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CONCISE COMMUNICATION

Ameliorating effect of dipotassium glycyrrhizinate on an IL-4- and IL-13-induced atopic dermatitis-like skin-equivalent model

Sung Hoon Lee[1,4] · Il-Hong Bae[1,2] · Hyangtae Choi[1] · Hyeong Won Choi[1] · Soojung Oh[1] · Paulo A. Marinho[1] · Dae Jin Min[1] · Dae-Yong Kim[2] · Tae Ryong Lee[1] · Chang Seok Lee[3] · Jongsung Lee[4]

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Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is not fully understood. Defects in skin barrier function and dysregulation of the Th2 immune response are thought to be pivotal in AD pathogenesis. In this study, we used keratinocytes and AD-like skin equivalent models using Th2 cytokines IL-4 and IL-13. The keratinocytes and AD-like skin model were used to investigate the effect of dipotassium glycyrrhizinate (KG), which is widely used as an anti-inflammatory agent for AD treatment. KG decreased AD-related gene expression in keratinocytes stimulated with Th2 cytokines. KG alleviated AD-like phenotypes and gene expression patterns and inhibited release of AD-related cytokines in the AD-like skin equivalent models. These findings indicate KG has potential effectiveness in AD treatment and AD-like skin equivalent models may be useful for understanding AD pathogenesis.

Keywords Atopic dermatitis · Dipotassium glycyrrhizinate · Skin equivalent

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disorder associated with Th2 cytokines [1]. However, the cause and mechanism of AD have not yet been fully elucidated. A three-dimensional human skin equivalent (SE) model has been used for biological studies to overcome of the limitations of AD animal models [2, 3]. For example, AD-like SE models with filaggrin mutations have been developed [4–7]. A method of inducing AD-like phenotypes by treating with *S. aureus* has been reported [8].

Acute AD is characterized by overexpression of Th2 cytokines such as IL-4 and IL-13 [9–11]. IL-4 and IL-13, representative Th2 cytokines, are key molecules for generating AD-like SE models [3, 12–14]. Kamsteeg et al. described a SE model with morphologic and molecular characteristics of AD obtained through IL-4 and IL-13 treatment [3]. Stimulation of SEs with these Th2 cytokines increased the expression of genes associated with AD such as neural epidermal growth factor-like 2 (NELL2) and carbonic anhydrase 2 (CA2). Histological analysis of SEs demonstrated that IL-4 and IL-13 induced spongiosis-like intercellular spaces between the cells, which is a hallmark of lesional AD skin.

Licorice (*Glycyrrhiza glabra*) is a traditional soothing herb that grows in several regions of the world. Licorice has antitumor, antimicrobial, antiviral, anti-inflammatory, and immunoregulatory properties [15]. The topical application of licorice extract shows protective effects in patients with AD [16]. Glycyrrhetinic acid and glycyrrhizic acid are specific compounds isolated from licorice. Dipotassium glycyrrhizinate (KG) is the dipotassium salt of glycyrrhizic acid, which is a widely used anti-inflammatory agent [15, 17–20]. KG is chemically stable, has high solubility, and is used as

✉ Chang Seok Lee
cslee2010@eulji.ac.kr

✉ Jongsung Lee
bioneer@skku.edu

1 Amorepacific Corporation R&D Center, Yongin-si, Republic of Korea

2 Department of Veterinary Pathology, College of Veterinary Medicine, Seoul National University, Gwanak-ro, Gwanak-gu, Seoul, Republic of Korea

3 Department of Beauty and Cosmetic Science, Eulji University, Sanseong-daero, Sugeong-gu, Seongnam-si, Gyeonggi-do 13135, Republic of Korea

4 Department of Integrative Biotechnology, College of Biotechnology and Bioengineering, Sungkyunkwan University, Suwon, Gyunggi-do 16419, Republic of Korea

a raw material for cosmetics because it has no reported side effects, even with continuous use [21].

In this study, to evaluate the anti-inflammatory efficacy of KG, we used IL-4 and IL-13-induced keratinocytes and AD-like SE models. KG decreased AD-related gene expression in keratinocytes stimulated with IL-4 and IL-13. We also demonstrated that KG alleviated AD-related structure and gene expression in AD-like SE models induced with IL-4 and IL-13. KG also inhibited the release of AD-related cytokines. Collectively, these data suggest that KG has potential effectiveness in AD treatment and AD-like SE models may be useful for understanding AD pathogenesis.

Materials and methods

Materials and treatment

KG was from Maruzen Pharmaceuticals Co., Ltd. (Hiroshima, Japan). KG was co-treated with IL-4 and IL-13 for 4 days in keratinocytes. KG was treated with IL-4 and IL-13 for 4 days after 10 days of air–liquid interface culture in SE.

Cell culture

Human neonatal epidermal keratinocytes were from Cascade Biologics (Portland, OR, USA). Keratinocytes were maintained in EpiLife medium (Cascade Biologics) supplemented with human keratinocyte growth supplement (Cascade Biologics) and penicillin–streptomycin 100× (Cascade Biologics). Keratinocytes were serially passaged at 70–80% confluence, and experiments were carried out using subconfluent cells at passage two, at which time they were proliferating actively. Normal human dermal fibroblasts, neonatal (NHDFn) were purchased from Cascade Biologics (Portland, OR, USA). Cells were incubated at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

Cell cytotoxicity assay

The viability of cultured keratinocytes was analyzed using a CCK-8 (Cell Counting Kit-8), as described by the manufacturer (CK04-05, Dojindo, Japan).

Production of skin equivalents

SEs were prepared as previously described [22]. The dermal layer was made by mixing type I collagen (Advanced biomatrix, San Diego, CA, USA) with human dermal fibroblasts (6.0×10^4 cells/well) in media cocktail [DMEM, F12, $NaHCO_3$ and NaOH]. The mixture was added to each insert of 12 mm Snapwell cell culture inserts (Corning Costar, NY, USA) and incubated for 2 h at 37 °C to allow polymerization. The dermal layer was then cultured in 106 media (Cascade Biologics) supplemented with 100 µg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MA, USA) and allowed to contract for 7 days at 37 °C and 5% CO_2. Human epidermal neonatal keratinocytes (2.0×10^5 cells/well) were seeded on the dermal layer. SEs were cultured for 1 day in EpiLife media and 1 day in 3D culture media, CnT-3D-PR (CELLnTEC, Bern, Switzerland), after which the SEs were fed strictly from the bottom in 3D culture media with surfaces exposed to air for 10 days to promote epidermal differentiation. SEs were fed 3D media with or without IL-4 and IL-13 (R&D Systems, MN, USA) for 4 days. For analysis, SEs were embedded in OCT compound for hematoxylin and eosin staining and immunohistochemical analysis. Epidermis was separated using forceps for analysis of mRNA expression by RT-PCR.

Analysis of mRNA expression by RT-PCR

To determine relative mRNA expression of selected genes, total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and 4 µg RNA was reverse-transcribed into cDNA using SuperScriptIII reverse transcriptase (Invitrogen). Aliquots were kept at − 20 °C. Quantitative PCR was performed using an ABI 7500 Fast Real-Time PCR System with commercially available TaqMan site-specific primers and probes (Applied Biosystems, Foster City, CA, USA). The cDNA samples were analyzed for NELL2 (Hs00196254_m1), CA2 (Hs01070108_m1), Has3 (Hs00193436_m1), filaggrin (Hs00856927_g1), AQP3 (Hs00185020_m1), keratin 1 (Hs00196158_m1), keratin 10 (Hs00166289_m1), IL-6 (Hs00174131_m1), and IL-8 (Hs00174103_m1). The results were normalized to RPL13A (Hs04194366_g1) level.

Western blots

SEs were lysed with RIPA lysis buffer (Millipore-Sigma, Billerica, MA, USA) containing protease inhibitors (Millipore-Sigma). Protein concentration was determined by BCA assay, and cell lysates were resolved by SDS-PAGE on 4–12% gradient Bis-Tris gels (Thermo Fisher Scientific, Rockford, IL, USA), transferred to nitrocellulose membranes (Thermo Fisher Scientific), and probed with antibodies against filaggrin (NCL-FILAGGRIN, Novocastra, Germany), AQP3 (ab125219, Abcam, Cambridge, MA, USA), GAPDH (14C10, Cell Signaling, Danvers, MA, USA), and horseradish peroxidase-conjugated secondary anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA). GAPDH was used to normalize the protein expression. Western

blotting ECL reagent (GE Healthcare, Piscataway, USA) was used to develop the signals.

Cytokine arrays

Cytokines released from skin equivalents were analyzed using a Human Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA, USA) according to the manufacturer's protocol. Samples were measured using a Luminex 200 dual laser apparatus (Luminex Corp., Austin, TX, USA) with MILLIPLEX Analysis software (Millipore, Billerica, MA, USA).

Statistical analysis

Data are expressed as mean ± standard deviation (SD), and statistical significance was determined by one-way analysis of variance. A p value < 0.05 was considered statistically significant.

Results

IL-4/IL-13-induced atopic dermatitis-like phenotypes and expression of AD-related genes

IL-4 and IL-13 are Th2 cytokines that are generally used to induce AD in SE models [2, 3, 9, 10, 12–14]. SEs were stimulated on the last 4 days of air–liquid interface culture with 15 or 30 ng/mL IL-4 and IL-13 to produce AD-like phenotypes with modified Kamsteeg et al. methods [3]. We examined the morphology of SEs supplemented with these cytokines. Treatment with IL-4/IL-13 caused spongiosis-like intercellular spaces between cells (Fig. 1a). Epidermal differentiation-protein expression was investigated in IL-4/IL-13-induced SEs. Filaggrin was expressed in the stratum granulosum (SG) and the interface between the SG and the stratum corneum (SC) in control SE. IL-4/IL-13-induced AD-like SEs showed reduced filaggrin staining than control. IL-4/IL-13-induced SEs showed reduced expression of keratin 1 and keratin 10 proteins. A study showed

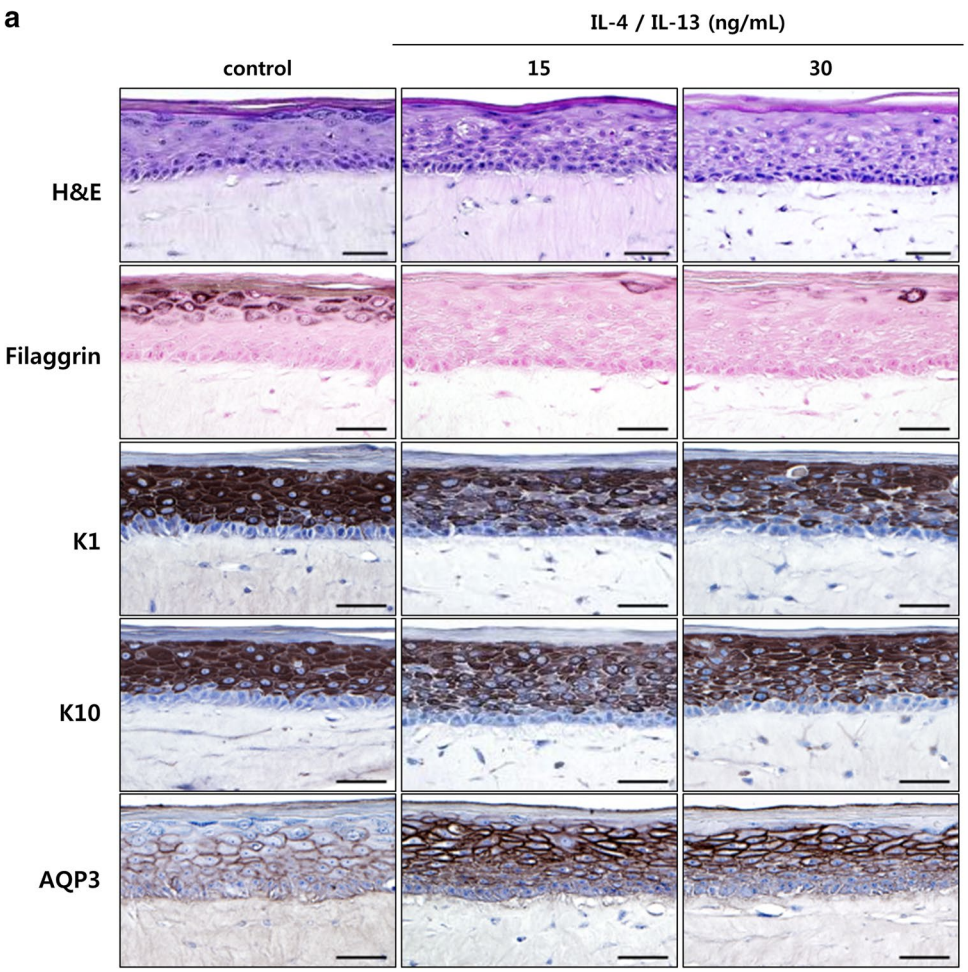

Fig. 1 IL-4/IL-13-induced atopic dermatitis-like phenotypes and expression of AD-related genes in skin equivalents. IL-4 and IL-13-induced atopic dermatitis-like phenotypes and expression of AD-related genes in skin equivalents. Skin equivalents (SEs) were treated on the last 4 days of the air–liquid interface culture with IL-4 (15 ng/mL) and IL-13 (15 ng/mL) or IL-4 (30 ng/mL) and IL-13 (30 ng/mL). **a** Histological section of SEs. Scale bar 50 μm. **b** Results of AD-related gene expression. ($n = 3$). **c** Western blot analysis of filaggrin in SEs. (*$p < 0.05$, **$p < 0.01$)

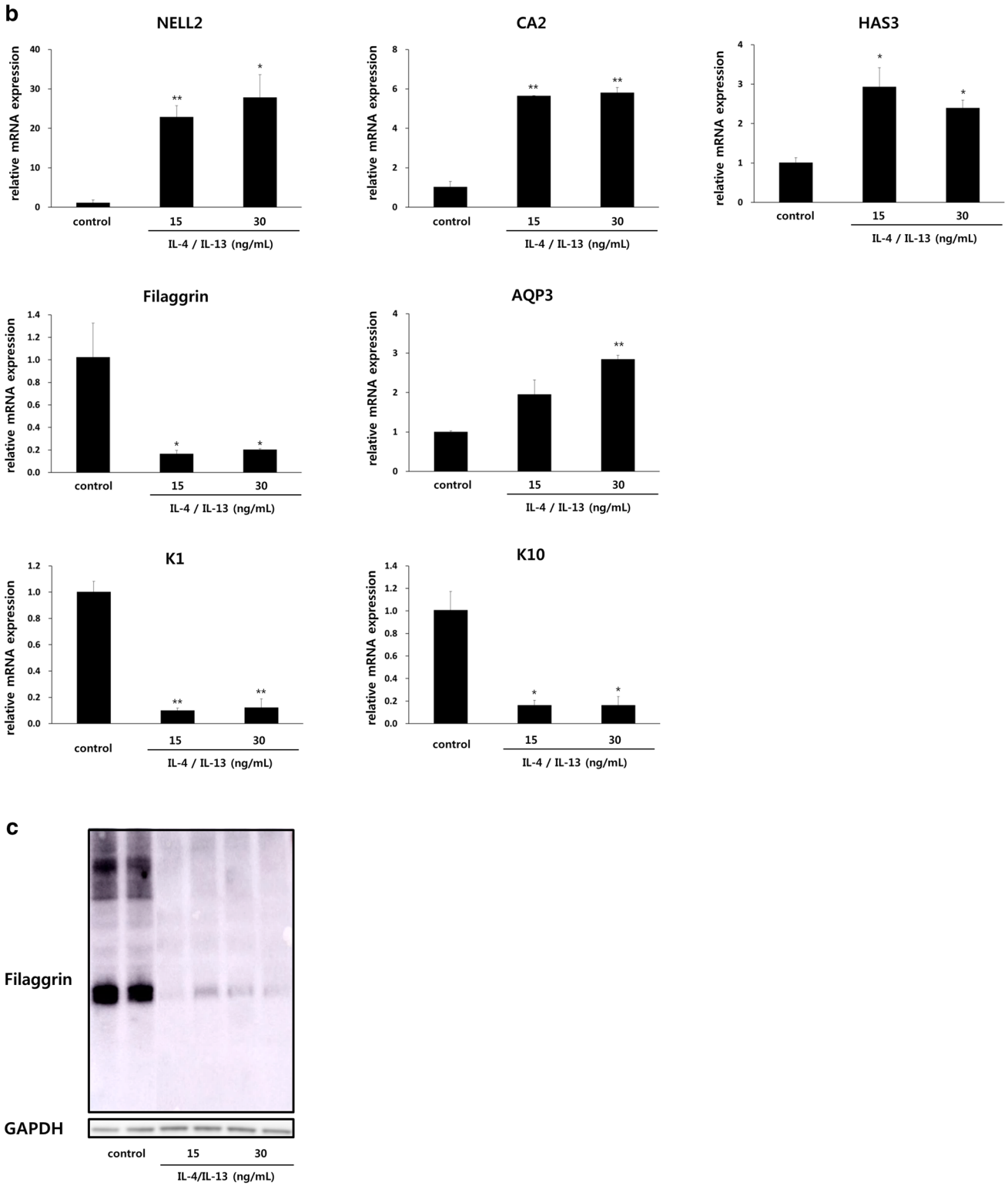

Fig. 1 (continued)

that mRNA and protein levels of AQP3 increase in AD patients, and AQP3 is involved in keratinocyte proliferation and epidermal hyperplasia [23]. Thus, we examined expression of AQP3 in our model. IL-4/IL-13-induced SEs showed increased AQP3 protein compared with control SEs (Fig. 1a).

We investigated the effect of IL-4/IL-13 treatment on expression of AD-related genes in SEs using real-time quantitative PCR (Fig. 1b). Stimulation with IL-4 and IL-13 significantly increased mRNA for NELL2 and CA2 (Fig. 1b). In addition, hyaluronan synthase 3 (HAS3) is highly expressed in the middle of the spongiotic epidermis [2, 13, 24, 25]. We found that IL-4/IL-13 increased mRNA for HAS3 (Fig. 1b). Similar to staining data in Fig. 1a, reduced expression of filaggrin, keratin 1, and keratin 10 genes were confirmed. We investigated filaggrin protein levels using western blot. IL-4/IL-13 reduced filaggrin protein level in our SE model (Fig. 1c). The results indicate that IL-4 and IL-13 contributed to changes in epidermal structure and expression of AD-related genes.

Dipotassium glycyrrhizinate regulated expression of AD-related genes in IL-4/IL-13-stimulated human epidermal keratinocytes

KG is a widely used anti-inflammatory agent extracted from licorice root (Fig. 2a) [15, 17–20]. To evaluate the effect of KG in AD, we treated keratinocytes with IL-4 (15 ng/mL), IL-13 (15 ng/mL) and KG for 4 days. No cytotoxicity was observed for KG up to 400 μM in keratinocytes (Fig. 2b). We examined expression of NELL2, CA2, AQP3, and HAS3 genes in IL-4/IL-13-stimulated keratinocytes. KG treatment significantly reduced mRNA from NELL2, CA2, AQP3, and HAS3 genes (Fig. 2c).

Dipotassium glycyrrhizinate repaired abnormal phenotypes in the IL-4- and IL-13-induced atopic dermatitis model

To evaluate the effect of KG on AD-like SE models, KG was co-treated with IL-4 and IL-13 for the last 4 days of air–liquid interface culture. KG treatment blocked the spongiosis-like intercellular spaces in AD-like SE (Fig. 3a). KG restored the reduced expression of filaggrin in a dose-dependent manner. Oppositely, IL-4/IL-13 treatment increased the expression of AQP3 in AD-like SEs and KG reduced the AQP3 protein expression in a dose-dependent manner (Fig. 3a). To examine if KG regulated expression of AD-related genes in AD-like SEs, SE epidermis was isolated, and mRNA expression was analyzed. In the AD-like SEs, expression level of the filaggrin gene was restored in a dose-dependent manner with KG treatment (Fig. 3b). Expression of the AQP3 gene decreased at more than 200 μM KG (Fig. 3b). The mRNA levels of pro-inflammatory cytokines such as IL-6 and IL-8, which increased in AD-like SEs, were reduced in response to KG treatment (Fig. 3b). Western blot showed that while IL-4/IL-13 treatment decreased filaggrin protein level, increased AQP3 protein level, KG restored the effects of IL-4/IL-13 treatment on filaggrin and AQP3 protein levels (Fig. 3c).

Expression of other AD-related genes including GM-CSF, MCP-3, IL-1α, IL-6, IL-8, RANTES, and TNFα increases in atopic lesions [26–29]. Therefore, we evaluated if KG treatment suppressed production of cytokines using cytokine array. IL-4/IL-13 treatment increased expression levels of the tested cytokines (Fig. 3d). KG significantly reduced production of all cytokines. These results indicate that KG has an alleviating effect on AD-like SEs through regulation of AD-related mRNA, proteins and pro-inflammatory cytokines.

Discussion and conclusion

SE models have been developed and used to study skin physiology for more than 30 years [30, 31]. Although SE models have limitations [32, 33], many groups have tried making biologically relevant skin models using genetically modified cells [6, 7] and with the help of organ-on-a-chip systems [34, 35]. SEs have also been used to study skin dysfunction caused by inflammatory diseases such as AD [2, 36, 37] and psoriasis [38, 39]. The most common method for studying AD is to treat Th2 cytokines in SEs. Th2 cytokines induce AD-like features in SEs [3, 12–14]. Most AD-like models are made of reconstructed human epidermis [12–14] or keratinocytes cultured on de-epidermized dermis [3]. To understand potential cross talk between fibroblasts and keratinocytes in AD pathology, SE models have generated [40]. Berroth et al. reported the functions of fibroblasts in skin proliferation and differentiation in AD patients using the fibroblasts derived from perilesional atopic skin and healthy keratinocytes as well as healthy fibroblasts and atopic keratinocytes, suggesting the involvement of fibroblasts in AD physiology.

Licorice has anti-inflammatory and immunoregulatory properties [15]. Topical application of licorice extract has ameliorating effects in AD patients [16]. KG isolated from licorice is reported to improve the skin of AD patients. However, results on the efficacy of KG using AD-like SEs have not been reported. We examined the effect of KG in AD-like SE developed by our group. KG ameliorated AD-like features in both epidermal keratinocytes and AD-like SEs. Specifically, KG regulated expression of AD-related genes in both IL-4/IL-13-stimulated-epidermal keratinocytes and SEs. In addition, KG partially repaired AD-like structures and reduced the production of pro-inflammatory cytokines in AD-like SEs. The similar results in epidermal keratinocytes to those in AD-like SEs suggest that our developed AD-like SE model may be applied to AD studies.

Several studies have shown that glycyrrhizin (the main compound in licorice roots) inhibited inflammation by suppressing expression of high mobility group box-1 (HMGB1), a DNA-binding protein that is released from

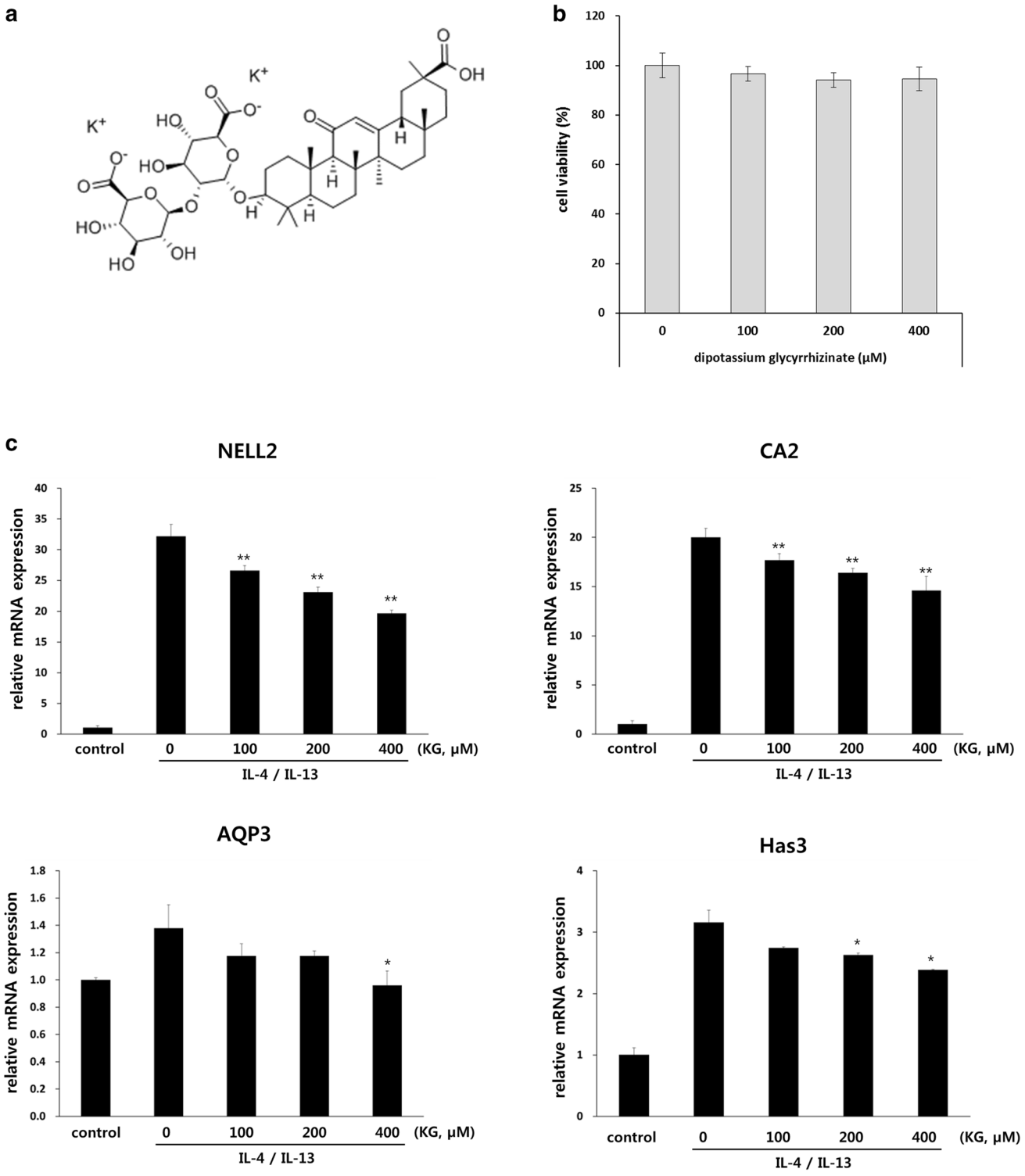

Fig. 2 Dipotassium glycyrrhizinate (KG) regulated expression of AD-related genes in IL-4/IL-13-stimulated human epidermal keratinocytes. Keratinocytes were treated with IL-4 (15 ng/mL) and IL-13 (15 ng/mL) for 72 h. **a** Structure of dipotassium glycyrrhizinate. **b** Effects of dipotassium glycyrrhizinate on the viability of human epidermal keratinocytes. **c** qPCR analysis of AD-related genes ($n=3$) (*$p<0.05$, **$p<0.01$)

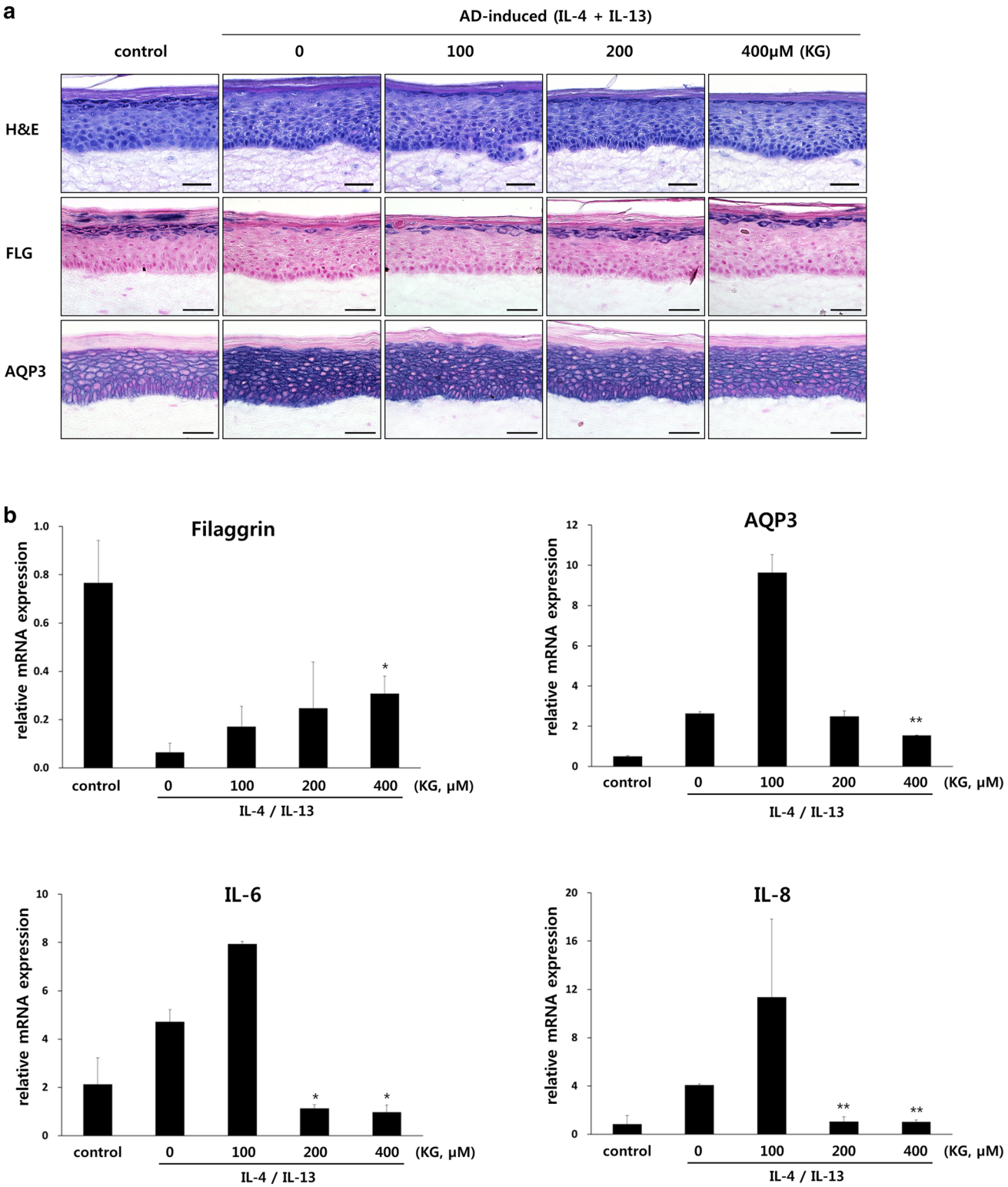

Fig. 3 Dipotassium glycyrrhizinate partially repaired the AD-like phenotype in the IL-4/IL-13-induced AD-like skin equivalent model. Skin equivalents (SEs) were treated on the last 4 days of the air–liquid interface culture with IL-4 (15 ng/mL), IL-13 (15 ng/mL) and different concentrations of KG. **a** Histological section of SEs. Scale bar 50 μm. **b** qPCR analysis of AD-related genes ($n=3$). **c** Western blot of filaggrin and AQP3 and **d** the cytokine array (*$p<0.05$, **$p<0.01$)

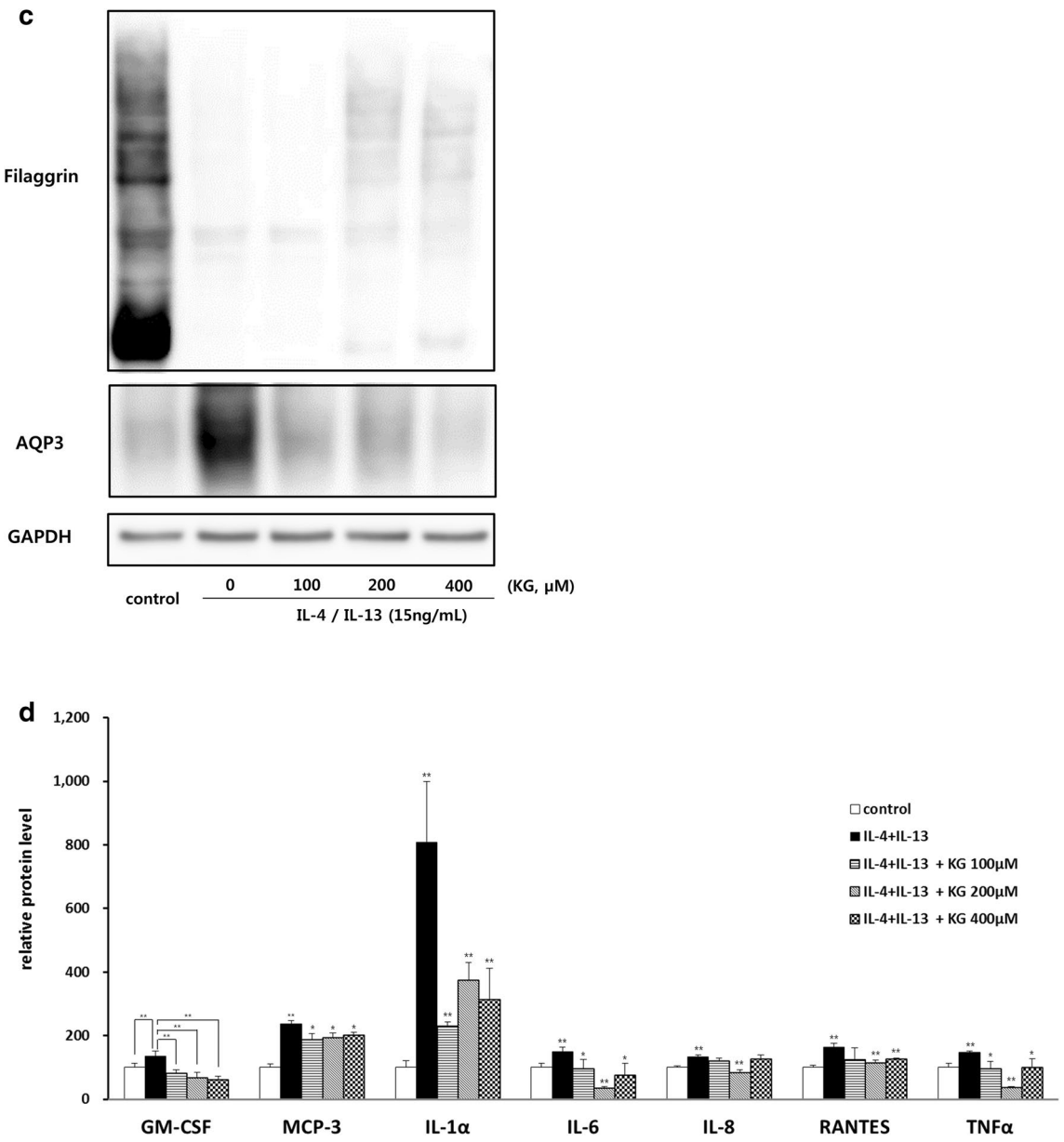

Fig. 3 (continued)

damaged cells during inflammation [41–43]. KG also inhibited HMGB1-dependent inflammation [42]. In addition, Matsui et al. reported that glycyrrhizin derivatives reduce production of eotaxin 1, which is highly expressed in AD lesions [44]. Eotaxin 1 is a typical eosinophil chemoattractant that is important in Th2-type diseases such as AD [45, 46]. Therefore, it is worthy to speculate relation of KG on the regulation of HMGB1 and eotaxin 1 in the aspect of anti-inflammatory effects of KG on IL-4/IL-13-induced AD-like SEs.

In conclusion, our results suggest that KG has potential effectiveness in AD treatment and AD-like SE models may be useful for understanding AD pathogenesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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