Effects of Gintonin-Enriched Fraction in an Atopic Dermatitis Animal Model: Involvement of Autotaxin Regulation

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Received February 13, 2017; accepted April 4, 2017

Regular Article

Ginseng extract has been used for prevention of atopic dermatitis (AD) in experimental animal models. However, little is known about its active ingredients and the molecular mechanisms underlying its anti-AD effects. Recently, we isolated a unique lysophosphatidic acid (LPA) receptor ligand, gintonin, from ginseng. Gintonin, the glycolipoprotein fraction of ginseng, contains LPAs, mainly LPA C18:2 with other minor lysosphopholipid components. A line of evidence showed that serum autotaxin (ATX) activity and level are significantly elevated in human AD patients compared to those in normal controls, which indicates that ATX may be involved in human AD. In a previous study, we demonstrated that gintonin exerted anti-inflammatory effects via inhibition of microglial activation and proinflammatory cytokine production by immune cells and that it strongly inhibited ATX activity. In this study, we investigated whether oral administration of the gintonin-enriched fraction (GEF) could ameliorate the symptoms of 2,4-dinitrofluorobenzene (DNFB)-induced AD in NC/Nga mice. We found that oral administration of GEF to DNFB-induced AD mice for 2 weeks reduced ear swelling and AD skin index. In addition, oral administration of GEF reduced the serum levels of immunoglobulin E, histamine, interleukin-4, and interferon-γ. Histological examination showed that oral administration of GEF attenuated skin inflammation and significantly reduced eosinophil and mast cell infiltration into the skin. Moreover, oral administration of GEF not only decreased serum ATX level but also reduced serum ATX activity. The present study shows that the anti-AD effects of ginseng might be attributed to GEF-induced anti-inflammatory activity and ATX regulation.

Key words Panax ginseng; gintonin; autotaxin; anti-atopic dermatitis

Ginseng, a traditional herbal medicine, has shown various beneficial immunomodulatory effects in humans.1 In addition, ginseng showed anti-allergic, anti-scratching, and anti-atopic dermatitis (AD) effects in an animal model.2–4) Ginseng extract or ginseng saponin fraction, isolated via diverse extraction methods, exhibited beneficial effects in many skin diseases, including anti-AD effects.5,6) However, relatively little is known about the active ingredients and molecular mechanisms underlying ginseng effects. Recently, we isolated a novel glycolipoprotein fraction from Panax ginseng, called gintonin, which does not contain ginseng polysaccharides and saponins. Gintonin mainly consists of lysophosphatidic acids (LPAs) and ginseng proteins, such as GLP151 and GMP.6) Gintonin contains approximately 9.5% LPAs, of which the major component is an unsaturated LPA C18:2.6) Gintonin activates six LPA receptor subtypes leading to induction of [Ca2+]i transient in the cells that express endogenous and heterologous LPA receptors by different magnitudes.8) In animals, LPAs are mainly produced from lysophosphatidylcholine by plasma autotaxin (ATX) (also called lysophospholipase D, lysoPLD).9) LPAs produced by plasma ATX bind to albumin or gelsolin and are delivered to the target cells or organs, where they exert their biological effects.8

In addition to gintonin-mediated LPA receptor regulations, we and other groups showed that LPAs, such as LPA C18:2, and gintonin exert a strong negative feedback inhibition on ATX activity.9,10) Thus, gintonin can be considered as a ginseng-derived ATX negative regulator as well as a LPA receptor ligand. Interestingly, a recent line of evidence showed that plasma ATX might be involved in skin diseases. ATX expression is up-regulated in allergic asthma.11,12) ATX could largely contribute to AD in humans. Kremer et al. reported that serum ATX activity was increased in patients with cholestatic pruritus.13,14) In addition, serum ATX activity or levels were found to be significantly increased in AD patients compared to those in normal controls.15–18) These findings suggest that ATX could be a target for human AD therapy in the future. However, it has not yet been determined whether gintonin, a herbal ATX regulator, exhibits in vivo anti-AD effects.

In this study, we induced AD in animals using the widely used 2,4-dinitrofluorobenzene (DNFB), which induces AD-like skin symptoms similar to those observed in human AD patients.19,20) In previous study, we developed a simple method for gintonin-enriched fraction (GEF) preparation using ethanol and H2O. The yield of GEF was approximately 6-fold higher than that obtained in the previous gintonin preparation and we found that GEF contains more than 90% gintonin in enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody against gintonin.21) Using GEF we investigated whether oral administration of the GEF could ameliorate the symptoms
of 2,4-dinitrofluorobenzene (DNFB)-induced AD in NC/Nga mice. In addition, we discuss the molecular mechanisms involved in GEF-mediated anti-AD effects.

MATERIALS AND METHODS

Materials

Given the scarcity of gintonin in animal experiments, we used the GEF. The GEF was prepared as follows. One kilogram of 4-year-old ginseng was ground into small pieces (>3 mm) and refluxed with 70% fermented ethanol eight times for 8 h each at 80°C. The extracts (340 g) were concentrated, dissolved in distilled, cold water at a ratio of 1:10, and stored at 4°C for 24–96 h. The supernatant and precipitate of water fractionation after ethanol extraction of ginseng were separated by centrifugation (3000 rpm, 20 min). The precipitate after centrifugation was lyophilized. This fraction was referred to as the GEF since this fraction contains most of the gintonin.21) DNFB and olive oil were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). ELISA kits for immunoglobulin E (IgE), histamine, interleukin-4 (IL-4), and interferon-γ (IFN-γ) were purchased from Komabiotech (Seoul, Korea), Abnova Chemicals (Taipei, Taiwan), and R & D Systems (Minneapolis, U.S.A.), respectively. ATX sandwich ELISA kit and FS-3 were purchased from Echelon Biosciences Inc. (Salt Lake City, UT, U.S.A.). Zoletil 50 was purchased from Virbac (Carros, France). Rompun was purchased from Bayer Korea (Seoul, Korea). All other reagents used were purchased from Sigma-Aldrich Co.

Animals

Six-week-old NC/Nga male mice (19–22 g) were purchased from Japan SLC (Shizuoka, Japan) and maintained under specific pathogen-free conditions in a temperature-controlled room (23±1°C) with a 12 h light/dark cycle and ad libitum access to food and water. Animal experiments were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Konkuk University (No. KU08095). All procedures were conducted in accordance with the United States National Institute of Health guidelines.

Induction of Atopic Dermatitis and GEF Treatment in Experimental Animals

Before the induction of AD, the skin of the dorsal area of the NC/Nga mice was shaved 2 d before DNFB treatment. AD induction was initiated by sensitizing the anesthetized mice with 100 µL of 0.15% DNFB in acetone–olive oil (3:1) applied on the shaved dorsal skin at the indicated times for 3 weeks19) (Fig. 1). Mice treated with acetone–olive oil (3:1) served as a control group for determination of histological and immunological baselines. After induction of atopic dermatitis, the mice were randomly divided into four groups. The first group included the normal mice treated with acetone–olive oil (3:1). The second group was the disease group containing mice that received oral saline (vehicle) after AD induction. The third and fourth groups were treated orally with GEF at 50 and 100 mg/kg, respectively, after AD induction. The vehicle or GEF was administered once daily for 2 weeks.

Evaluation of Dermatitis Lesion and Ear Thickness

The severity of dermatitis was assessed using the method described by Yamamoto et al.22) The severity of dermatitis was evaluated once, just before euthanization of the experimental animals. The development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion was evaluated using the following scoring system: 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The sum of the individual scores was used as a dermatitis score. The dermatitis scoring was performed in blind test and the ear thickness of each mouse was measured using a micrometer on the start day (21st day) of administration of vehicle or GEF and on the sacrifice day of the experimental animals. To minimize variation, the measurements were performed by single investigator.

Histopathological Evaluation of Skin

The skin of the back from each group was removed, fixed in 4% paraformaldehyde solution, dehydrated and cleared in serial dilutions of
alcohol and xylene, and embedded in paraffin.\textsuperscript{23} To investigate the histopathological changes in the skin, sections of 5-µm thickness were stained with hematoxylin–eosin (H&E) and toluidine blue dyes and observed under a light microscope (BX50; Olympus, Tokyo, Japan). To quantify the change in skin thickness, five images, including the epidermis and dermis, of one section per skin strip in each group were captured under a 10× or 20× objective lens using a DP70 image analysis system (Olympus). The epidermal and dermal thickness was measured using the line measure option in NIH Image J program (http://rsbweb.nih.gov/ij/) and expressed as the mean thickness±standard error of the mean (S.E.M.). To quantify the infiltration of mast cells and inflammatory cells, including eosinophils and lymphocytes, 5 images of the dermis per section for each animal in each group were captured under a 20× objective lens. The number of inflammatory and mast cells was counted manually and expressed as the mean number of cells±S.E.M. per mm\textsuperscript{2}. Mast cells and inflammatory cells were confirmed via detection of metachromasia in the cytoplasm and hematoxylin-stained small dark nuclei, respectively.

**Measurement of IgE and Histamine Levels in Serum**

The concentrations of total IgE and histamine were measured by ELISA. Serum samples were immediately prepared after the mice were sacrificed. IgE concentrations were measured using a mouse IgE ELISA kit (Koma Co., Seoul, Korea) according to the manufacturer’s protocol. Histamine concentrations were measured using a histamine ELISA kit (Abnova Chemicals).

**Measurement of IL-4 and IFN-γ Levels in Serum**

The concentrations of IL-4 and IFN-γ were measured using a mouse IL-4 Quantikine ELISA Kit and a mouse IFN-γ Quantikine ELISA Kit (R & D Systems), respectively, according to the manufacturer’s protocols.

**Assay of ATX Activity and Levels in Serum**

ATX activity was evaluated in the serum using an ATX substrate, FS-3, according to the manufacturer’s instructions. ATX-mediated hydrolysis of the fluorogenic ATX substrate, FS-3, produces the fluorescent fluorescein due to a fluorescence resonance energy transfer.\textsuperscript{10,24} Briefly, the sera were diluted in Tris-buffered saline solution (TBS: 140 mM NaCl, 5 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 50 mM Tris–HCl, pH 8) and incubated with FS-3 in a 96-well plate. FS-3 in TBS containing 0.1% charcoal-treated bovine serum albumin (BSA) was then added to each well of the 96-well plate (final concentration of FS-3=2.5 µM). Then, the resulting fluorescence was measured over time (excitation wavelength: 485 nm, emission wavelength: 528 nm). The percent of ATX activity was calculated from the slopes of the fluorescence versus time graphs of each sample and blank. Serum ATX concentrations were measured using an autotaxin sandwich ELISA kit (Echelon Biosciences Inc.) according to the manufacturer’s protocol.

**Ethics Statement**

Animal experiments were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Konkuk University (No. KU08095).

**Statistical Analysis**

The data are expressed as means±S.E.M. One-way ANOVA followed by Tukey multiple comparison test or student’s t-test when appropriate. Significance was set at \( p<0.05 \).

**RESULTS**

**Effects of GEF on AD-Related Inflammatory Responses in AD Animal Model**

According to the experimental schedule in Fig. 1, we first induced AD in mice using DNFB. Next, we examined the therapeutic effects of GEF by comparing ear swelling or thickness and skin dermatitis index between the control mice and mice treated with DNFB alone, and DNFB+GEF, using previously described methods.\textsuperscript{25} As shown in Fig. 2, DNFB treatment induced an increase in the ear thickness and dermatitis index compared to that in the control group; however, oral GEF administration for 14 d after induction of AD significantly reduced the ear thickness and dermatitis index. Interestingly, these effects were independent of the administered GEF dosage. We examined the effects of GEF on various inflammatory indexes in DNFB-induced AD animals because the levels IgE, histamine, IL-4, and IFN-γ were found to be elevated in AD.\textsuperscript{25} As shown in Fig. 3, DNFB treatment induced an increase in serum IgE, histamine, IL-4, and IFN-γ compared to those in control mice; however, oral GEF administration for 14 d attenuated DNFB-induced increase in IgE, histamine, IL-4, and IFN-γ. These results indicate that oral GEF administration to DNFB-induced AD mice reduced the skin changes and dermatitis index as well as the inflammatory responses.

**Effects of GEF on Skin Thickness and Inflammatory and Mast Cell Infiltration in AD Mouse Model**

Because AD is characterized by hyperkeratosis, large ulcers, and infiltration of inflammatory cells, such as eosinophils and mast cells,\textsuperscript{26,27} we examined the effects of GEF on the histopathological changes of the skin after DNFB treatment. In the DNFB-treated group, the epidermal thickness was significantly increased (175±13.2 µm) compared to that in the normal control group (53±6.1 µm), whereas it was significantly decreased in DNFB+GEF group at a dose of 100 mg/kg (102±5.3 µm), which corresponds to changes in dermal thickness for each group (280±11.2 µm in normal control, 610±31.5 µm in DNFB, and 407±21.2 µm in GEF100 group) (Figs. 4A–D, left insets, 1, and 3).

Inflammatory cells, including eosinophils and lymphocytes, can be activated by inflammatory mediators, including IL-5, granulocyte macrophage colony-stimulating factor (GM-CSF), and CC chemokines. The activated inflammatory cells can produce cytokines and chemokines that contribute to the major pathological features of AD.\textsuperscript{28} Therefore, we determined whether the anti-AD effects of GEF were associated with a decrease in the infiltration of inflammatory cells in the dermis. The number of inflammatory cells was significantly increased in DNFB-treated group (63±3.4/mm\textsuperscript{2}) compared to that in the normal control group (28±2.3/mm\textsuperscript{2}), whereas this increase was attenuated in the DNFB+GEF group at a dose of 100 mg/kg (39±2.5/mm\textsuperscript{2}) (Figs. 4A–D, right insets, and K).

In addition, mast cells can be activated by certain inflammatory mediators, such as neuropeptides and cytokines. The activated mast cells release a number of important signaling molecules. Particularly, a representative molecule, histamine, has potent proinflammatory activities.\textsuperscript{28} Therefore, we investigated the effect of GEF on mast cell infiltration. As shown in Figs. 4E–H and L, greater mast cell infiltration was observed in the dermis (29±2.1/mm\textsuperscript{2}) of the DNFB-treated group than in the normal control group (9±1.3/mm\textsuperscript{2}); however, this in-
that gintonin strongly inhibited ATX activity at low concentrations, thus, we examined whether oral administration of gintonin inhibited microglial activation and serum ATX activity and levels were significantly elevated in DNFB-induced AD mice. However, the molecular mechanism(s) underlying the anti-AD effects of ginseng extract or ginseng saponin fraction were not fully elucidated. In addition, most of the previous studies mainly focused on the prevention rather than treatment of AD.

In AD mouse model using DNFB, it is currently unknown whether DNFB-mediated skin inflammations are directly linked to the increase of serum ATX activity and level. It is likely that DNFB-mediated increase of the cytokine secretions might be indirectly coupled to an increase of ATX activity and level, since previous reports showed that increased secretions of cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α) are associated with increases of autotaxin levels. Based on these reports, we first examined whether DNFB-induced inflammation on skin can induce up-regulation of the serum ATX activity and levels in AD animal model, similar to that observed in AD patients. Then, we investigated the therapeutic effects of GEF in DNFB-induced AD mice. We found that oral administration of GEF for 14 d attenuated ear swelling, reduced epidermal and dermal thickness, and decreased eosinophil and mast cell infiltrations in histopathological examination. In addition, oral administration of GEF inhibited the increase in the expression of inflammatory mediators, such as IgE, histamine, IL-4, and IFN-γ in the serum. Moreover, GEF reduced serum ATX activity and levels. These results show that anti-AD effects of GEF in AD mouse model might involve regulation of both the immune system (inflammatory response) and serum ATX activity and levels.

The possible explanations underlying the anti-AD effects of GEF could be based on the dual action of GEF on the immune system. The first possibility involves the anti-inflammatory actions of GEF. In previous studies, we demonstrated that oral administration of gintonin inhibited microglial activation and attenuated the expression of ionized calcium-binding adaptor molecule-1 (Iba-1) in the brain cortex and hippocampus in Alzheimer’s disease animal model. In addition, treatment of RAW264.7 cells with gintonin strongly inhibited lipopolysaccharide-induced production of proinflammatory cytokines, such as IL-6, IL-1β, and TNF-α via mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) and levels might contribute to its anti-AD effects in mice.

DISCUSSION

AD is a chronic and incurable skin inflammation. It is characterized by itchy, red, swollen, and thickened skin. In addition, exudation of a clear fluid from the affected areas may occur. The causes of AD are not completely known; however, they may involve genetic factors, abnormal immune system functions, and various environmental exposures. Interestingly, AD may start during childhood and become severe over time if not treated appropriately. Current treatments of AD mainly aim to alleviate the symptoms by increasing the skin moisture, applying a steroid cream, or simply avoiding allergenic food or clothes. Because modern therapies, except steroids, have a limited capacity to treat AD, many investigators have focused on alternative or complementary medicines for AD treatment. The ability of the extract of ginseng, one of these medicines, to attenuate the symptoms of AD in experimental animal models was investigated. In addition, ginseng saponin fraction was found to attenuate experimentally induced AD in animals. However, the molecular mechanism(s) underlying the anti-AD effects of ginseng extract or ginseng saponin fraction were not fully elucidated. In addition, most of the previous studies mainly focused on the prevention rather than treatment of AD.

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pathways due to activation of LPA receptors. In addition, gintonin restored the levels of miR-34a and miR-93.\(^{35}\) On the other hand, AD has been considered as an allergic T-helper 2 (Th2)-mediated disease that is characterized by abnormal IgE production, peripheral eosinophilia, mast cell activation, and induction of Th2 lymphocytes expressing IL-4, IL-5, and IL-13. Especially, IL-4 and IgE in AD causes mast cells to migrate into inflamed tissues.\(^{36–38}\) Infiltrated mast cells that are activated by IgE release histamine, leukotriene, heparin and chemokines. In the present study, we could observe that GEF inhibited the increase in IL-4 and IgE expression in serum from AD. Thus, GEF-mediated inhibition of mast cell infiltration could be partially achieved by the attenuation of IL-4 and IgE production (Fig. 3).

We and other groups demonstrated that LPAs isolated from various seeds and gintonin strongly inhibited ATX activity in MDA-MB-435 cells.\(^{9,10}\) Thus, the other possible mechanism that can mediate the anti-AD effects of GEF in mice involves the regulation of plasma ATX levels and activity, which is closely related to its anti-inflammatory effects. The fact that LPA, produced by ATX, induces plasma exudation and accelerates development of human mast cells supports this hypothesis.\(^{39}\) ATX was shown to play a role in eosinophil trafficking into inflamed skin tissues\(^{40}\) and lymphocyte homing in allergic airway inflammation and asthma.\(^{49}\) Thus, GEF-mediated inhibitory effects on the serum ATX activity and levels could be further linked to suppressive effects on AD-induced skin inflammation. Finally, the GEF-mediated dual actions on the immune system and serum ATX activity and levels might contribute to its anti-AD effects in animals. However, further studies are required to elucidate the exact molecular mechanisms responsible for the anti-inflammatory effects of GEF and the regulation of serum ATX levels and activity.

On the other hand, Shimizu \textit{et al.} reported that intradermal injection of free LPA exhibited scratching-like behaviors in mice.\(^{16}\) There is a discrepancy between the previous report and our present findings. However, the previous report is different from the present report in two points of view. First, we administered GEF to AD mouse model through oral route to examine anti-AD effects, whereas Shimizu \textit{et al.} administered free LPA to mice via intradermal route.\(^{16}\) Second, previous report used free LPA, whereas we used a complex of ginseng protein, carbohydrate and lipids including LPAs. Thus, different administration route of GEF and different compositions of GEF compared to previous free LPA might exhibit different effects from that of free LPA. GEF, which consist of ginseng proteins, carbohydrates and several lipids including LPAs,\(^{13}\) was administered via oral route and orally administered GEF might be digested and modified before absorption through gastrointestinal systems. Thus, one possibility is that ginseng proteins or other lipid components in GEF might be also involved in GEF-mediated anti-AD effects, although further

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**Fig. 3. Effects of GEF on IgE, Histamine, IL-4 and IFN-γ Levels**

The serum concentrations of (A) IgE, (B) histamine, (C) IL-4 and (D) IFN-γ collected was determined by ELISA kits according to the manufacturer’s protocols. Data represent the mean±S.E.M. (n=5–6). \(^{*}\)p<0.05, significantly different from the control group; \(^{*}\)p<0.05, significantly different from the DNFB-treated group.
studies will be required to elucidate exact molecular mechanisms on which components of GEF and how orally administered GEF exhibits anti-AD effect.

In conclusion, this study showed that oral administration of GEF attenuated the inflammatory responses and decreased serum ATX activity and levels in DNFB-induced AD mice. This dual action of GEF might explain the molecular mechanisms underlying the anti-AD effects of ginseng. Finally, GEF might be a useful candidate for prevention and treatment of AD.

Acknowledgments This work was supported by the Brain Research Program through the NRF of Korea funded by the Ministry of Science, ICT and Future Planning (NRF-2016M3C7A1913845), the Bio & Medical Technology Development Program of the NRF funded by the Korean gov-
Fig. 5. Effects of GEF on Autotaxin Activity and Levels in Serum
(A) ATX activity determination was performed using the FS-3 as described in Materials and Methods. (B) Autotaxin levels were measured using an autotaxin sandwich ELISA kit. Data represent the mean ± S.E.M. (*p<0.05, significantly different from the control group).