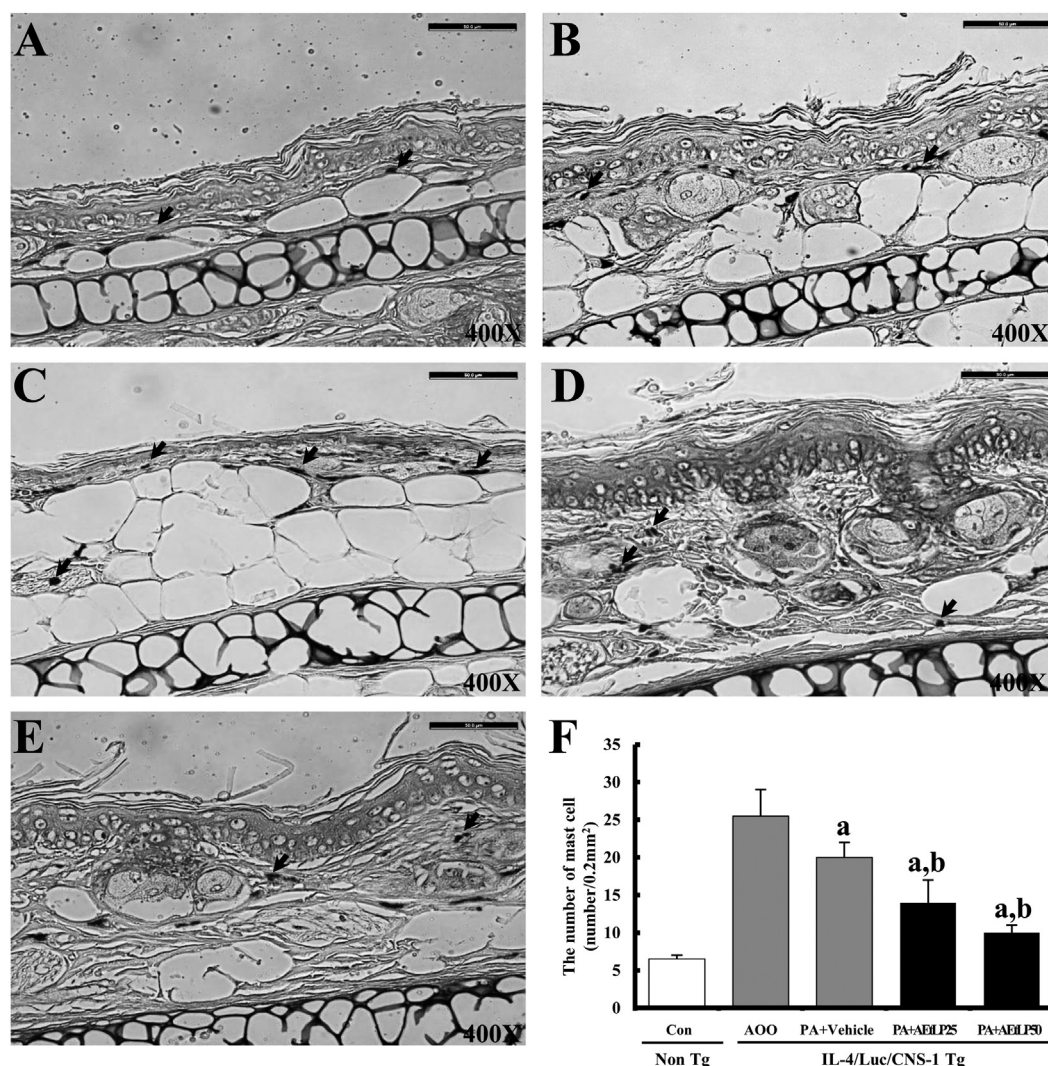


Fig. 5. Infiltration of mast cells in non-Tg (A) or IL-4/Luc/CNS-1 Tg mice treated with AOO (B), PA+Vehicle (C), PA+AETLP25 (D) and PA+AETLP50 (E). Slide sections of ear tissue were stained with 0.25% toluidine blue and observed at magnifications of 100 $\times$ or 200 $\times$. Arrowhead indicates infiltrated mast cells in the dermis of ears. (F) Numbers of infiltrated mast cells. Data shown are the mean $\pm$ SD ($n=5$). a, $p < 0.05$ is the significance level compared to the AOO-treated group. b, $p < 0.05$ is the significance level compared to the PA+Vehicle-treated group.

3.3.5. Effect of AETLP treatment on cytokine expression

Finally, to determine whether or not AETLP could induce alteration of atopic response-related cytokine expression, the expression levels of VEGF and IL-6 were measured in lymph nodes of IL-4/Luc/CNS-1 Tg mice. High expression of VEGF protein was observed in the PA+Vehicle-treated group, whereas a low level was detected in the AOO-treated group. Reduction of VEGF protein expression was observed in the PA+AETLP-treated group (Fig. 6A and B). Furthermore, similar results were observed in the analysis of IL-6 expression. Increased IL-6 expression after PA treatment was reversed by AETLP treatment, although not to the level of the AOO-treated group (Fig. 6A and C). The above results suggest that AETLP treatment may relieve the allergic response induced by PA treatment through regulation of VEGF and IL-6 expression.

4. Discussion

Allergic responses are characterized by infiltration of specific cells, including inflammatory cells such as T lymphocytes, leukocytes, neutrophils, eosinophils, macrophages, mast cells, and regulatory and natural killer T cells (Dubois et al., 1994; Zhang et al., 1997). The activation of inflammatory cells in this infiltration

process is mediated by various cytokines and chemokines at the site of injury. Of these cytokines, IL-4 is the hallmark cytokine for Th2 cells, although it is also produced by NK cells, basophils, eosinophils, and mast cells, and is tightly related with its role in immunity against extracellular allergens (Voehringer et al., 2004; Gessner et al., 2005; Croxford and Buch, 2011). Based on this role of IL-4, some transgenic animal models featuring an IL-4-regulated reporter gene have been developed for assessing immunity *in vivo*. Knock-in mice expressing IL-4 linked via a viral IRES element with enhanced EGFP has been used to demonstrate the evolution of type 2 immunity from tissue lymphocytes after *Nippostrongylus* infection (Mohrs et al., 2001). Further, IL-4 dual-reporter mice, which permit the simultaneous analysis of IL-4 transcripts via GFP and IL-4 protein secretion by use of huCD2, have been applied to reveal a two-step process for cytokine production under conditions of challenge with strictly enteric helminth (Mohrs et al., 2005). Meanwhile, IL-4/Luc/CNS-1 Tg mice used in this study demonstrate higher luciferase activity in immune-related organs such as the lymph nodes, thymus, and lung after treatment with allergens, including respiratory sensitizer, vaccine additives, and crude extracts of natural allergens (Bae et al., 2011). Therefore, we selected IL-4/Luc/CNS-1 Tg mice in order to quantify the relieving effects of *Liriope platyphylla*. The current data provide additional

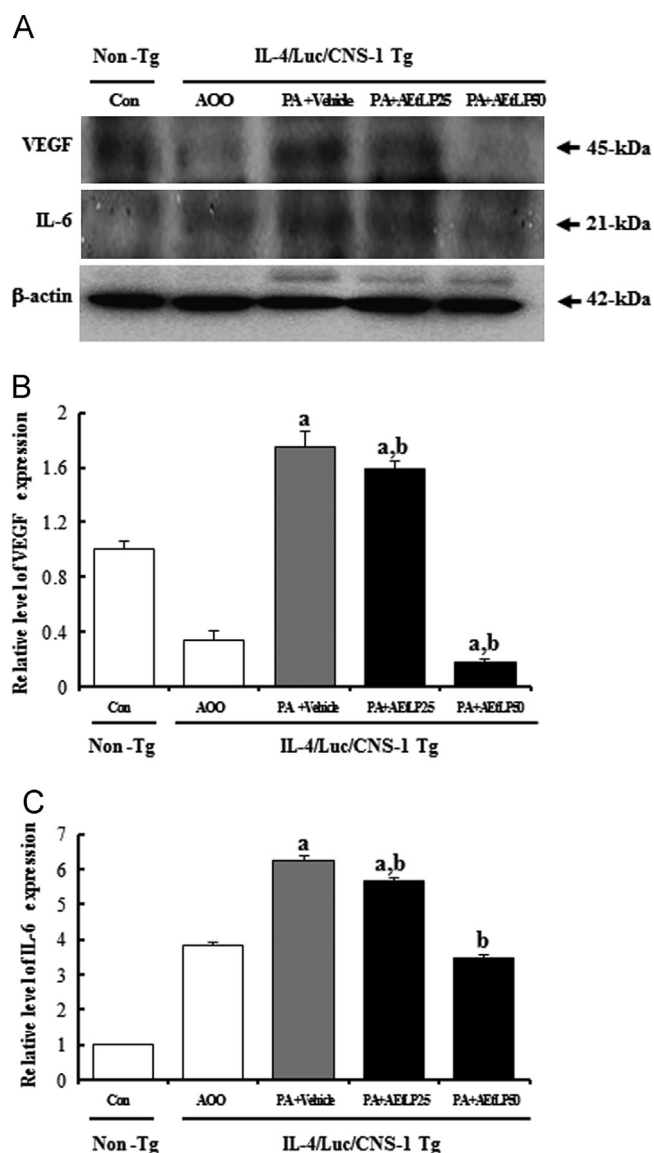


Fig. 6. Expression level of VEGF and IL-6 in lymph node. Alteration of the expression of the two proteins was measured by Western blotting. After the intensity of each band was determined using an imaging densitometer, the relative level of each protein was calculated based on the intensity of actin protein. Data represent the mean $\pm$ SD from three replicates. a, $p < 0.05$ is the significance level compared to the AOO-treated group. b, $p < 0.05$ is the significance level compared to the PA+Vehicle-treated group.

evidence that IL-4/Luc/CNS-1 Tg mice may facilitate the evaluation and prediction of human body responses against a variety of environmental allergens. However, in our study, IL-4/Luc/CNS-1 Tg mice showed difference responses to PA treatment. As shown in Fig. 2, the highest luciferase signal was detected in the thymus, followed by the pancreas and SL, in response to application of PA on the ear dorsum. This result is slightly different from previous results in which the highest luciferase signal was observed in ML, followed by the thymus, lung, pancreas, and SL, after PA was spread on the dorsum of ears and the shaved dorsal region. We believe that this difference is attributable to differences in spreading site and PA volume.

Recently, our group investigated the therapeutic effect of LP on PA-induced atopic dermatitis using NC/Nga mice after AEtLP administration for 2 weeks. The pathological phenotypes of atopic dermatitis, such as erythema, ear thickness, edema, scab, and

discharge, were significantly reduced upon PA+10% AEtLP treatment compared to PA treatment only. Further, weights of lymph nodes and thymus in immune organs gradually decreased in the AEtLP-treated group, whereas reduced mast cell infiltration into the dermis was statistically observed upon PA+5% AEtLP and PA+10% AEtLP cotreatment. Especially, reduction of the IgE concentration was detected only upon PA+10% AEtLP cotreatment, although this level was maintained upon PA+5% AEtLP cotreatment (Kim et al., 2010b, 2010c). The results of the current study were in agreement with the above report, although the rates of increase and decrease varied. However, one important difference is that the current study used IL-4/Luc/CNS-1 Tg mice as a model of the allergic response, whereas the above study utilized NC/Nga mice model for AD.

Meanwhile, mast cells are considered as a key effector cell in IgE-mediated immediate hypersensitivity and allergic disorders, as well as in the protection of immune responses to parasites and bacteria (Galli, 2000; Kawakami et al., 2009). However, recent studies have proposed a greater role for mast cells from innate defense against bee and snake venoms (Metz et al., 2006) to multiple aspects of adaptive immune responses, including antigen presentation, leukocyte recruitment, and draining of lymph nodes (McLachlan et al., 2003). Furthermore, these roles have been extended to allergic diseases and helminth and bacterial infection, autoimmune diseases (Secor et al., 2000), carcinogenesis (Soucek et al., 2007), allograft tolerance (Lu et al., 2006), and angiogenesis (Heissig et al., 2005). Therefore, we investigated the alteration of mast cells in ears of IL-4/Luc/CNS-1 Tg mice after PA+AEtLP treatment. The number of mast cells infiltrated into the dermis significantly increased in the PA-treated group compare to the non-Tg group. However, the number of mast cells decreased in the PA+AEtLP-treated group. This result was very similar to a previous study that investigated the therapeutic effect of AEtLP in NC/Nga mice after PA treatment. However, in the AOO-treated group, the number of mast cells was higher in IL-4/Luc/CNS-1 Tg mice than in NC/Nga mice. Further studies are required to identify the main causes of this difference.

Taken together, the above results demonstrate two novel findings about *Liriope platyphylla* and IL-4/Luc/CNS-1 Tg mice. Firstly, IL-4/Luc/CNS-1 Tg mice may be successfully applied to screen for therapeutic effects of various herbal medicines. Secondly, AEtLP can effectively relieve atopic dermatitis induced by PA treatment. Furthermore, these findings indicate that AEtLP could be considered as a new drug for the treatment of AD.

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