

Topical application of a phospholipid mixture purified from pig lungs ameliorates 2,4-dinitrofluorobenzene-induced allergic contact dermatitis in BALB/c mice

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Abstract This work was designed to assess the pharmacological effectiveness as a novel anti-atopic dermatitis remedy of a phospholipid mixture purified from pig lung tissues, named KT&G101, using the BALB/c mouse model of allergic contact dermatitis. Allergic contact dermatitis was induced by applying 2,4-dinitrofluorobenzene (DNFB) epicutaneously onto the dorsal skins of mice, and KT&G101 was topically applied onto the skin areas with the lesions. The topical application of KT&G101 (0.05 ml of 10 mg/ml and 20 mg/ml KT&G101, twice a day for 15 days) decreased the total IgE level elevated in the sera of mice undergoing allergic contact dermatitis. KT&G101 was also able to decrease the 2,4-dinitrophenyl (DNP)-specific IgE level elevated in the sera of the model mice. It reduced the incidences of scratching behaviors in the mice undergoing DNFB-induced allergic contact dermatitis. It attenuated some histopathological changes, such as pustule, epidermal hyperplasia, dermatitis and fibroplasia, while it could enhance the recovery of epidermis, in the damaged skin tissues within a relatively short period after the topical application of KT&G101. KT&G101 lessened the

expression of cytokines mRNAs, such as Th1-specific IL-2, TNF- β and IFN- γ , and Th2-specific IL-4, in the mouse skin tissues showing the lesions. In brief, it is concluded that KT&G101 alleviates the symptoms involved in induced allergic contact dermatitis in BALB/c mice.

Keywords Allergic contact dermatitis · Cytokine · 2,4-Dinitrofluorobenzene · IgE · Phospholipid

Abbreviations

AD	Atopic dermatitis
DEXA	Dexamethasone
DNFB	2,4-Dinitrofluorobenzene
DNP	Dinitrophenyl
DPPC	Dipalmitoylphosphatidylcholine
PC	Phosphatidylcholine
PS	Phosphatidylserine

Introduction

Allergic contact dermatitis is a prototypic T-cell-mediated cutaneous inflammatory and a contact hypersensitivity reaction that occurs after contact with allergens. It consists of two distinct phases, i.e., the clinically silent sensitization phase and the clinically apparent elicitation phase. In the sensitization phase, haptens, such as low molecular weight molecules or metal ions, are captured by skin dendritic cells, including epidermal Langerhans cells, and activated Langerhans cells migrate to regional lymph nodes and activate antigen-specific T-cell precursors [1]. Allergic contact dermatitis caused by DNFB, one of the strong haptens, is mediated by CD8+ cytotoxic T cells and downregulated by CD4+ T cells [2]. In the elicitation

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phase induced by re-exposure to the same hapten, the reactive haptens activate resident skin cells, such as keratinocytes, fibroblasts, endothelial cells and mast cells, which release proinflammatory substances including cytokines (IL-1 β , IL-6, TNF- α , etc.) and chemokines (CCL20, etc.) [3]. Furthermore, the hapten molecules become immunogenic after covalently attaching to certain amino acid residues of cell surface proteins and then cause the self-proteins to induce allergic contact dermatitis [4]. Similarly to contact dermatitis, it is known that atopic dermatitis (AD) is attributed to chronic exposure to environmental allergens, such as house dusts or mites [5]. Although haptens are generally used to induce allergic contact dermatitis via primarily evoking a Th1-dominated response, the multiple application of haptens onto the skin of hairless mice over extended period has recently shown to cause the skin inflammation to shift from a typical Th1-dominated delayed-type hypersensitivity response to a chronic Th2-dominated inflammatory response that resembles human AD [6, 7]. The repeated application of several haptens, such as oxazolone, DNFB and 2,4,6-trinitrochlorobenzene (TNCB), caused a variety of changes, observed in AD patients, on the mouse skin lesions, which did not appear after only a single application [7]. Accordingly, the animal model made by repeated hapten sensitization process can be useful for evaluating underlying action mechanisms of plausible anti-atopic therapies, chiefly due to its reproducibility, low cost and time-saving.

Pulmonary surfactant is a unique mixture of surfactant lipids and surfactant-specific proteins. Phosphatidylcholine (PC) comprises about 80% of the total surfactant lipids, about half of which is dipalmitoylphosphatidylcholine (DPPC) [8]. The lipid-rich surfactant preparations and DPPC, at physiological concentrations, could reduce lipopolysaccharide (LPS)-induced IL-8 expression at both mRNA and protein levels in lung epithelial cells, resulting in inhibited translocation of toll-like receptor 4 into membrane lipid raft microdomains [9]. PC, a major component of surfactant lipids, contains its anti-inflammatory property through its capability to suppress arthritic symptoms in the carrageenan-induced rat arthritis model [10]. Orally administered PC reduced the collagen-induced arthritis-induced hypersensitivity, decreased the number of leukocyte-endothelial cell interactions and the extent of functional capillary density, and decreased the expression of inducible nitric oxide synthase (iNOS) [11]. PC was also able to induce a prolonged inhibition of TNF- α -induced pro-inflammatory signaling in intestinal epithelial cells through a shift of the TNF- α receptors at the surface to lipid rafts [12]. Liposomes made of phosphatidylserine (PS), another component of surfactant lipids, were suggested to exhibit an anti-inflammatory activity through activation of peroxisome proliferator-activated receptors (PPARs) [13].

In rat microglial cultures, PS liposomes reduced the release of proinflammatory molecules such as nitric oxide (NO), IL-1 β , and TNF- α enhanced by LPS [14]. Phospholipids in pulmonary surfactant are secreted from alveolar Type II cells to an external part of body where oxygen is plenty. Due to being highly saturated in the hydrocarbon chains, they may maintain stability against lipid peroxidation induced by oxygen. Based on some desirable characteristics of surfactant phospholipids described above, KT&G101, a mixture of chemically stable and metabolically favorable phospholipids purified from pig lung tissues, is being developed as a novel topical remedy in order to treat AD. The clinical trial phase III of KT&G101 is currently in progress and this study was designed to support the clinical effectiveness of KT&G101 in the aspects of pharmacology. In this work, we demonstrate that KT&G101 improves the symptoms related with DNFB-induced allergic contact dermatitis in BALB/c mice.

Materials and methods

Chemicals

2,4-Dinitrofluorobenzene (DNFB), dexamethasone (DEXA), phosphatidylcholine (PC, egg yolk), phosphatidylinositol (PI, soybean), phosphatidylserine (PS, bovine brain) and dipalmitoylphosphatidylcholine (DPPC, synthetic) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MI, USA). *i*-MAX II DNA polymerase was from iNtRON Biotechnology (Sungnam, Korea). TRIZOL[®] reagent and M-MLV reverse transcriptase were obtained from Invitrogen (Eugene, OR, USA) and Promega (Madison, WI, USA), respectively. Mouse IgE ELISA core kit and mouse anti-DNP IgE ELISA kit were from Koma Biotech (Seoul, Korea) and Alpha Diagnostic International (San Antonio, TX, USA), respectively. PCR primers were from COSMO Co. (Seoul, Korea). All other chemicals used were of reagent grade or better.

Experimental animals

Five-week-old male BALB/c mice (21 $\pm$ 2 g) were obtained from Daehan Biolink Co., Eumsung, Korea. The animal room was maintained at 25 $\pm$ 2°C with a 12-h light/dark cycle. Food and tap water were supplied ad libitum. The ethical guidelines, described in the NIH Guide for Care and Use of Laboratory Animals, were followed throughout the experiments. Animal experiments performed in this work were approved by the Ethical Committee, Kangwon National University, Chuncheon, Korea.

Preparation of KT&G101

Fresh pig lung tissues were obtained from a slaughterhouse having HACCP (Hazard Analysis Critical Control Point) criteria in Hongchun, Kangwon-do, Korea. The pig lungs were kept in an ice-water bath as soon as the tissues were taken out, and used within 5 h after the slaughter.

Pig lung tissues were diced and homogenized in 3 volumes of ice-cold saline containing 5 mM CaCl_2 using a metal-blade blender (Model HM-390, Hanil Electric Co., Seoul, Korea) for 1 min, and incubated in an ice-water bath for 1 h and centrifuged at $5000\times g$ for 45 min at 4°C to get rid of blood and to collect the precipitate. The precipitate was re-suspended in 6 volumes of hypotonic 70 mM NaCl solution containing 5 mM CaCl_2 , and homogenized again using a metal-blade blender for 1 min. The homogenate was incubated in an ice-water bath for 1 h. and centrifuged at $5000\times g$ for 45 min at 4°C to obtain the precipitate. Calcium ions were added to induce the aggregation of surfactant lipids and proteins in the homogenates to facilitate their precipitation in low centrifugal force. The final pellets were dissolved in two volumes of chloroform/methanol (2:1 by volume) mixture at room temperature. The aqueous phase and the materials insoluble in the organic solvent phase were discarded. To eliminate a portion of neutral lipids from the organic solvent phase, the chloroform/methanol mixture was thoroughly evaporated using a rotary vacuum evaporator (Model N-1000S, Tokyo Rikakakai Co., Tokyo, Japan) at 65°C and an excess amount of cold acetone was poured into the dried total lipid mixture. The pool of polar lipids containing phospholipids was recovered as the precipitate, insoluble in acetone.

The pool of polar lipid mixture, dissolved in pure chloroform, was further purified using an open silica gel column [Kieselgel 60 (230–400 mesh, Merck No. 7729), diameter/height (1:2–1:2.5)] pre-washed and saturated with pure acetone. To get rid of neutral lipids thoroughly from the pool of polar lipids, non-polar solvents such as acetone and chloroform were applied into the column first. A polar solvent mixture of chloroform, methanol and water was employed later to obtain the pure phospholipids. The eluting solvents were applied into the column in the following order; one bed volume of pure acetone, one bed volume of pure chloroform, one-third bed volume of chloroform/methanol (9:1 by volume) mixture, one-third bed volume of chloroform/methanol (4:1 by volume) mixture and 5–6 bed volumes of chloroform/methanol/water (6:8:1 by volume) mixture. Among the fractions eluted by the chloroform/methanol/water mixture, the fractions showing the R_f values between 0.3 and 0.55 on the thin-layer chromatography developed by chloroform/methanol/water (65:25:4 by volume) mixture were collected. The collected fractions were concentrated using a rotary vacuum evaporator to remove

both solvents and moisture and then freeze-dried for 20 h at -98°C (FDU-8606, freeze-dryer, Operon Co. Kimpo, Korea) to generate the white powder of a phospholipid mixture, named KT&G101. The purity of KT&G101 was determined by TLC. To identify both phospholipids and other neutral lipids on a TLC plate, the charring procedure using 5% (w/v) sulfuric acid in ethanol was employed. 0.2% (w/v) ninhydrin in ethanol spraying was employed to visualize either peptide or protein spots on a TLC plate in the sample. To determine the concentration of total phospholipids, a modified Bartlett assay using Fiske and Subbarow reagent [15] was employed. The HPLC–ELS (Evaporative Light Scattering) Detector system (Waters e2695 Module System, USA) was used to quantify the content of DPPC in total phospholipids.

KT&G101 was suspended in 5 mM citrate buffer, pH 5.8 containing 1.5 mM CaCl_2 , and used in this work. As a control, 5 mM citrate buffer, pH 5.8 containing 1.5 mM CaCl_2 was used. The experimental samples of KT&G101 were designed to contain 1.5 mM CaCl_2 which is adopted from the concentration of Ca^{2+} ions on the surface of lung [16, 17], and 5 mM citrate buffer, pH 5.8 which is exploited as a pH buffer and as substances preventing the aggregation of phospholipids in the presence of Ca^{2+} ions.

Treatment with DNFB and/or tested materials

After the mice were acclimatized to their housing environment for 1 week, they were topically applied with 100 μl of 0.15% DNFB in acetone onto the clipped abdominal skin for initial sensitization. From one week later, 50 μl of 0.15% DNFB in acetone was topically applied onto the shaved dorsal skin of sensitized mouse once per 3 days for 30 days, 10 times in total. Mice in the control group were treated with acetone only. Fifty microliters of vehicle, 0.05% dexamethasone, 10 and 20 mg/ml KT&G101 was topically applied onto the DNFB-painted skin twice a day on the days of the sixth to tenth DNFB challenge. The experimental method was referred from the procedure reported by Inagaki et al. [18] and slightly modified for this experiment.

Measurement of total and specific IgE in serum

Blood samples were collected from the heart under ether anesthesia 24 h after the tenth application of DNFB, stored at room temperature for 30 min, and centrifuged at $3000\times g$ for 5 min to separate sera. The concentrations of total and DNP-specific IgE in sera were quantified using mouse IgE ELISA core kit (Koma Biotech, Seoul, Korea) and mouse anti-DNP IgE ELISA kit (Alpha Diagnostic International, San Antonio, TX, USA) according to the manufacturers' protocols, respectively.

Histopathological analysis

Dorsal skin tissues were taken 24 h after the tenth application of DNFB and fixed in 10% formaldehyde solution. After the routine tissue process, the tissues were embedded in paraffin and sectioned at 3 μ m for hematoxylin and eosin staining. Degrees of pustule, epidermal hyperplasia, dermatitis and fibroplasia in the fixed tissues were semi-quantitatively determined based on histopathological criteria of skins. Epidermal hyperplasia was evaluated as mild (1+; 3–5 layers), moderate (2+; 5–8 layers) and severe (3+; 8–10 layers) compared to normal (0; 2–4 layers). Pustule, dermatitis and fibroplasia in dermis were evaluated as no lesion (0), slight (1+), mild (2+), moderate (3+) and severe (4+).

Detection of scratching behavior

The scratching behavior was evaluated after topically applying KT&G101 or dexamethasone on the day of the tenth application of DNFB. Six mice were separately put into an acrylic cage (9 $\times$ 13 $\times$ 16 cm) divided into six compartments, and their behaviors were then recorded for 1 h using a HANDYCAM video camera (HDR-SR11; Sony, Tokyo, Japan) under unmanned conditions. Scratching behaviors by the hind paws were counted for 1 h playing back the videotapes. An average of the scratching numbers obtained with counting twice was regarded as a scratching number for each mouse.

RNA isolation and determination of cytokine mRNA levels

The tissue specimens for cytokine mRNA quantification were excised 4 h after the last application of DNFB. The frozen tissues were homogenized in TRIZOL[®] reagent (Molecular Research Center, Cincinnati, USA) using tissue tearor (Model 985-370, Biospec Products, Bartlesville, OK, USA). The homogenate was mixed with 0.2 ml of chloroform per 1 ml TRIZOL[®] reagent, and the mixture was vigorously shaken for 15 min at 4°C, and centrifuged at 12000 $\times$ g for 15 min. RNA in the upper aqueous phase was precipitated by mixing with 0.5 ml isopropanol per 1 ml TRIZOL[®] reagent, and the mixture was centrifuged at 12000 $\times$ g for 8 min after storing at room temperature for about 5 min. The RNA pellet was washed with 75% ethanol, centrifuged at 7500 $\times$ g for 5 min and dissolved in RNase-free water. The mRNA levels of IL-2 (forward primer: 5'-GCA GGA TGG AGA ATT ACA GGA-3', reverse primer: 5'-TCA GAG CCC TTT AGT TTT AC-3'), TNF- β (forward primer: 5'-AGG GAT GAG AAG TTC CCA A-3', reverse primer: 5'-GGT ATG AGA TAG CAA ATC GG-3'), IFN- γ (forward primer: 5'-TAC TGC CAC

GGC ACA GTC ATT GAA-3', reverse primer: 5'-GCA GCG ACT CCT TTT CCG CTT CCT-3'), IL-4 (forward primer: 5'-ACG GAG ATG GAT GTG CCA AAC GTC-3', reverse primer: 5'-CGA GTA ATC CAT TTG CAT GAT GC-3'), and β -actin (forward primer: 5'-GTG GGC CGC TAG GCA CCA-3', reverse primer: 5'-CGG TTG GCC TTA GGG TTC AGG GGG G-3') were determined by RT-PCR.

Statistical analysis

The results were expressed as mean $\pm$ SEM. Statistical comparisons between experimental groups were performed by unpaired Student's *t* test. *P* values less than 0.05 were considered to be significant.

Results

Composition of KT&G101

About 4 g KT&G101 could be obtained from 2 kg of pig lung tissues using the procedure described in “[Materials and methods](#)”. To determine the composition of KT&G101, it was analyzed by thin-layer chromatography using chloroform/methanol/water (65:25:4 by volume) mixture as a developing solvent. As shown in Fig. 1, KT&G101 was identified to chiefly consist of phosphatidylcholine (PC) and phosphatidylserine (PS). However,

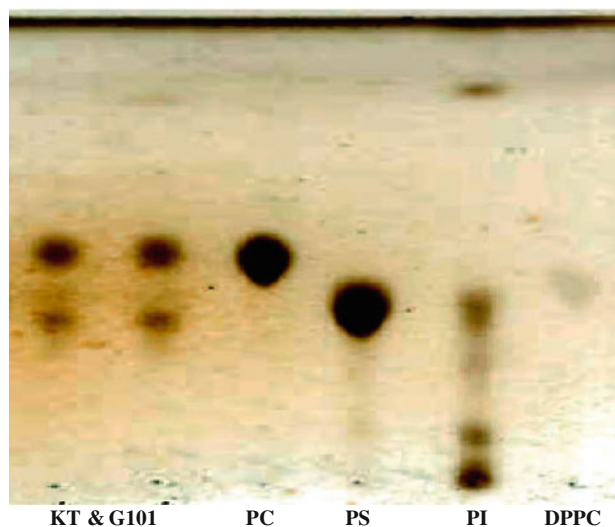


Fig. 1 Composition analysis of KT&G101, a phospholipid mixture purified from pig lung tissues. KT&G101 was analyzed by thin-layer chromatography using Merck Silica Gel 60 plate. The lipid spots on the plate were visualized by spraying 5% sulfuric acid in ethanol and charring at 190°C for 30 min. The first two lanes indicate KT&G101. The four phospholipids, such as PC, PS, PI and DPPC, were used as standard components

dipalmitoylphosphatidylcholine (DPPC), a major constituent of pulmonary surfactant, was not efficiently stained by spraying 5% sulfuric acid in ethanol and heating at 190°C (Fig. 1, last lane). The inefficient staining of DPPC was caused by its reluctance to associate with 5% sulfuric acid in ethanol and subsequently less charred at the high temperature (Fig. 1). Neither cholesterol nor triacylglycerol in KT&G101 was visualized on the TLC plate with spraying 5% sulfuric acid in ethanol. Any proteins and peptides were not detected when the TLC plate was stained with 0.2% ninhydrin spray. The DPPC content in KT&G101 was determined using an HPLC–ELSD (Evaporative Light Scattering Detector) system. Capcell Pak C18 (4.6 mm × 250 mm, Shiseido Co., Tokyo, Japan) column was employed to analyze phospholipids at 50°C and the detection was carried out at 65°C in 350 kPa. The HPLC analysis showed that KT&G101 contained 34.2% DPPC on a weight basis. Taken together, the major components of KT&G101 are PC and PS, and its predominating specific phospholipid is DPPC.

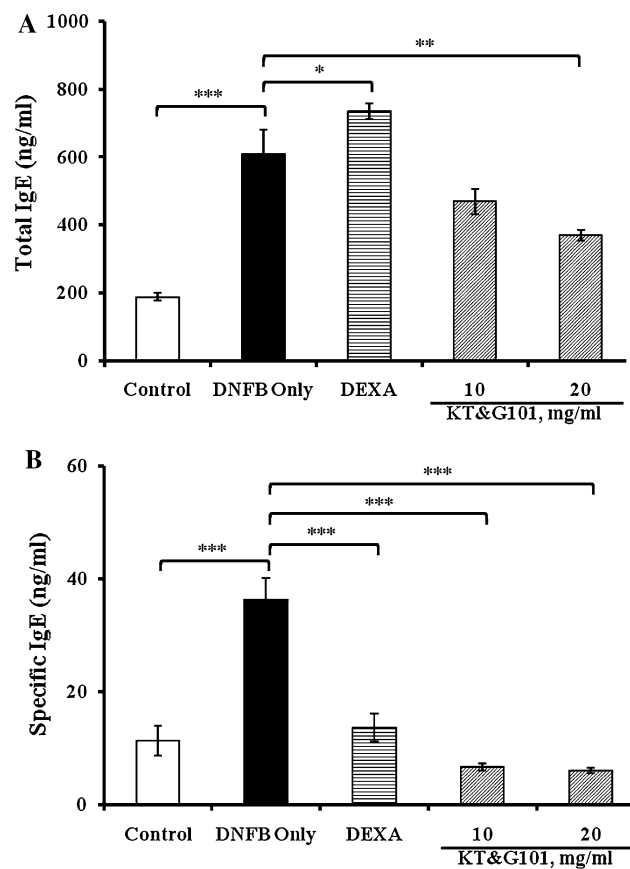


Fig. 2 Effect of KT&G101 on the total (a) and specific (b) serum IgE levels in BALB/c mice treated with DNFB. Total and DNP-specific IgE were determined in the mouse serum by ELISA. Each column shows the mean ± SEM of 7 mice in each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the corresponding group

Total and specific IgE levels in serum

DNFB, dissolved in acetone, was topically applied onto the shaved dorsal skins of sensitized mice in order to induce contact allergic dermatitis. Effects of KT&G101 on total and DNP-specific IgE levels were examined in the sera of mice with DNFB-induced contact allergic dermatitis, as shown in Fig. 2. The mice treated with DNFB showed 3.2-fold enhanced serum total IgE levels (Fig. 2a). Topical application of KT&G101 at the concentrations of 10 and 20 mg/ml decreased serum total IgE levels to approximately 70.0 and 60.6%, respectively, compared to that of DNFB only (Fig. 2a). However, its effect at 10 mg/ml appeared to be statistically non-significant. Dexamethasone (DEXA) was unable to diminish serum total IgE level of DNFB only group (Fig. 2a).

Application of DNFB only enhanced serum DNP-specific IgE level 3.2-fold (Fig. 2b), which is similar to an increase in serum total IgE level. Application of KT&G101 at the concentrations of 10 and 20 mg/ml significantly diminished serum DNP-specific IgE level elevated by DNFB only (Fig. 2b). The diminution by KT&G101 in the specific IgE level was much higher than that in the total IgE level. Dexamethasone also reduced the DNP-specific IgE level elevated by the treatment with DNFB (Fig. 2b). Taken together, topical application of KT&G101 reduces serum total and specific IgE levels elevated in the mouse with DNFB-induced contact allergic dermatitis.

Histopathological changes

In the control group treated with acetone only, the mice preserved normal morphology of skin tissues. Painting with DNFB markedly gave rise to skin lesions, characterized by wound healing processes such as pustule formation (Fig. 3a), epidermal hyperplasia (Fig. 3b), dermatitis (Fig. 3c) and fibroplasias (Fig. 3d). The notable effects of KT&G101 at the concentrations of 10 and 20 mg/ml were found in the recovering of epidermis and reduced dermatitis; the recovering of epidermis indicated 91.6 and 96.1% to completion, respectively (Fig. 3e), and dermatitis, mainly composed of neutrophils and macrophages, was markedly alleviated compared with that of DNFB and dexamethasone (Fig. 3c). Pustule formation was marginally removed in both KT&G101 treatment groups (Fig. 3a). No favorable effects of KT&G101 were found in epidermal hyperplasia compared with dexamethasone (Fig. 3b). The values of epidermal hyperplasia and fibroplasia in the dexamethasone treatment group appeared to be relatively low, but these results were most likely associated with the retarded skin recovery in the group, as shown in the low degree of the recovering of epidermis (Fig. 3e). The exemplified histopathological changes of mouse skin lesions of each experimental group were shown in Fig. 4.

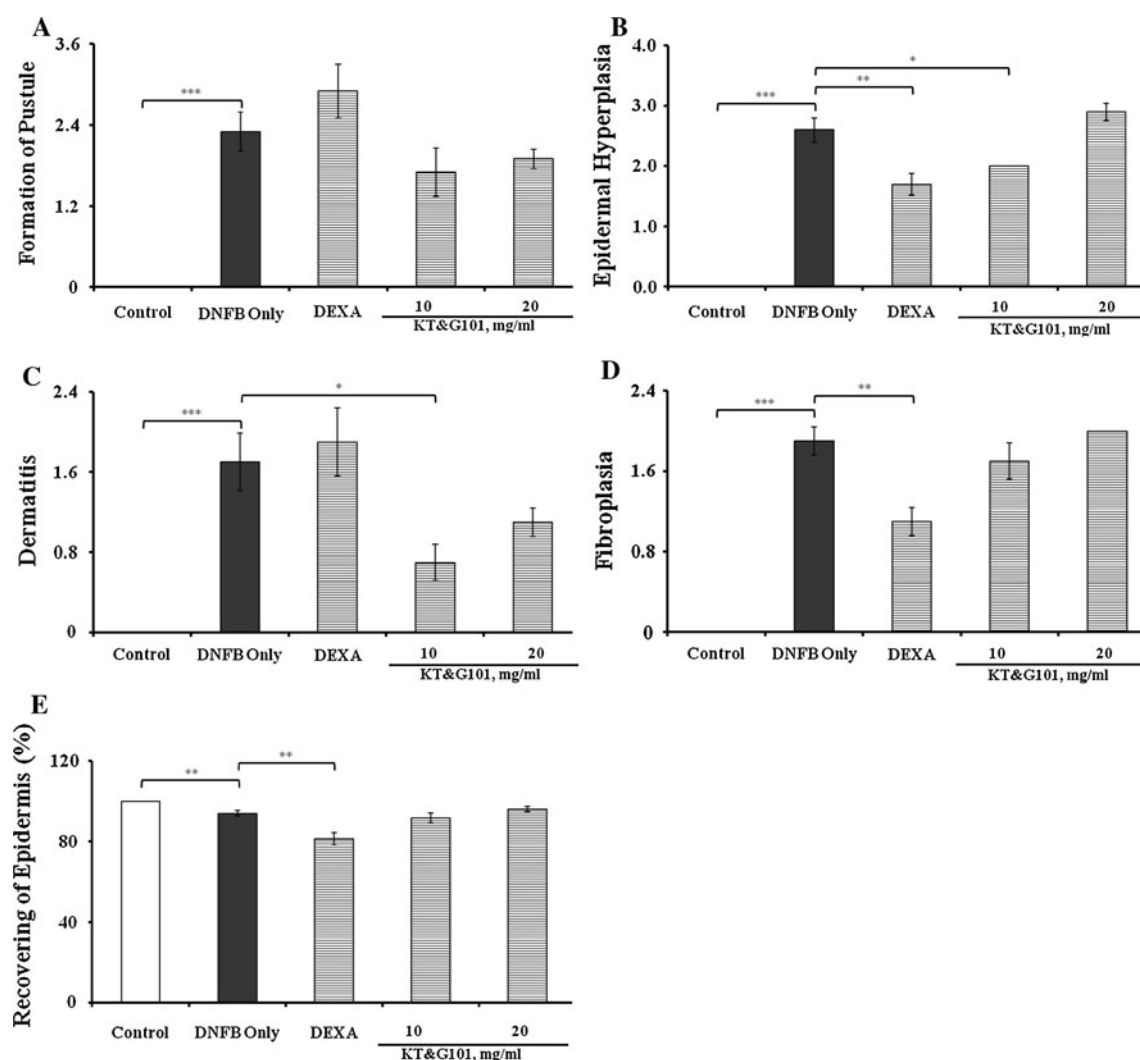


Fig. 3 Effect of KT&G101 on histopathological changes in mouse skin lesions caused by repeated application of DNFB. The skin samples were taken out 24 h after the tenth application of DNFB. Pustule (a), epidermal hyperplasia (b), dermatitis (c), fibroplasia

(d) and recovering of epidermis (e) were scored in the fixed skin tissues under microscope. Each column shows the mean $\pm$ SEM of 7 mice in each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the corresponding group

Scratching behavior

The frequencies of scratching behaviors of mouse corresponded with the severity of the skin lesions caused by the repeated application of DNFB (Fig. 5). KT&G101 at a dosage of 20 mg/ml was able to reduce the DNFB-induced scratching behavior, while that at a dose of 10 mg/ml also reduced the DNFB-induced scratching behavior but statistically non-significantly (Fig. 5). Its suppressive effect at 20 mg/ml on the scratching behaviors of mice was similar with that of dexamethasone (Fig. 5). In brief, KT&G101 possesses anti-scratch property in the animal models of contact allergic dermatitis.

Cytokine mRNA

In order to evaluate changes in the levels of some cytokine mRNAs in the skin tissues with the lesions by contact allergic dermatitis, three Th1 cytokines, such as IL-2, IFN- γ and TNF- β , and one Th2 cytokine, IL-4, were chosen to be examined. RT-PCR synthetic primers for them were described in “Materials and methods”. The four cytokine mRNA levels increased significantly in the skin lesions of contact allergic dermatitis triggered by repeated application of DNFB (Fig. 6, second columns). KT&G101 at both 10 and 20 mg/ml dosages, respectively, reduced the mRNA levels of IL-2 elevated in the skin lesions of DNFB-induced contact allergic dermatitis (Fig. 6a). Its

Fig. 4 Representative histopathology of mouse skin lesions in the experimental groups. **a** Control group; **b** DNFB group; **c** dexamethasone group; **d** KT&G101 (20 mg/kg) group. Note the recovered epidermis (E) with pustule (arrows) and epidermal hyperplasia. Compare the epidermis thickness (E) of Fig. 4b and d with that of the normal skin (Fig. 4a). For pustule formation and epidermal hyperplasia, Fig. 4b was graded as 2+ for both, and Fig. 4d was regarded, respectively, as 3+ and 2+. For fibroplasia, both Fig. 4b and d were graded as 2+. The recovery of skin injury was poor in the dexamethasone group (Fig. 4c). Lines in Fig. 4a and c indicate the dermis layer. H&E stain. Magnification = $\times 100$ for all figures

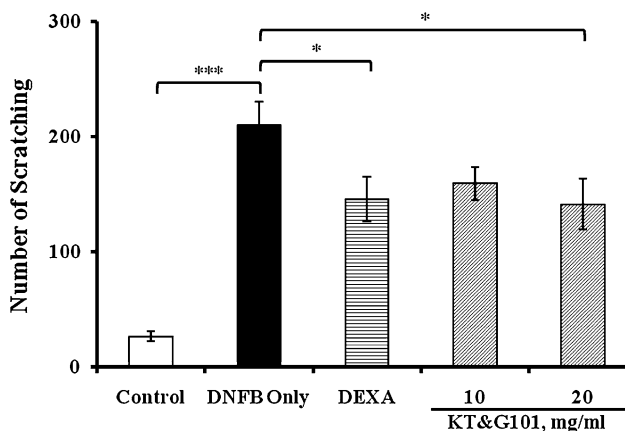
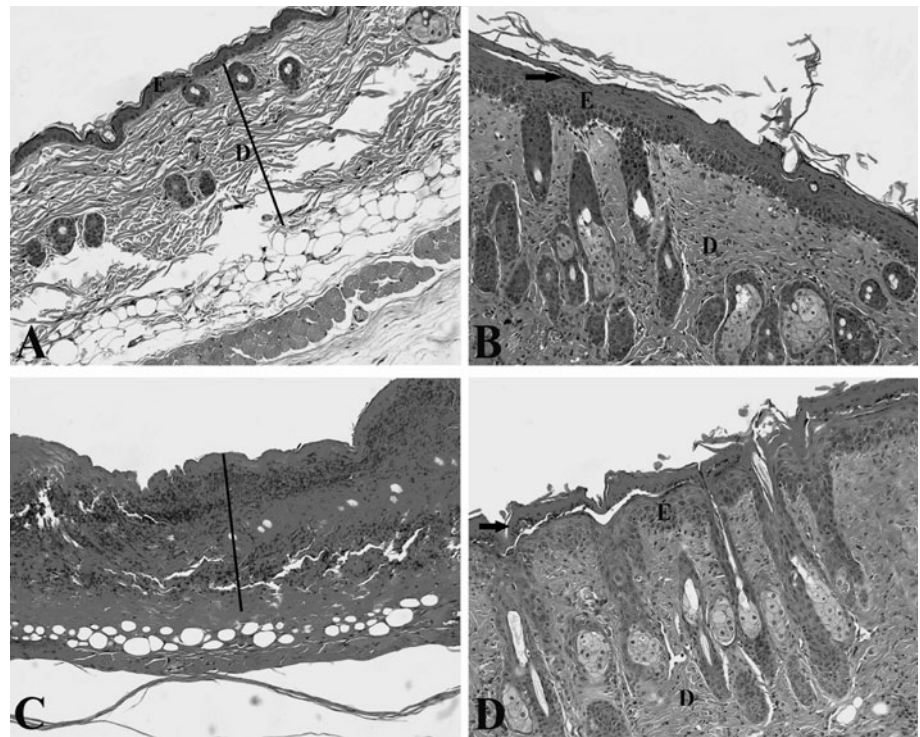


Fig. 5 Effect of KT&G101 on the DNFB-induced scratching behavior in BALB/c mice. Each mouse was separately videotaped 1 h after the tenth application of DNFB. Scratching with hind paws was twice counted for 1 h viewing individually videotaped mouse and the two counting numbers were averaged. Each column shows the mean $\pm$ SEM of 6 mice in each group. * $P < 0.05$; *** $P < 0.001$, compared with the corresponding group

suppressive effect was bigger than that of dexamethasone (Fig. 6a, third column). KT&G101 decreased the mRNA level of TNF- β elevated by DNFB in the damaged skin tissues (Fig. 6b). The suppressive effect of KT&G101 was bigger than that of dexamethasone, and the mRNA levels of TNF- β in the group treated with KT&G101 was similar to or lower than that in the control group (Fig. 6b). As shown in Fig. 6c, KT&G101 at the two dosages brought

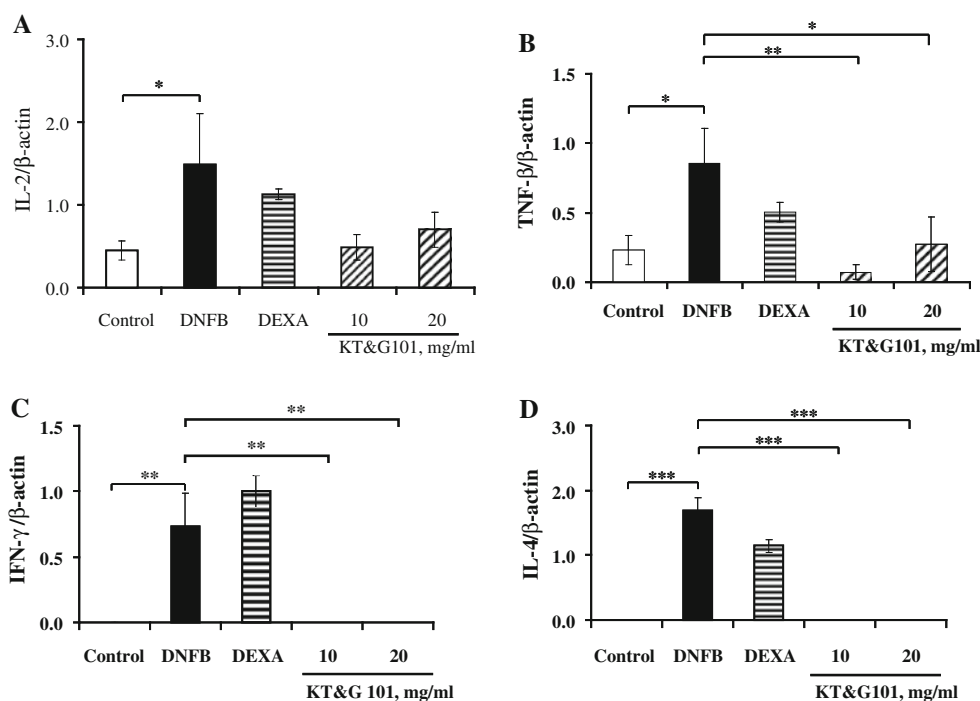
down the mRNA levels of IFN- γ almost to that in the control group. However, dexamethasone was unable to reduce the mRNA level of IFN- γ elevated by repeated application of DNFB (Fig. 6c). The similar suppressive effect of KT&G101 was observed in the mRNA level of IL-4 in the skin lesions artificially triggered by contact allergic dermatitis (Fig. 6d). Dexamethasone also reduced the IL-4 mRNA level elevated by DNFB (Fig. 6d). However, its reducing effect on the IL-4 mRNA level was lower than that of KT&G101. Taken together, KT&G101 enables to suppress the mRNA levels of the Th1 and Th2 cytokines, tested in this work, in the skin tissues with the lesions triggered by contact allergic dermatitis in BALB/c mice.

Discussion

Atopic dermatitis (AD), usually being the initial manifestation of atopic systemic diseases, is a chronic and relapsing skin disorder with allergic inflammation, and its occurrence involves a complex interplay between genetic and environmental factors. It is frequently associated with elevated IgE antibodies against many kinds of allergens. AD is sub-classified into extrinsic and intrinsic types according to the presence or absence of allergen-specific IgE antibodies. The extrinsic type of AD is particularly characterized by IgE-mediated sensitization mechanisms, while the intrinsic AD is associated with decreased IL-4

Fig. 6 Effect of KT&G101 on cytokine mRNA expression in the mouse skin repeatedly treated with DNFB.

Th1-specific (IL-2, **a**; TNF- β , **b**; IFN- γ , **c**) and Th2-specific (IL-4, **d**) cytokine mRNA levels were determined in the skin lesions using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Each cytokine mRNA level was represented as a relative ratio compared to β -actin mRNA used as an internal standard. Each column shows the mean $\pm$ SEM of 5 mice in each group. * P < 0.05; ** P < 0.01; *** P < 0.001, compared with the corresponding group



and IL-13 production but not with IgE-mediated mechanisms [19]. Although topical corticosteroids are frequently used to alleviate the symptoms of AD and to reduce skin inflammation, they aggravated or did not influence the scratching behavior in some animal allergic models [18, 20]. Terreic acid, an inhibitor of Tec family kinases crucial to immune cell activation, was able to suppress the skin lesions induced by repetitive epicutaneous applications of a house dust mite allergen and Staphylococcal enterotoxin B [21]. Oral administration of heat-killed *Lactobacillus brevis* inhibited IgE production and ear swelling, and suppressed the development of dermatitis in the NC/Nga AD mouse model [22]. Oral administration of *Astragalus membranaceus*, an herbal medicine used to treat allergic disease, suppressed AD-like skin lesions in NC/Nga mice treated with 2,4-dinitrofluorobenzene (DNFB) under specific pathogen free (SPF) conditions by reducing IFN- γ production [23]. Oral administration of dihomogamma-linolenic acid, an essential fatty acid, prevented development of AD-like skin lesions, including the reduction of the occurrences of the scratching behavior, the reduction of the plasma total IgE level and histopathological lesions, in the animal AD model, suggesting that dihomogamma-linolenic acid in phospholipids is a key component in the development and prevention of AD [24]. Recently, it has been reported that DNA vaccine against macrophage migration inhibitory factor, a proinflammatory cytokine implicated in the pathogenesis of AD, not only prevents the development of AD but also improves the symptoms of pre-existing AD [25]. Gastrin-releasing peptide receptor (GRPR) mediates

the itch sensation in the dorsal horn of the spinal cord, and thus may provide a new target for antipruritic drug development [26].

In the present work, we employed a mouse allergic contact dermatitis model [18] accompanied by frequent scratching behaviors using a contact sensitizer, DNFB, to evaluate the pharmacological efficacies of KT&G101 which is being developed as an effective remedy for the treatment of atopic dermatitis (AD). The repeated application of DNFB expectedly gave rise to an increase in the total and specific IgE levels in the serum, enhanced scratching behaviors and elevated the levels of IL-2, TNF- β , IFN- γ and IL-4, respectively, in the damaged skin tissues. It also brought about histopathological changes, such as pustule formation, epidermal hyperplasia, dermatitis and fibroplasia, in the skin tissues with the lesions. There appeared to be no significant differences in the effects between the lower dose (10 mg/ml) and the high dose group (20 mg/ml) in the histopathological examination, implying that the effects reached the peak at 10 mg/ml. This reasoning might be supported by the facts that suppressive effects of KT&G101 at 20 mg/ml are lower than those at 10 mg/ml, in cases of pustule formation (Fig. 6a), epidermal hyperplasia (Fig. 6b), dermatitis (Fig. 6c) and fibroplasias (Fig. 6d). Although KT&G101 at 20 mg/ml would produce unexpected side effects in some characters of the skin tissues, the precise understanding remains elusive. Further study is warranted to elucidate the dose-response relationship of KT&G101. KT&G101 remarkably reduced the total and specific serum IgE levels, which were

elevated by the repeated application of DNFB, and relieved the histopathological changes in the skin tissues with the lesions caused by DNFB. First of all, the ability of KT&G101 to reduce the serum IgE levels in DNFB-induced dermatitis animal models strongly proves its plausible candidate as a novel remedy to treat AD, since the elevated level of IgE could be one of the determinate factors in the episode and development of AD. The polyclonal mouse IgE molecules in sera from mice with AD-like allergic skin inflammation and human polyclonal IgE molecules present in sera from AD patients enhanced the survival and production of cytokine in mast cell cultures, implying that polyclonal IgE in AD might modulate the numbers and function of mast cell [27]. In addition, the level of total serum IgE has been proposed to be regarded as a useful parameter between extrinsic AD and intrinsic AD [28].

Itching, one of the most important symptoms of allergic skin diseases, such as AD, leads to a desire to scratch. Scratching behavior, the first sign of skin changes and the most important symptom in AD, provokes the destruction of skin barriers, resulting in worsening of the skin lesions and more itching [29]. Hence, the control of itching and scratching becomes a crucial strategy for the treatment of AD. KT&G101 was able to reduce the scratching behavior in the skin lesions caused by the repeated application of DNFB. It might attenuate the aggravation of AD as a result of frequent scratching behavior. In a recent report, fermented barley extract, prepared from barley shochu (a Japanese spirit produced from rice, barley, sweet potato, and so on) residue, was shown to inhibit the development of AD-like skin lesions and also suppress the scratching behavior in NC/Nga mice probably by inhibiting the cytokine production involved in chronic inflammation [30].

KT&G101 significantly diminished the levels of Th1 cytokines, such as IL-2, TNF- β and IFN- γ , in the AD-like skin lesions of mice. It also diminished the mRNA level of IL-4, a Th2 cytokine, in the skin lesions. According to the previous report [31], the production of total IgE and the expression of IFN- γ in the DNFB-induced skin lesion were significantly suppressed by IL-4 gene deletion in BALB/c mice, suggesting that IL-4 plays an important role in the onset of DNFB-induced hypersensitivity in BALB/c mice. However, *Uncariae rhynchophylla*, an herb which has blood pressure lowering and anti-inflammatory effects, suppresses the development of AD-like dermatitis in DNFB-treated NC/Nga mice by reducing the production of IFN- γ [32]. The number of cells expressing Th2 cytokines, such as IL-4 and IL-5, significantly increased in acute lesions of AD, whereas delayed-type allergic reaction and Th1 immune response are involved in pathogenesis of AD [33]. The expression of IFN- γ , a Th1 cytokine,

predominates in chronic skin lesions of some patients with AD [34]. It is expected that KT&G101 play a curing role in both intrinsic AD and extrinsic AD.

DPPC, a principal PC enriched in KT&G101, may play a key role in KT&G101's improving effects against DNFB-induced AD-like lesions in BALB/c mice. Lungs of animals including humans contain a peculiar phospholipid, DPPC. The proportion of DPPC accounts for 40% of the total lipids in the lung tissue, which is significantly higher than those in any other tissues. DPPC is well known to have a key role in lowering the surface tension by forming monolayer at the air-liquid interface on the surface of alveoli [35]. The application of DPPC on the atopic skin lesions may have several beneficial effects. DPPC, due to its saturated fatty acid tails, is more stable than the lipids having unsaturated fatty acid tails against lipid peroxidation which is triggered by exposing unsaturated fatty acids on the surface of skin to oxygen molecules. Phospholipids containing DPPC may form lipid films on the surface of the affected skin and obstruct the interaction of DNFB with the target cells. It is deemed that KT&G101's masking the interactions between the hapten and the target cells would be one of the mechanisms for KT&G101 to alleviate the symptoms of contact dermatitis including AD. DPPC may form a protective layer seen on the surface of the alveoli because of its peculiar molecular properties. It has amphiphilic properties so that it can mix with water and keep more water in the skin than neutral lipids. Accordingly, the saturated phospholipids isolated from lung may keep moisture in the epidermis and play a protective role on the surface of the skin. It is also expected to fill the intercellular spaces in the skin and mask non-specific immune reactions especially on the skin affected by atopic diseases. On the basis of the current findings, it is considered that some phospholipids applied to dorsal backs of mice may form protective layers on the surface of skin and mask the contact of DNFB to the cells which initiate immune responses and block a series of the cascade of immune reactions. It is also thought that a portion of phospholipids were utilized by both subepidermal and epidermal cells as building blocks, which subsequently facilitates the recovery of the skin affected by dermatitis. The results, obtained in this work, imply that KT&G101 improving the signs and symptoms of dermatitis may have a different mechanism to alleviate the symptoms of dermatitis compared to dexamethasone. DPPC, as a phospholipid non-toxic to skin, may be utilized as a source of palmitoyl-CoA by the epithelial cells which is a starting building block for the synthetic pathway of ceramide, a crucial lipid in the skin barrier.

In conclusion, KT&G101 improves unfavorable symptoms and parameters, such as serum IgE level, scratching behavior, and enhanced inflammatory cytokine expression

in the mouse model with allergic contact dermatitis. It also lessened undesirable histopathological changes brought about by the skin lesions in the model animals. These results assure that KT&G101 is considered to be a compelling and novel candidate remedy for the treatment of AD.

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