Improvement of Atopic Dermatitis-Like Skin Lesions by *Platycodon grandiflorum* Fermented by *Lactobacillus plantarum* in NC/Nga Mice

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Atopic dermatitis (AD) is characterized as a multi-factorial inflammatory skin disease that has been increasing worldwide. Previously, we demonstrated that FPG, which is *Platycodon grandiflorum* (PG) fermented by *Lactobacillus plantarum* (LP), increases the level of interferon (IFN)-gamma in mouse splenocytes in vitro. In this study, we investigated the effects of FPG in an animal model of AD, with a particular emphasis on its effects on Th helper (Th)1 and Th2 immune responses. To assess the potential use of FPG for the inhibition of AD, we established a model of AD-like skin lesions in NC/Nga mice. Immunoglobulin isotypes (Igs) and Th1/Th2 cytokines in the sera and spleens of AD-like mice were examined. In addition, histological examination was also performed. AD symptoms in skin lesions improved following oral administration of FPG. IgE secretion was significantly down-regulated, and this was accompanied by decreased levels of interleukin (IL)-4 and IgG1 and increased serum levels of IL-12p40 and IgG2a in FPG-treated animals. In splenocytes, the production of the Th1 cytokines IL-12p40 and IFN-gamma was up-regulated, while the levels of the Th2 cytokines IL-4 and 5 were down-regulated by FPG treatment. These results suggest that FPG inhibits the development of AD-like skin lesions in NC/Nga mice by suppressing the Th2 cell response and increasing the Th1 cell responses. Our results indicate that FPG is safe and effective for the prevention of AD-like skin lesions.

Key words: atopic dermatitis; *Lactobacillus plantarum*; NC/Nga mouse; T helper 1; T helper 2; *Platycodon grandiflorum*

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritic and eczematous skin lesions that is a multifactorial disease with genetic and environmental components, such as infectious agents, food allergens, and aeroallergens.1–3 The incidence of AD has increased dramatically in recent years, especially in industrialized countries, and it now affects up to 20% of children and 1–3% of adults worldwide.4 AD in infancy is the most frequent chronic skin disorder in childhood, and can affect an individual throughout their lifetime through its persistence or the onset of serious problems in adulthood.5 While the pathogenesis of AD has not yet been fully defined, it has been reported that clinical signs and symptoms are associated with underlying immunological changes. Allergic diseases such as rhinitis, asthma and atopic eczema are dominated by allergen-specific Th helper 2 (Th2) cell immune responses, leading to immunoglobulin E (IgE) production and the accumulation of eosinophils.5,6 A breakdown in the balance between Th1 and Th2 cytokines has been reported to be central to the pathology of AD.7–8 Specifically, it is generally believed that the immune response in AD is skewed toward a Th2 response, thus resembling a Th1 deficiency. Studies of patients with AD have shown that Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-13 are over-produced, while the production of the Th1 cytokine interferon (IFN)-gamma is reduced when compared to non-AD control subjects.9,10

Until recently, steroid therapy was widely applied for the treatment of AD; however, because such treatment causes severe side effects, including atrophy, striae, rosacea, perioral dermatitis, acne, and purpura.11–13 It cannot be used for long periods of time. More importantly, steroid treatment has general immunosuppressive effects that occur via a reduction in the cytokine production associated with both Th2 and Th1.14 To circumvent the toxicity of steroid-based drugs, several groups have conducted clinical trials of complementary or alternative medicines for the treatment of dermatological disorders, as this general class of therapeutics is used in over 80% of the world’s population.15 However, despite numerous trials evaluating the effects of complementary or alternative medicines on allergic diseases, including AD, the true efficacy and safety of complementary or alternative medicines, as well as their therapeutic mechanisms remain unclear.16,17

The root of *Platycodon grandiflorum* (PG) has been used as a traditional oriental medicine for the treatment of pulmonary and respiratory disorders, such as bronchitis, tonsillitis, and asthma. In the concept of oriental medicine, lung is believed to be involved in the development of AD-like skin lesions in NC/Nga mice by suppressing the Th2 cell response and increasing the Th1 cell responses.18 As a follow-up study, we evaluated that the effects of *Lactobacillus plantarum* (LP) fermented *Platycodon grandiflorum* (FPG) and we
also compared the efficacy among PG, LP and LP FPG. Using microbial fermentation, the fine structure of low-molecular biologically active substances can be broken down into the active structure of the bio-absorption of beneficial enzymes.\(^{19}\)

In the present study, we evaluated the effects of PG, which is widely distributed in northeast Asia and has long been used as a traditional Oriental medicine for the treatment of pulmonary and respiratory disorders, such as bronchitis, tonsillitis, and asthma.\(^{20,22}\) Pharmacological studies of the root of PG have revealed that it possesses immunomodulatory\(^{22–24}\) and anti-inflammatory\(^{25,26}\) properties. The lactic acid bacteria (LAB), LP, which are non-invasive and non-pathogenic Gram-positive commensal microorganisms, are recognized as health-promoting microorganisms.\(^{27}\) LAB strains have been reported to reduce some allergic manifestations in mice and humans.\(^{28–31}\)

NC/Nga mice originated from Japanese fancy mice at Nagoya University (Japan) in 1957. These mice develop AD-like skin lesions and IgE hyperproduction under conventional conditions with itching, erythema and hemorrhage, followed by edematous superficial erosion, deep excoriation, scaling, dryness of the skin and retarded growth.\(^{32,33}\) These pathophysiological observations in AD of NC/Nga mice highly resemble those in human AD; therefore, this strain of mouse has been considered a useful animal model for evaluation of pathologic mechanisms of human AD.\(^{34}\)

In the current study, we examined whether FPG exerted anti-atopic properties both in vitro and in vivo. Here, we report that FPG selectively up-regulated IgG2a and Th1 cytokines in AD-like NC/Nga mice. In addition, the levels of IgE, IgG1, and Th2 cytokines were reduced by treatment with FPG. These results suggest that FPG possesses preventative potential for AD, and that it might also serve as an effective immunomodulatory agent for patients with AD.

**MATERIALS AND METHODS**

**Preparation of FPG** The medicinal herbs of PG were purchased from a herbal market (Hallym Farm, Chungbuk, Korea) and authenticated by Professor Kyoo-seok Ahn, at Kyung Hee University. A total of 1500 g of PG was boiled in 10L distilled water (DW) at 100°C for 2 h, after which it was passed through filter paper (No. 2 Whatman, United Kingdom). Prior to experimental use, LP was propagated twice in an inoculation room and allowed to adapt for at least 1 week prior to use. Animal studies were approved by the institutional animal care and use committee of Kyung Hee University. To induce AD-like symptoms in NC/Nga mice, we used 2,4-dinitrobenzene (DNFB) (Sigma, St. Louis, MO, U.S.A.).\(^{36}\) Briefly, splenocytes from Balb/c mice were seeded into 96-well plates at a density of 5x10^4 cells/well and then incubated at 37°C for 24h. The cells were then treated with 0, 2, 10, 50, 250, 500, and 1000 μg/mL FPG. After the addition of 20 μL/well of CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI, U.S.A.), cells were cultured for an additional 4h, and the absorbance was then recorded at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Model 680; Bio-Rad Laboratories Inc., Hercules, California, U.S.A.). All experiments were performed in triplicate.

**Murine AD Model** Male NC/Nga mice were purchased from the Shizuoka Laboratory Animal Center (SLC) (Tokyo, Japan). Mice (6 weeks old) were maintained in an air-conditioned room and allowed to adapt for at least 1 week prior to use. Animal studies were approved by the institutional animal care and use committee of Kyung Hee University. To induce AD-like symptoms in NC/Nga mice, we used 2,4-dinitrofluorobenzene (DNFB) (Sigma, St. Louis, MO, U.S.A.).\(^{30}\) Briefly, dorsal hair was removed using an electronic clipper two days before DNFB treatment. DNFB was prepared at a concentration of 0.15% in acetone–olive oil (3:1), after which it was applied to the dorsal skin at the indicated times over the course of 4 weeks (Fig. 1). The mice were divided into the following four treatment groups (n = 6–7 per group): 50 mg/kg/d FPG; 50 mg/kg/d PG; 1x10^6 CFU/mL/d LP; and 200 mL of DW once a day from 3 to 10 weeks of age (Fig. 1). As a negative control for AD, NC/Nga mice were maintained without DNFB treatment under specific pathogen free (SPF) conditions.

**Histological Analysis** The backs of mice were subjected to repeated topical application with a 0.15% DNFB, and then removed on the final day of the experiment (week 10). The dorsal skin was then removed and fixed in 4% paraformaldehyde (Sigma, St. Louis, MO, U.S.A.). Next, the paraffin embedded skin sections were heat immobilized, deparaffinized by immersing in xylene (Sigma, St. Louis, MO, U.S.A.),
Fig. 1. Establishment of a DNFB-Induced Mouse Model of AD-Like Skin Lesions in NC/Nga Mice

Oral administration of LP, PG and FPG: 0.15% DNFB in acetone–olive oil (3:1) was applied to the dorsal skin of mice seven times over the course of four weeks to induce AD-like skin lesions. Prior to applying the DNFB, dorsal hairs were removed. Oral administration of LP, PG and FPG was begun at the same time as the first administration of DNFB on day three. The experimental groups were as follows: DW 200 mL/kg/d; 1×10⁷ CFU/mL/d LP; 50 mg/kg/d PG; and 50 mg/kg/d FPG. On week 10, the mice were sacrificed and the samples were collected to measure the immunological responses.

RESULTS

Statistical Analysis Results are expressed as means± S.D. Statistical analysis was performed using one-way analysis of variance in GraphPad Prism 5.0 (GraphPad Software, San Diego, California, U.S.A.). A Bonferroni post-hoc test was used to estimate the reliability interval. A p value <0.05 was considered significant.

RESEARCH ARTICLE

The Effects of FPG on Balb/c Splenocytes and Cytotoxicity To assess the therapeutic potential of FPG, we analyzed IFN-gamma as a biomarker based on its pivotal role in murine model of AD pathogenesis. IFN-gamma is a major cytokine produced by Th1 cells, and decreased levels of IFN-gamma are one of the symptoms of AD. To determine the effect of FPG on the production of IFN-gamma, we measured the release of IFN-gamma from Balb/c splenocytes treated with increasing concentrations of FPG. IFN-gamma release increased slightly following treatment with 10 µg/mL of FPG when compared to PG. The production of IFN-gamma increased in a dose-dependent manner with increasing FPG concentration, peaking at 250 µg/mL FPG (Fig. 2A). These results indicated that FPG induces the production of the Th1 cytokine IFN-gamma, and that the optimal concentration for maximum activity is 50 µg/mL.

To determine the effect of FPG on cell viability, we performed an MTS assay to assess toxicity in splenocytes. Cells were treated with 2, 10, 50, 250, 500, and 1000 µg/mL of FPG, and the cell viability was unaffected by FPG treatment at all concentrations (Fig. 2B). These results suggest that FPG is not-cytotoxic to cells.

Improvements in Epidermal Skin Lesions in FPG-Treated NC/Nga Mice To assess the therapeutic potential of FPG in vivo, we established a mouse model of AD-like skin lesions in NC/Nga mice. Briefly, animals were sensitized with 0.15% DNFB seven times over four weeks, after which they were simultaneously administered DW (200 mL/d), LP (1×10⁷ CFU/mL/d), PG (50 mg/kg/d), or FPG (50 mg/kg/d), as shown in Fig. 3a. From 5 week, treatment with DNFB was stopped, but oral administration of the LP, PG and FPG continued. One week after the cessation of DNFB treatment, the symptoms of AD-like skin lesions were markedly different in the control and FPG-treated mice. Specifically, there were obvious signs of AD-like skin lesions in control mice administered DW, including bleeding, severe itching and rash. However, in mice treated with LP, PG or FPG, the AD-like skin lesions symptoms were milder when compared to the control group. There were no significant differences in phenotype...
Alleviation of DNFB-Induced AD-Like Skin Lesions and Change in Serum IgE, IgG1, IgG2a Levels in NC/Nga Mice Treated with FPG

We next compared the pattern of IgG1 and IgG2a production in each treatment group to determine if FPG affected Th2-mediated or Th1-mediated immune responses. Ten weeks after the initiation of DNFB treatment to induce AD-like skin lesions, IgG1 levels in the 50 mg/kg/d FPG group declined from 144710±5239 ng/mL to 55722±15485 ng/mL (61.5%). Conversely, LP decreased the IgG1 levels by 34.7% and PG resulted in a 40.3% decrease to 94563±7829 ng/mL and 86357±11887 ng/mL respectively (Fig. 4B). As with IgE, treatment with FPG at 50 mg/kg/d also showed the highest reduction. Unlike IgE and IgG1, IgG2a belongs to the Th1 specific immunoglobulin isotype. When compared to the control group, FPG 50 mg/kg/d increased the production of Th1-associated IgG2a by approximately 5 times from week 10 (Fig. 4C). LP and PG induced an increase in IgG2a of approximately 3.4 times (Fig. 4C). FPG had statistical differences on IgE, IgG1 and IgG2a when compared to the LP and PG (the mark of statistical differences was not depicted in the figures). Thus, FPG alleviated AD-like skin symptoms through the up-regulation of IgG2a and the concomitant down-regulation of IgE and IgG1.

Regulation of the Balance between Th1 and Th2 Cytokines by FPG in DNFB-Induced AD-Like Skin Lesions in Mice

Given that FPG treatment differentially affected the production of IgGs related to Th1 and Th2 responses, we next examined the effect of FPG on Th1 and Th2 cytokine responses. To determine the effect of FPG on Th1 or Th2 cytokines, we measured the levels of IL-12p40 and IL-4, which represented Th1 and Th2 immune responses, respectively, in sera isolated from 16-week old NC/Nga mice. When compared to control mice, the levels of IL-4 were decreased by 35.7% in response to FPG at 50 mg/kg/d and were similar to those of negative control mice grown under SPF conditions (Fig. 5). In contrast, the levels of IL-12p40 were increased by approximately 2.5 times in response to FPG at 50 mg/kg/d and were similar to those of negative control mice grown under SPF conditions (Fig. 5).

The results indicated that FPG changes skin hypertrophy in DNFB-induced NC/Nga mice.

Figure 2. Effects of FPG on Balb/c Splenocytes

(A) Balb/c splenocytes were stimulated with PG (50 µg/mL) or FPG (2, 10, 50, 250 µg/mL), after which they were incubated for 48 h. Secreted IFN-gamma levels were measured by ELISA. (B) Cytotoxicity of FPG on splenocytes. Cells were treated with FPG (2, 10, 50, 250, 500, 1000 µg/mL) for 24 h, after which the cell viability was assessed by MTS cell proliferation assay. Data shown represent the mean±S.D. **p<0.01 when compared with control group (one-way analysis of variance).

among mice treated with different concentrations of LP, PG and FPG extracts (50 mg/kg/d). Control NC/Nga mice with fully developed AD-like skin lesions showed hypertrophy and hyperkeratosis (Fig. 3A). Dense infiltration of inflammatory cells was also observed in the epidermis in this group of mice (Fig. 3A). To analyze the effect of FPG on skin hypertrophy infiltration in DNFB-induced NC/Nga mice, FPG-treated skin was stained with hematoxylin and eosin, after which it was examined with an optical microscope. As shown in Fig. 3A, acanthosis was clearly suppressed in FPG-treated NC/Nga mice when compared with control NC/Nga mice. The thickness of the dorsal skin was 41±4 µm for the SPF mice, 152±15 µm for the control mice, 78±10 µm for the LP mice, 69±9 µm for the PG mice, and 55±6 µm for the FPG 50 mg/kg/d mice. Oral administration with 50 mg/kg/d FPG resulted in a 63.8% decrease in thickness when compared to the control group, which was the greatest decrease of all groups (Fig. 3B).

These results indicated that FPG changes skin hypertrophy in DNFB-induced NC/Nga mice.

Regulation of the Balance between Th1 and Th2 Cytokines by FPG in DNFB-Induced AD-Like Skin Lesions in Mice

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The results indicated that FPG changes skin hypertrophy in DNFB-induced NC/Nga mice.

Alleviation of DNFB-Induced AD-Like Skin Lesions and Change in Serum IgE, IgG1, IgG2a Levels in NC/Nga Mice Treated with FPG

In addition to clinical features, we assessed the levels of IgE, IgG1 and IgG2a in the sera from NC/Nga mice to characterize the immunological response during disease progression. Altered IgE levels are one of the most common indicators of AD. At week 10, the concentration of soluble serum IgE appeared to be lessened in extract-treated mice. The IgE levels of LP were 5961±758 ng/mL, while they were 18032±126 ng/mL in the control group. Mice treated with PG at 50 mg/kg/d had IgE levels of 3863±139 ng/mL, which represented a 78.6% reduction when compared to those of the control group. Mice treated with FPG at 50 mg/kg/d had IgE levels of 2309±164 ng/mL, representing an 87.2% reduction when compared to the control group. As a negative control, non-AD NC/Nga mice maintained under SPF conditions produced almost no IgE (Fig. 4A). Together, these results suggest that the release of IgE is correlated with the onset of AD-like skin lesions, and that FPG than the PG and LP, which inhibits the symptoms of AD-like skin lesions through the down-regulation of IgE production.

We also investigated the effect of FPG on splenocytes isolated from 16-week old NC/Nga mice. FPG at 50 mg/kg/d reduced the production of IL-4 and IL-5 by 70.6% and 60%, respectively, when compared to control cells (Fig. 6A). In all groups, IL-4 and IL-5 were inhibited, but treatment with FPG 50 mg/kg/d had the greatest effect, while the level of the Th1 cytokines IL-12p40 and IFN-gamma increased by 7.4 times
and 19.3 times when compared to the control (Fig. 6B). In IFN-gamma, PG and FPGs were noticeably increased, but LP did not increase significantly. FPG had statistical differences on IL-5 and IL-12 p40 production when compared to the LP and PG (the mark of statistical differences was not depicted in the figures). These results suggested that FPG plays an important role in the balance of Th1 and Th2 cytokines, increasing the levels of Th1 cytokines and decreasing the level of Th2 cytokines.

DISCUSSION

AD is a major allergic disease that results from dermal inflammation. Although the etiology and pathology of AD are not fully understood, many data suggested that typical symptoms of AD involve increased levels of Th2-mediated cytokines and a deficiency in Th1-derived cytokines. However, it has been shown that Th1 is also significantly involved in AD lesion. In such a model, a key element is the sequential activation of the Th2-cell subsets during the initiation phase, followed by the Th1-cell subset to account for the persistence of the inflammatory response. Therefore, enhancement of Th1 seems not necessarily beneficial to the treatment for human AD. In addition, as for NC/Nga AD model, it was reported that Th2 was not required to establish AD-like lesion. Yagi et al., showed that the skin microenvironment that favored IFN-γ production tightly correlated with the skin disease in NC mice through the infiltration of eosinophils. Even though there is a controversy in surrounding pathogenesis of AD, given the immunological imbalance in AD, one of the goals of AD therapy is to manipulate the cytokine network to selectively promote the Th1 response or preferentially inhibit the Th2 response. Many studies have suggested that the Th1 and Th2 types of reactions can reciprocally regulate one another. Therefore, balancing the Th1/Th2 types of reactions may be a fundamental approach to AD treatment. Recently, herbal
therapy has become increasingly popular due to its successful use over extended time periods in Asia and Europe. In addition, several studies have shown that the fermentation of herbal extracts with lactic acid bacteria may improve the therapeutic benefits of AD. The present study investigated the effects of FPG, which is composed of a medicinal herb, PG, fermented by LP, in vitro and in vivo in a NC/Nga AD-like animal model. FPG increased the levels of IFN-gamma in Balb/c splenocytes in vitro (Fig. 2A). In DNFB-sensitized NC/Nga mice, FPG treatment resulted in an increase in Th1-mediated IgG2a, IL-12p40, and IFN-gamma in sera and splenocytes (Figs. 4C, 5, 6B). These results confirm that FPG can restore the impaired balance between Th1 and Th2 responses by enhancing the secretion of Th1 cytokines.

In contrast to its effects on Th1 cytokines, FPG induced a significant decrease in soluble IgE, IgG1, and the Th2-mediated cytokines (IL-4, IL-5) in NC/Nga mice sera (Figs. 4A, B, 5) and splenocytes (Fig. 6A). The results of the present study indicate that oral administration of FPG to NC/Nga mice treated with DNFB suppressed the development of AD-like skin lesions. Macroscopic analysis showed severe hemorrhage, acanthosis and excoriation in the control group, whereas FPG treatment prevented these skin changes. Histologically, FPG administration decreased hypertrophy, hyperkeratosis and infiltration of inflammatory cells in the skin (Fig. 3A). To examine effective doses range and doses dependency of FPG, we performed the experiment at two different doses of FPG (50 mg/kg/d and 250 mg/kg/d). Although FPG was effective at two different doses, the effect of FPG at 50 mg/kg/d was slightly more effective than that of FPG at 250 mg/kg/d.
6 µm vs. 78 ± 8 µm). So we supposed that the optimal concentration for maximum activity would be 50 mg/kg/d.

Early chemical studies on PG revealed that triterpenoid saponins were the main active chemical components, and the saponins termed platycodins (A, D, D2, and D3), 2- and 3-O-acetyl polygalacin D2, platyconic acid A, and platycosides (A, B, C, D, E, and F) were found in the roots. Some saponins from root of PG have been known to have several beneficial effects, including anti-inflammation. A previous study also reported that platycodin D inhibited the induction of COX-2 by TPA, with a resultant inhibition of the production of PGE2 in rat peritoneal macrophages. Although we could not identify the beneficial ingredients in PG by fermentation, it is suggested that saponin would be one of the active components since the amount of total saponin was substantially increased by fermentation of PG (data shown in Materials and Methods).

In conclusion, FPG down-regulated the levels of IgE in AD-like skin lesions of mice through modulation of Th1 and Th2 immune responses. FPG treatment resulted in an increase in Th1-mediated cytokines and a decrease in Th2-mediated cytokines. These results suggest that FPG has beneficial preventive effects on AD symptoms and represents an effective drug candidate for the treatment of AD without severe side effects.

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