Possible new therapeutic strategy to regulate atopic dermatitis through upregulating filaggrin expression

Atsushi Otsuka, MD, PhD,[a,b] Hiromi Doi, MS,[a] Gyohei Egawa, MD, PhD,[a] Akiko Maekawa, PhD,[c] Tomoko Fujita, MD, PhD,[c] Satoshi Nakamizo, MD,[a] Chisa Nakashima, MD,[a] Saeko Nakajima, MD, PhD,[a] Takeshi Watanabe, MD, PhD,[b] Yoshiki Miyachi, MD, PhD,[a] Shuh Narumiya, MD, PhD,[c] and Kenji Kabashima, MD, PhD[a] *Kyoto, Japan*

Background: Nonsense mutations in filaggrin *(FLG)* represent a significant genetic factor in the cause of atopic dermatitis (AD).
Objective: It is of great importance to find drug candidates that upregulate FLG expression and to determine whether increased FLG expression controls the development of AD.
Methods: We screened a library of bioactives by using an *FLG* reporter assay to find candidates that promoted *FLG* mRNA expression using a human immortalized keratinocyte cell line (HaCaT). We studied the effect of the compound on keratinocytes using the human skin equivalent model. We examined the effect of the compound on AD-like skin inflammation in NC/Nga mice.
Results: JTC801 promoted *FLG* mRNA and protein expression in both HaCaT and normal human epidermal keratinocytes. Intriguingly, JTC801 promoted the mRNA and protein expression levels of FLG but not the mRNA levels of other makers for keratinocyte differentiation, including loricrin, keratin 10, and transglutaminase 1, in a human skin equivalent model. In addition, oral administration of JTC801 promoted the protein level of Flg and suppressed the development of AD-like skin inflammation in NC/Nga mice.
Conclusion: This is the first observation that the compound, which increased FLG expression in human and murine keratinocytes, attenuated the development of AD-like skin inflammation in mice. Our findings provide evidence that modulation of FLG expression can be a novel therapeutic target for AD. (J Allergy Clin Immunol 2014;133:139-46.)

Key words: *Atopic dermatitis, filaggrin, JTC801, keratinocyte differentiation*

Abbreviations used
AD: Atopic dermatitis
AP-1: Activator protein 1
BMDC: Bone marrow–derived dendritic cell
C_T: Cycle threshold
FLG: Filaggrin
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
K10: Keratin 10
NHEK: Normal human epidermal keratinocyte
ORL1: Opioid receptor-like 1
SC: Stratum corneum
TCR: T-cell receptor
TEWL: Transepidermal water loss
TGM1: Transglutaminase 1

The main function of the skin is to form a protective barrier against external stimuli, such as irritants and allergens. In the epidermis terminal differentiation of keratinocytes leads to the formation of the stratum corneum (SC), an impenetrable physical barrier consisting of flat anucleated corneocytes imbedded in an intercellular matrix rich in nonpolar lipids.[1] The cross-linking of filaggrin (FLG) monomers derived from profilaggrin produced in the keratinocytes with keratin filaments induces an aggregation of the keratin filaments into tight bundles.[2] In the upper layers of the SC, FLG monomers are further processed to hydroscopic amino acids and their derivatives by proteases[3] called natural moisturizing factors, which play a major role in SC hydration.[4]

Barrier disruption and continuous percutaneous exposure to allergens presumably initiate and drive the development of atopic dermatitis (AD).[5-7] AD is a common skin condition characterized by a complex and heterogeneous pathogenesis.[6-9] Direct evidence of a link between the incidence of AD and nonsense mutations in the gene encoding *FLG* has been discovered.[5] *FLG*-null mutations are seen in approximately 20% to 30% of patients with AD.[5,10-12] Additionally, irrespective of mutations in *FLG*, FLG expression is downregulated in almost all cases of moderate-to-severe AD.[13] This finding suggests that barrier dysfunction is a primary cause of AD.

We and others have previously demonstrated that flaky tail mice, namely those deficient in the *Flg* gene, elicited a severe contact hypersensitivity reaction compared with wild-type mice and exhibited spontaneous AD-like skin lesions.[14-18] Consistently, mice with mutations in *Flg* allowed the penetration of both haptens and protein antigens through the SC, which resulted in exaggerated immune responses.[19] Moreover, a large-scale study proved that intragenic copy number variation within *FLG*, with alleles encoding 10, 11, or 12 repeats of FLG monomers, contributes to the risk of AD.[20] Moreover, a recent highlight in the allergy field is that the mutations in the *FLG* gene are associated with not only skin but also other allergic

From [a]the Department of Dermatology, [b]the Center for Innovation in Immunoregulative Technology and Therapeutics, and [c]the Department of Pharmacology, Kyoto University Graduate School of Medicine.
Supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor, and Welfare of Japan and a clinical research grant from the AK project at Kyoto University.
Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.
Received for publication February 21, 2013; revised July 11, 2013; accepted for publication July 19, 2013.
Available online September 20, 2013.
Corresponding author: Kenji Kabashima, MD, PhD, Department of Dermatology, Kyoto University Graduate School of Medicine, 54 Shogoin Kawara, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: kaba@kuhp.kyoto-u.ac.jp.
0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2013.07.027

diseases. The biology of this molecule in its altered function offers new insights into a range of conditions not previously thought to be related.[21] Therefore a therapeutic strategy through modulation of FLG expression is promising not only to control AD but also to prevent the development of other allergic diseases.

In this study we screened a library of bioactives using an FLG reporter assay and found some candidates that promoted *FLG* mRNA expression in a human immortalized keratinocyte cell line (HaCaT). Among the candidates, JTC801 promoted *FLG* expression with normal human epidermal keratinocytes (NHEKs) independent of opioid receptor-like 1 (ORL1) receptor signaling. The human skin equivalent model revealed that JTC801 promotes the expression level of FLG but has no marginal affect on other differentiation markers of keratinocytes. Furthermore, oral administration of JTC801 promoted the protein level of Flg and suppressed the development of AD-like skin inflammation in NC/Nga mice.

METHODS

Cell culture and reagents

Second-passage neonatal foreskin NHEKs were purchased from Kurabo Industries (Osaka, Japan) and cultured in the serum-free keratinocyte growth medium HuMedia-KG2 (Kurabo Industries) containing human epidermal growth factor (0.1 ng/mL), insulin (10 μg/mL), hydrocortisone (0.5 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 ng/mL), and bovine brain pituitary extract (0.4% vol/vol) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The Ca^{2+} concentration in HuMedia-KG2 was 0.15 mmol/L. Cells were passaged at 60% to 70% confluence to avoid differentiation, and the experiments were conducted with subconfluent cells at passage 3 or 4 in the proliferative phase at 60% to 80% confluence. Induction of keratinocyte differentiation was achieved by culturing the keratinocytes for 48 hours in a culture medium containing 1.35 mmol/L Ca^{2+}. Keratinocyte differentiation was confirmed based on morphologic changes determined by using microscopy. The wild-type and activator protein 1 (AP-1)–deleted profilaggrin constructs were generated as in previous reports.[22] NHEKs were transfected with pGL4.17 plasmid by using TransIT-LT1 reagent (Panvera, Madison, Wis), according to the manufacturer's protocol.

JTC801 was purchased from Tocris Bioscience (Bristol, United Kingdom). Recombinant human IL-4 and IL-13 were purchased from R&D Systems (Minneapolis, Minn).

Mice

C57BL/6NCrSlc (B6) mice were purchased from SLC (Shizuoka, Japan). Pure flaky tail mice were backcrossed to B6 mice for 10 generations to have the single homozygous Flg mutation from flaky tail mice. Female mice were used in all experiments, unless otherwise stated. They were maintained on a 12-hour light/dark cycle at a temperature of 24°C and a humidity of 50% ± 10% under specific pathogen-free conditions at Kyoto University Graduate School of Medicine. NC/Nga mice were purchased from SLC, maintained under conventional conditions, and orally administrated JTC801 (30 mg/kg in 0.5% methyl cellulose) or vehicle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Human skin equivalent model

A 3-dimensional human dermal model kit (TESTSKIN LSE-high; Toyobo, Osaka, Japan) derived from human normal keratinocytes and fibroblasts was cultured in the presence of JTC801 for 5 days, according to the manufacturer's protocol.

Immunohistochemistry

Deparaffinized sections were immersed in 0.3% H_2O_2 to abolish endogenous peroxidase activity and incubated with a monoclonal mouse anti-human FLG (clone, AKH-1; Santa Cruz Biotechnology, Dallas, Tex) overnight. The secondary antibody and 3,39-diaminobenzidine staining were added according to the manufacturer's protocol (R&D Systems). The skin sections were counterstained with hematoxylin.

Transepidermal water loss and SC conductance

Measurements of transepidermal water loss (TEWL) and SC conductance were performed at room temperature (22°C-26°C) and 40% to 60% humidity. TEWL was measured on the lesional skin by using a Vapo Scan AS-VT100RS machine (Asahi Biomed, Tokyo, Japan). SC hydration was evaluated by analyzing skin electrical impedance with a Corneometer SKICON-200 (IBS, Hamamatsu, Japan). All data are presented as the median of 3 repeated recordings.

Quantitative PCR analysis

Quantitative PCR analysis was performed, as reported previously.[23] Briefly, total RNAs were isolated from NHEKs with Trizol (Life Technologies, Gaithersburg, Md). cDNA was reverse transcribed with a PrimeScript RT reagent kit (Takara Bio, Otsu, Japan). Quantitative RT-PCR with a Light Cycler real-time PCR apparatus was performed (Roche Diagnostics, Foster City, Calif) by using SYBR Green I (Roche, Basel, Switzerland). The primer sequences are described in the Methods section in this article's Online Repository at www.jacionline.org. Expression of mRNA (relative) was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* mRNA by the change in the Δ cycle threshold (ΔC_T) method and calculated based on $2^{-\Delta CT}$.

Western blotting

Proteins were obtained from ear skin, as reported previously.[24] These samples were used for immunoblotting with polyclonal antibodies against FLG, loricrin, and keratin 10 (K10; COVANCE, Berkeley, Calif). Horseradish peroxidase−conjugated secondary anti-mouse IgG antibody (GE Healthcare, Piscataway, NJ) was used for detection with ECL (GE Healthcare).

Clinical observation

The clinical severity of skin lesions was scored according to the macroscopic diagnostic criteria that were used for the NC/Nga mouse.[25] In brief, the total clinical score for skin lesions was designated as the sum of individual scores of the right ear, left ear, dorsum, and face graded as 0 (none), 1 (mild), and 2 (severe) for the symptoms of erythema/hemorrhage, edema, crust, excoriation/erosion, and scaling/dryness.

Statistical analysis

Unless otherwise indicated, data are presented as means ± SDs and are representative of 3 independent experiments. *P* values were calculated with the Wilcoxon signed-rank test. *P* values of less than .05 are considered significantly different.

RESULTS

JTC801 promotes the transcription level of profilaggrin

To explore candidate compounds that promote the *FLG* mRNA, we screened the 1120-compound library of bioactives (Tocriscreen Mini) using HaCaT cells by means of real-time PCR.[26] This screening revealed that several compounds, such as a histone deacetylase inhibitor (Scriptaid, Tocris Bioscience), actinomycin D, and JTC801, potently promoted the *FLG* mRNA expression (see Table E1 in this article's Online Repository at www.jacionline.org). Because HaCaT is an immortalized keratinocyte cell line, we further examined whether these compounds increase *FLG* mRNA expression with NHEKs