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Effect of 6,7-dimethoxy-2,2-dimethyl-2H-chromene (agerarin) on the recovery of filaggrin expression through targeting of Janus kinases in the inflammatory skin

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Abstract

Filaggrin (FLG) is a structural component of the stratum corneum that is essential for maintaining the barrier function of the skin and for the formation of natural moisturizing factors. 6,7-Dimethoxy-2,2-dimethyl-2H-chromene (Agerarin) is a bioactive compound derived from Ageratum houstonianum, a plant that is used as a traditional medicine to treat skin diseases. This study aimed to evaluate the effect of agerarin on skin inflammation in a dinitrochlorobenzene (DNCB)-induced atopic dermatitis mouse model. We found that the topical administration of agerarin ameliorates atopic dermatitis-like skin lesions. We also showed that agerarin restores the reduced filaggrin (FLG) expression in DNCB-applied skin sections. Moreover, agerarin decreased phosphorylation of JAK1 and JAK2 kinases to enhance FLG expression, which was reduced by TNFα+IFNγ and IL4+IL13 treatment, in HaCaT keratinocytes. These results demonstrate the feasibility of agerarin as a possible therapeutic against conditions of skin inflammation, such as atopic dermatitis, by improving the upregulation of FLG expression.

Keywords: Atopic dermatitis, 6,7-Dimethoxy-2,2-dimethyl-2H-chromene, Filaggrin, Janus kinase, Keratinocyte

1. Introduction

Filaggrin (FLG) is a filament-associated protein found predominantly in keratin fibers in epidermal keratinocytes [1]. The FLG gene is part of the Epidermal Differentiation Complex (EDC) locus, located in the human chromosome 1q21 region, which encodes various structural proteins involved in epidermal differentiation [2,3]. A large precursor protein (>400 kDa) called profilaggrin (proFLG) consists of multiple, repeating FLG monomers (~37 kDa each). The cleavage of proFLG is mediated by various proteases, including kallikrein 5, caspase-14, elastase-2, matriptase, and prostatin, in the granular layer. Monomeric FLG binds tightly to keratin filaments to promote flattening of corneocytes [4] and constitutes an epidermal barrier to prevent water loss and invasion of foreign pathogens [5]. FLG monomers undergo further processing into an arginine-, histidine-, and glutamine-rich hygroscopic amino acid pool, including urocanic acids and pyrrolidone carboxylic acid, that functions as an essential part of the moisturizing factor (NMF) in the stratum corneum [6]. The FLG-NMF system plays multifunctional roles in epidermal homeostasis and
maintenance of skin barrier function [7]. Various genetic studies have shown that loss-of-function of the FLG gene is strongly associated with dysfunction of the skin barrier [8,9].

The Janus kinase (JAK) is a family of nonreceptor tyrosine kinases associated with type I and II cytokine receptors. Upon ligand binding, JAKs phosphorylate cytokine receptors and transduce signaling through multiple signaling proteins, including signal transducer and activator of transcription (STAT), which functions as a transcription factor [10]. The JAK family includes JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2), and the STAT family includes seven family members, STAT1–4, STAT5α–β, and STAT6. JAK-STAT signaling plays a crucial role in transmitting inflammatory responses in the skin [11], and inhibition of the JAK-STAT pathway ameliorates allergic skin inflammation by suppressing T helper (Th) 2 cell responses in the NC/Nga mouse model [12]. Various studies have demonstrated the clinical efficacy of JAK inhibitors for the treatment of AD [13]. Moreover, treatment with the JAK inhibitor JTE-052 was shown to increase FLG expression and improve skin barrier function in a human skin graft model [14].

These studies suggest that the JAK-STAT signaling pathway plays a crucial role in regulating FLG expression and skin barrier function.

6,7-Dimethoxy-2,2-dimethyl-2H-chromen (called agerarin; Fig. 1A) is a biologically active compound (e) derived from the ethanolic extract of Ageratum houstonianum [15]. It has been shown to upregulate the expression of aquaporin-3, a water/glycerol transport protein in the skin, by stimulating the expression of the circadian gene CLOCK [15]. In addition, we previously showed that agerarin downregulates STAT3 expression in melanocytes [16]. However, it is not known whether agerarin modulates FLG expression in keratinocytes.

This study aimed to evaluate whether agerarin modulates the expression of FLG and affects the skin barrier function in an inflammatory environment. Our results showed that agerarin ameliorates skin lesions in a 2,4-dinitrochlorobenzene (DNCB)-applied atopic dermatitis mouse model. Biochemical studies demonstrated that agerarin restored the reduced FLG expression induced by both TNFz+IFNy and IL4+IL13 cytokines by inhibiting JAK1/2, two upstream kinases of STAT3, in HaCaT keratinocytes.

2. Methods

2.1. Materials

Human primary neonatal keratinocyte HEKn cells were obtained from Thermofisher Scientific Korea (Seoul, Korea). HaCaT cells were maintained in DMEM medium (1.8 mM CaCl2) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and HEKn cells were cultured in EpiLife medium supplemented with Human Keratinocyte Growth Supplement (HKGS) (Thermofisher Scientific Korea). Agerarin was isolated as described previously [15]. 2,4-Dinitrochlorobenzene (DNCB) was obtained from Sigma–Aldrich (St. Louis, MO, USA). TNFz and IFNy were obtained from Prospec-Tany Technogene Ltd. (Ness-Ziona, Israel). The hematoxylin and eosin (H&E) stain kit and toluidine blue dye were obtained from Sigma–Aldrich. The Firefly Luciferase Assay System was from Promega (Madison, WI, USA). Antibodies against phospho-JAK1 (Y1034/1035), JAK1, phospho-JAK2 (Y1007/1008), JAK2, phospho-STAT3 (Y705), and STAT3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against FLG and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-FLG antibody for immunofluorescent staining was from BioLegend (San Diego, CA, USA).

2.2. Induction of atopic dermatitis-like skin lesions in Balb/c mice

Seven-week-old male Balb/c mice were obtained from Orient Bio, Inc. (Seongnam, Korea). The mice were divided into three groups: (i) naive control (n = 5), (ii) DNCB + vehicle (n = 7), and (iii) DNCB + agerarin (n = 7). DNCB and agerarin were dissolved in a 1:3 (v/v) mixture of acetone/olive oil and 70% ethanol, respectively. On day 0, the hair on the dorsal skin of the mice was shaved. The skin barrier was disrupted by applying 4% SDS 4 h before the application of 1% DNCB once a day, which was repeated for 3 days. On day 3, mice in groups ii and iii were sensitized with 1% DNCB. After a 4-day break, 0.5% DNCB was applied to the mice in groups ii and iii, followed by additional treatment with vehicle (group ii) or agerarin (group iii) once a day for 14 days (days 8–21). Mice were sacrificed on day 22. All animal experiments were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals and approved by the Konkuk University Institutional Animal Care and Use Committee (Approval number; KU19080).

2.3. Serum IgE

Mouse blood samples were collected through cardiac puncture immediately following euthanasia.
with CO2 gas inhalation, and serum IgE concentrations were measured with an ELISA MAX Standard Set Mouse IgE kit (BioLegend, San Diego, CA, USA) according to the manufacturer’s instructions. Color development at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (SoftMax Pro; Molecular Devices, Sunnyvale, CA, USA).

2.4. Histology

For histologic examination, dorsal AD-like skin samples were fixed in 10% neutralized formalin for 24 h and embedded in paraffin. Each section (4 μm) was stained with H&E. Mast cells were stained with 0.1% toluidine blue. Images of each section were captured with a light microscope (EVOS FL Auto, Bothell, WA, USA), and epidermal and dermal thicknesses were measured in the digital images using ImageJ version 1.52a (National Institutes of Health, Bethesda, MD, USA).

2.5. Immunofluorescence microscopy

The paraffin-embedded dorsal AD-like skin sections were deparaffinized with xylene, followed by graded ethanol. The tissue sections were immersed in 1 mM EDTA (pH 8.0) for 20 min at 70 °C, rinsed in PBS, and then placed in a blocking buffer containing 7% goat serum for 1 h. The primary anti-FLG antibody (1:300 dilution) was added overnight at 4 °C. After washing with PBS, rhodamine red-X-conjugated secondary antibody (Jackson ImmunoResearch Lab, 1:500 dilution) was incubated for 1 h at 25 °C. The nuclei were stained with Hoechst 33258 solution for 10 min. After washing with PBS, the slides were mounted with a fluorescence mounting medium (ProLong Gold antifade reagent; Thermo Fisher Scientific, Waltham, MA, USA).

Fig. 1. The beneficial effect of agerarin on the amelioration of DNCB-induced skin lesions in Balb/c mice. (A) Chemical structure of agerarin. (B) Experimental schedule for the induction of AD-like lesions. DNCB, dinitrochlorobenzene. (C) Histological features of the DNCB-induced skin lesions. Paraffin-embedded sections were stained with hematoxylin and eosin. The areas in the boxes are magnified in the right panels. Scale bars, 400 μm. (D–F) The thicknesses of the epidermis (D) and dermis (E) were measured by ImageJ software. (E) Serum IgE levels were measured on day 22. Data are presented as mean ± SD. *** P < 0.001 by Dunnett’s multiple comparisons test (n = 7).
Invitrogen, Carlsbad, CA, USA). Fluorescent images were examined under an EVOS FL fluorescence microscope (Advanced Microscopy Group; Bothell, WA, USA).

2.6. Immunoblot analysis

Keratinocyte cells were lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% Na-Deoxycholate, 500 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 10 μg/mL leupeptin, and 1 mM PMSF. The protein extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After incubation with the appropriate primary and secondary antibodies, the blots were developed using an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA). The relative intensities of the immunoreactive bands were measured using ImageJ version 1.52a software. Relative band intensity was expressed as a ratio to GAPDH.

2.7. Quantitative real time-PCR (qR-PCR)

A qR-PCR was performed using iCycler iQ system with an iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s recommendations. Validated commercial qR-PCR primers and SYBR Green-based fluorescent probes specific for FLG (id: qHsaCEP0039328) and GAPDH (id: qHsaCEP0041396) were obtained from Bio-Rad. PCR conditions were as follows: denaturation at 95 °C for 2 min, followed by 40 cycles using a step program (95 °C for 10 s and 60 °C for 45 s). The relative expression levels of FLG mRNA were normalized to those of GAPDH using the software program provided by the manufacturer.

2.8. In vitro JAK kinase assay

JAK kinase activity was determined using a fluorescence resonance energy transfer (FRET)-based in vitro kinase assay (Z’-LYTE Kinase Assay Kit, Invitrogen) adapted from a previous study [17]. Briefly, the assay mixtures, containing 50 mM HEPES (pH7.5), 0.01% Bris-35, 10 mM MgCl2, 1 mM EGTA, 2 μM Z’-LYTE peptide substrate (Tyr 6 for JAK1, JAK2, and JAK3; Tyr3 for TYK2), and JAK enzyme [22 ng JAK1 (PV4774, Invitrogen), 0.05 ng JAK2 (PV4210, Invitrogen), or 0.5 ng JAK3 (PV4790, Invitrogen)], were preincubated with serially diluted agerarin or tofacitinib (for reference) for 10 min at 25 °C. The kinase reaction was initiated by adding ATP (for JAK1, 75 μM; for JAK2, 25 μM; for JAK3, 10 μM; for TYK2, 25 μM). Following 60 min incubation, the development reaction was initiated by the addition of Z’-LYTE development reagents. After 60 min, Coumarin and Fluorescein emissions were determined at 450 nm and 520 nm, respectively, after excitation at 400 nm using a fluorescence microplate reader Gemini EM (Molecular Devices; Hampton, NH, USA). The curve fitting and data analysis were carried out using IDBS XLfit5 software (Guildford, Surrey, UK).

2.9. Molecular docking simulation

The X-ray crystallographic structures of the JAK1 and JAK2 proteins were obtained from the RCSB Protein Data Bank as 5WO4.pdb and 4JIA.pdb, respectively. For the in silico docking experiments, the apo-proteins and the ligand were prepared using the Sybyl program (Tripos, St. Louis, MO, USA). The 3D structure of the agerarin was obtained from PubChem (CID: 12565). UCSF Chimera (https://www.cgl.ucsf.edu/chimera/) was used to prepare proteins and analyze the docking results. The AutoDock tools and AutoDock Vina (http://vina.scripps.edu/) were used to generate the pdbqt files and for the docking experiments, respectively. LigPlot software (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/) was used to analyze the interactions between ligand and protein. Three-dimensional structures were prepared using the PyMOL program (PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC).

2.10. Statistical analysis

Data are presented as means ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test using GraphPad Prism version 8.0.2 software (GraphPad Software, Inc., La Jolla, CA, USA). In all analyses, P values less than 0.05 were considered significant.

3. Results

3.1. Topical application of agerarin ameliorates DNCB-induced skin inflammation in Balb/c mice

To evaluate the effect of agerarin on skin inflammation, we used a mouse model of skin inflammation induced by DNBC. In the experiment, DNBC was repeatedly applied to the dorsal skin of Balb/c mice in the presence or absence of agerarin (Fig. 1B). DNBC caused AD-like skin lesions, such as erythema and keratinization, which were substantially
improved by the topical administration of agerarin (Fig. 1C). H&E staining showed that agerarin reduced DNCB-induced hyperkeratosis (Fig. 1C). The morphometric analysis confirmed the beneficial effects of agerarin on DNCB-induced epidermal (Fig. 1D) and dermal thickness (Fig. 1E). High serum IgE level is a typical marker of DNCB-induced AD-like skin inflammation. Agerarin also significantly reduced serum IgE levels when compared to DNCB-challenged mice (DNCB, 1481 ± 181 ng/mL vs. agerarin, 924 ± 205 ng/mL; \( P < 0.001 \)) (Fig. 1F). These results suggest that agerarin improves AD-like clinical manifestations.

### 3.2. Agerarin reduces the infiltration of mast cells

Chronic skin inflammation is characterized by the infiltration of immune cells, such as CD4\(^+\) T cells, eosinophils, and mast cells, to the skin lesions. As mast cells are indispensable for maximal skin inflammation in the allergen-induced mouse model [18], we examined the infiltration of mast cells by toluidine blue staining. Consistent with a previous study, DNCB caused a remarkable increase in the population of mast cells in comparison to that of the naive control; in contrast, agerarin substantially reduced the infiltration of mast cells (Fig. 2A). Quantitative analysis showed that the topical application of agerarin significantly decreased the number of TB-positive cells compared to DNCB-challenged mice (DNCB, 124 ± 32 cells/1.8 cm\(^2\) vs. agerarin, 46 ± 11 cells/1.8 cm\(^2\); \( P < 0.001 \)) (Fig. 2B). These results suggest that agerarin can ameliorate DNCB-induced AD-like skin inflammation.

### 3.3. Agerarin restores FLG expression in DNCB-induced skin lesions

FLG is a major component of the skin barrier, and loss-of-function mutation of the FLG gene is a major predisposing factor for AD [19]. We evaluated the effect of agerarin on FLG expression in DNCB-challenged mice skin. Immunofluorescence analysis showed that FLG staining was detected in the epidermal layer of naive skin, but rarely in DNCB-applied skin (Fig. 3A). However, the topical application of agerarin substantially increased the number of FLG-stained cells, suggesting that agerarin upregulates FLG expression in DNCB-induced skin lesions.

As FLG is only expressed in differentiated keratinocytes, we asked whether agerarin induces keratinocyte differentiation. It is known that a high concentration of extracellular calcium (\( \geq 1 \) mM) induces keratinocyte differentiation [20]. We observed that exposure to high concentrations of CaCl\(_2\) (1.2 and 1.8 mM) induced changes in cell morphology of primary neonatal keratinocytes (HEKn) (Fig. 3B). When HEKn cells were treated with agerarin in the presence of 0.06 mM CaCl\(_2\), the cell morphology was similar to that of control cells (Fig. 3C). In addition, agerarin did not induce the expression of epidermal differentiation marker proteins proFLG and loricrin, but high concentrations of extracellular CaCl\(_2\) resulted in accumulation of proFLG proteins (Fig. 3D). These data suggest that agerarin did not induce differentiation of keratinocyte and did not accumulate proFLG proteins in undifferentiated keratinocytes, suggesting that agerarin exerts an

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**Fig. 2.** Effect of agerarin on the inhibition of mast cell infiltration in DNCB-induced skin lesions. (A) Paraffin-embedded skin sections were stained with 0.1% toluidine blue. The areas in the dashed boxes are magnified in the right panels. Scale bars, 400 \( \mu \)m. (B) The number of toluidine blue-positive cells per 1.8 cm\(^2\) in six random fields was counted. Data are presented as mean ± SD. ***, \( P < 0.001 \) by Dunnett’s multiple comparisons test \((n = 6)\).
ability to restore FLG expression reduced by the inflammatory response. Thus, to further analyze the effect of agerarin on FLG expression in the subsequent experiments, we used differentiated HaCaT keratinocytes cultured in DMEM medium containing a high concentration of CaCl$_2$ (1.8 mM), in which FLG is highly expressed.

3.4. Agerarin abrogates TNF$\alpha$+IFN$\gamma$- and IL4+IL13-induced suppression of FLG expression

Th2-type cytokines, such as IL4 and IL13, play an essential role in the pathogenesis of the acute phase of AD [21], whereas Th1-type cytokines, such as IFN$\gamma$, and pro-inflammatory cytokine TNF$\alpha$ are highly expressed in chronic AD [22]. Previous studies have demonstrated that Th2/Th1-type cytokines and TNF$\alpha$ decreased FLG expression in human keratinocytes [23–28]. We investigated whether agerarin can restore FLG expression suppressed by TNF$\alpha$+IFN$\gamma$ and IL4+IL13. Consistent with previous studies, TNF$\alpha$+IFN$\gamma$ decreased FLG expression in HaCaT keratinocytes in a time-dependent manner; mRNA levels were decreased within 3 h, as revealed by RT-PCR (Fig. 4A) and qR-PCR (Fig. 4C), and protein levels were reduced after 24 h, based on immunoblot analysis (Fig. 4E). Similarly, Th2-type cytokines (IL4+IL13) also reduced proFLG levels in a time-dependent manner (Fig. 4G). Notably, agerarin abrogated TNF$\alpha$+IFN$\gamma$-induced suppression of FLG expression in a dose-dependent manner, as revealed by RT-PCR (Fig. 4B), qR-PCR (Fig. 4D), and immunoblot analysis (Fig. 4F). Agerarin also recovered IL4+IL13-induced reduction of proFLG levels (Fig. 4H). These results suggest that agerarin may exert a beneficial effect on the recovery of FLG expression reduced by both Th2 and Th1 responses.

3.5. Inhibition of JAK kinase abrogates downregulation of FLG expression by TNF$\alpha$+IFN$\gamma$

The JAK-STAT3 pathway is considered as an upstream activator for the downregulation of FLG expression in response to various inflammatory cytokines [23]. We confirmed the effects of TNF$\alpha$+IFN$\gamma$ and IL4+IL13 on the activation of the JAK-STAT3 signaling pathway. As expected,
phosphorylation of JAK1 at Tyr-1034/1035, JAK2 at Tyr-1007/1008, and STAT3 at Tyr-705, which is a downstream target of both JAK1 and JAK2, increased within 10 min following stimulus with TNFα + IFNγ (Fig. 5A) and IL4 + IL13 (Fig. 5B).

To determine whether inhibition of JAK kinase is associated with the downregulation of FLG expression, we examined the effect of tyrphostin B42 (AG490), a known JAK2 inhibitor [29], on TNFα + IFNγ-induced suppression of FLG expression. We found that AG490 dose-dependently recovered FLG expression, which was reduced by TNFα + IFNγ stimulation, as revealed by RT-PCR (Fig. 5C), qR-PCR (Fig. 5D), and immunoblot analysis (Fig. 5E). These data suggest that inhibition of JAK kinase can restore FLG expression suppressed by inflammatory cytokines.

3.6. Agerarin inhibits both TNFα + IFNγ- and IL4 + IL13-induced JAK-STAT3 signaling

To investigate the mechanism underlying the effects of agerarin on the recovery of FLG expression, we examined whether agerarin modulates the JAK-STAT3 signaling pathway. We found that agerarin attenuated TNFα + IFNγ-induced phosphorylation of JAK1 (Tyr-1034/1035), JAK2 (Tyr-1007/1008), and STAT3 (Tyr-705) (Fig. 6A). When the cells were treated with IL4 + IL13, similar results were observed (Fig. 6B). These results suggest that agerarin inhibits the JAK-STAT3 signaling pathway activated by both Th2 and Th1 responses.

To validate whether agerarin targets JAK kinases, we performed a FRET-based in vitro kinase assay with each family member of JAK kinases. Tofacitinib, a selective JAK1 and JAK3 inhibitor [30], was used as a reference compound. We observed that tofacitinib inhibited JAK activities with IC50 values of 3.86, 15.6, and 4.76 nM against JAK1, JAK2, and JAK3, respectively. Under this experimental condition, we found that agerarin inhibited JAK1 (IC50, 0.473 μM), as well as JAK2 (IC50, 4.92 μM) and JAK3 (IC50, 3.12 μM) to a lesser extent, but did not inhibit TYK2 at up to 100 μM concentration. Tofacitinib inhibited JAK1 and JAK3 at similar concentrations, while agerarin is 10.4- and 6.60-fold more potent in...

Fig. 4. Effect of agerarin on the restoration of FLG expression reduced by inflammatory cytokines. HaCaT cells were treated with TNFα + IFNγ (each 10 ng/mL) (A–F) or IL4 + IL13 (each 100 ng/mL) (G and H) for indicated times in the absence or presence of different concentrations (0–40 μM) of agerarin. Total RNA was extracted and FLG mRNA levels were examined with RT-PCR (A and B) and quantitative real-time PCR (C and D). Whole-cell lysates were prepared and immunoblotting was performed using anti-FLG antibody (E and F) or IL4 + IL13 (each 100 ng/mL) (G and H) for indicated times in the absence or presence of different concentrations (0–40 μM) of agerarin. The graph data are presented as mean ± SD (n = 3). REL, relative expression level; proFLG, prolaggrin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase (loading control). NS, not significant (P > 0.05); *, P = 0.0287; **, P < 0.01; ***, P < 0.001 by Dunnett’s multiple comparisons test (n = 3).
inhibiting JAK1 than JAK2 and JAK3, respectively (Fig. 6C). These results suggest that agerarin is a JAK inhibitor that is more selective for JAK1 than JAK2 and JAK3.

3.7. Agerarin binds to JAK1 and JAK2 in silico

To predict the possible binding mode of agerarin to JAK kinases, we performed in silico docking simulations. The binding affinities between JAK1 and agerarin ranged between −6.7 kcal/mol and −5.6 kcal/mol. Fourteen hydrophobic interactions were observed involving residues L881, G882, E883, V889, A906, E957, F958, L959, G962, R1007, N1008, L1010, G1020, and D1021 (Fig. 7A, left). It has been reported that interactions with L959 may be essential for the docking of a potent inhibitor [31]. As agerarin is smaller than the original ligand, it may be possible that more water molecules participate in the interactions.

In the crystal structure of JAK2 with the potent inhibitor 2-aminio-[1,2,4]triazolo[1,5-alpyridine derivative [32], there were twelve hydrophobic interactions involving L855, G856, V863, A880, V911, M929, E930, Y931, P933, G935, and L983, and one H-bond involving L932. Like JAK1, after docking the ligand with JAK2, the apo-protein and ligand complex with a binding affinity of −8.5 kcal/mol was selected and compared with the crystal structure. Then, in silico docking of agerarin with JAK2 was conducted, and nine binding modes were produced (Fig. 7A, right). The binding affinities of the nine complexes ranged from −6.0 kcal/mol to −5.6 kcal/mol. The binding mode with the best binding affinity was selected, which showed nine hydrophobic interactions involving residues L855, V863, M929, Y931, L932, G935, S936, L983, and D994 of JAK2.

The binding modes of agerarin with JAK1 and JAK2 were compared (Fig. 7B), which showed that the pyran rings were oriented differently, but the
benzyl groups were in similar positions. The different orientation of the compound resulted in more hydrophobic interactions with JAK1. Most inhibitors of JAKs share aromatic heterocyclic structures that include the nitrogen atom necessary for the H-bond with L959 of JAK1 or L932 of JAK2. Although the hydrogen bond with L959 (JAK1) or L932 (JAK2) was not observed in the binding modes of agerarin with JAK1 and JAK2 because of the absence of the nitrogen atom, the hinge region may play an essential role in binding by participating in hydrophobic interactions with both kinases. Collectively, these results suggest that agerarin might bind to JAK1 and JAK2, leading to inhibition of kinase activity and resulting in the inhibition of STAT3.

4. Discussion

In this study, we showed that the topical application of agerarin, a natural bioactive compound isolated from A. houstonianum, ameliorated skin inflammation in a DNCB-challenged mouse model. Histological examination demonstrated that agerarin reduced the epidermal and dermal thicknesses of AD-like skin lesions. According to biochemical studies, agerarin recovered the expression of FLG mRNA and protein levels, reduced by IL4+IL13 and TNFα+IFNγ.

The epidermis is the outermost layer of the skin that forms the first line of protective defense barrier against invading pathogens and allergens. Disruption of skin barrier function is strongly associated with increased allergen sensitization, trans-epidermal water loss, and pathogenic skin inflammation, which can augment the allergic inflammatory responses [33]. Therefore, it appears likely that the epidermal barrier dysfunction and cutaneous hyperimmune responses are crucial for the pathogenesis of AD [34]. FLG is a key protein that forms a cornified cell envelope and maintains a proper skin barrier function [7]. FLG deficiency is observed in AD patients [35] and is strongly associated with the development of AD [8,9].

In the present study, we found that agerarin increases FLG expression in DNCB-induced skin lesions. As FLG is only expressed in differentiated keratinocytes, we tested whether agerarin facilitates...
keratinocyte differentiation using HEKn primary keratinocytes. We first checked FLG expression in differentiated cells. In accordance with previous studies, HEKn cells were differentiated by high concentration (≥1 mM) of calcium. We also confirmed that proFLG was not induced in medium containing a low concentration of calcium (0.06 mM), but strongly expressed, along with another epidermal differentiation marker loricrin, in medium containing a high concentration (≥1 mM) of calcium. Under these experimental conditions, agerarin did not alter morphological changes and did not induce proFLG and loricrin expression in the medium containing a low concentration of calcium, demonstrating that agerarin itself is not capable of inducing keratinocyte differentiation and expressing FLG in undifferentiated keratinocytes.

Based on these data, we hypothesized that agerarin does not affect the expression of FLG in healthy keratinocytes but could prevent inflammatory cytokine-induced suppression of FLG expression. To test this hypothesis, we used a HaCaT keratinocyte cell line cultured in DMEM medium containing a high concentration of CaCl₂ (1.8 mM). In this culture condition, differentiation of HaCaT cells is induced and cells strongly express FLG mRNA and proFLG protein. When HaCaT cells were treated with TNFα + IFNγ, FLG expression was substantially reduced. Under these experimental conditions, we tested whether agerarin prevents the reduction of FLG expression induced by inflammatory cytokines. We found that agerarin dose-dependently recovered FLG expression suppressed by both TNFα + IFNγ and IL4+IL13. These results suggest that
Agerarin is not capable of inducing FLG expression but restores FLG expression reduced by inflammatory cytokines.

FLG expression is regulated by multiple factors, including genetic and environmental factors, as well as cutaneous inflammatory cytokines [36]. Th2-type cytokines, such as IL4, IL5, IL9, and IL13, are increased in the acute phase of AD [27], while TNFα and Th1-type cytokines, such as IFNγ and IL1β, dominate the chronic phase of AD [22]. TNFα is a major pro-inflammatory cytokine produced by various cell types, including Th1 and Th2 cells as well as dendritic cells, macrophages, and keratinocytes [37]. Suppression of FLG expression is induced by Th2- and Th1-type cytokines and TNFα [23–28]. Th2- and Th1-type cytokines utilize the JAK-STAT pathway to transmit signals from the membrane to the nucleus. Therefore, JAK inhibitors can provide an effective treatment strategy that can simultaneously inhibit multiple cytokine pathways important for AD development as well as inflammatory diseases such as rheumatoid arthritis [11]. Indeed, the topical application of the JAK inhibitor JTE-052 restored the downregulated FLG expression induced by IL4+IL13 [14]. Also, several JAK inhibitors are currently being evaluated in clinical trials with AD patients [13]. Thus, a therapeutic strategy to inhibit the JAK-STAT3 signaling could be potentially advantageous for the restoration of FLG expression and the improvement of the skin barrier function.

In this study, we found that agerarin inhibited both IL4+IL13 and TNFα+IFNγ-induced tyrosine phosphorylation of JAK1/2 and STAT3. The in silico molecular docking approach supported the inhibitory activity of agerarin by predicting the hydrophobic interaction with the kinase domain, suggesting that agerarin may interact with JAK1 and JAK2. To test the hypothesis that agerarin interacts directly with JAK1 and JAK2, we conducted FRET-based in vitro JAK kinase assays. As a result, agerarin preferentially inhibited JAK1 (IC50, 0.473 μM) with weak inhibition on JAK2 (IC50, 4.92 μM) and JAK3 (IC50, 3.12 μM), but had no effect on TYK2. Taken together, agerarin may be a JAK inhibitor that exhibits a more favorable property against JAK1. A previous study demonstrated that the topical application of a JAK inhibitor increases FLG expression in an atopy-like mouse model [14]. We also observed that JAK2 inhibitor AG490 restores FLG expression suppressed by TNFα+IFNγ. Similar to other JAK inhibitors, agerarin restores FLG expression reduced by Th2- and Th1-type cytokines. However, we cannot rule out the possibility that agerarin also targets the upstream molecules of JAKs, causing a similar inhibitory effect on JAK phosphorylation at the cellular level.

Negative regulation of the JAK-STAT pathway by agerarin might be useful for ameliorating damaged skin barrier function through the recovery of FLG expression in keratinocytes. Given the critical roles of the JAK-STAT pathway in mediating inflammatory responses, agerarin may provide therapeutic benefits as an adjuvant to conventional chemotherapies against various skin inflammatory diseases such as AD and psoriasis. As JAK kinases are activated by multiple cytokines, we cannot rule out the possibility that agerarin does not limit the IL4+IL13 and TNFα+IFNγ responses proposed in this study. Further studies will aim to identify additional molecular targets involved in skin inflammation that are downregulated by agerarin.

In conclusion, agerarin restores FLG expression by inhibiting the JAKs activated by IL4+IL13 and TNFα+IFNγ in keratinocytes and ameliorates DNCB-induced AD-like skin lesions. These findings support that agerarin may be beneficial for alleviating inflammatory skin disorders by improving skin barrier function. Th2-type cytokines trigger chronic itch, a typical feature of AD, by directly activating sensory neurons through neuronal JAK1 signaling [38]. Thus, beyond restoring skin barrier function, agerarin may also be beneficial for treating chronic itch and subsequently enabling further exploration and therapeutic applications for various inflammatory disorders.

Conflict of interest

The authors declare no conflict of interest.

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References


