Defects of filaggrin-like proteins in both lesional and nonlesional atopic skin

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Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by a disturbed epidermal barrier. In a subset of patients, this is explained by nonsense mutations in the gene encoding filaggrin (FLG).

Objectives: We sought to evaluate the respective role of *FLG* mutations and proinflammatory cytokines and to assess the expression of FLG, hornerin (HRNR), and FLG2, 2 FLG-like proteins, which are involved in epidermal barrier functions, in normal skin and both lesional and nonlesional skin of patients with AD.

Methods: An *FLG*-genotyped cohort of 73 adults with AD and 73 aged-matched control subjects was analyzed by using immunohistochemistry and immunoblotting. Normal primary human keratinocytes were differentiated in either the absence or presence of IL-4, IL-13, and IL-25.

Results: Compared with control subjects, FLG, HRNR, and FLG2 were detected at significantly lower levels in the skin of patients with AD, irrespective of their *FLG* genotype. The reduction was greater in lesional compared with nonlesional skin. In addition, the proFLG/FLG ratio was found to be higher in the skin of wild-type patients than in control subjects. Cytokine treatment of keratinocytes induced a dramatic reduction in FLG, FLG2, and HRNR expression both at the mRNA and protein levels.

Conclusion: The stratum corneum of lesional but also clinically unaffected skin of adults with AD is abnormal, with reduced expression of FLG and FLG-like proteins. In addition to nonsense mutations, proinflammatory cytokines and some defects in the proFLG processing can contribute to the FLG downregulation. Our study suggests that skin inflammation reduces the expression of FLG-like proteins, contributing to the AD-related epidermal barrier dysfunction. (J Allergy Clin Immunol 2013;131:1094-102.)

Key words: *Atopic dermatitis, skin, keratinocytes, filaggrin, hornerin, stratum corneum, skin barrier, cytokine*

Atopic dermatitis (AD; OMIM #603165) is one of the most common chronic inflammatory skin diseases. It usually begins in early childhood and affects up to 20% of children and 3% of adults in industrialized countries. AD is characterized by erythematous skin lesions, pruritis, altered epidermal barrier, and marked mononuclear cell infiltrate in the dermis.[1] AD results from complex interactions between genetic and environmental factors. Two nonexclusive pathophysiologic models have been proposed and remain debated. Historically, it was thought that the primary defect resides in the immune system, leading to excessive inflammation and a secondary local epidermal barrier disruption (the inside-outside theory).[2] Loss-of-function mutations in the gene encoding filaggrin (FLG) are the strongest and most widely replicated risk factor for the disease (see Palmer et al,[3] Brown and McLean,[4] and the references cited therein), suggesting an alternative view of AD pathophysiology because FLG is an essential component of the stratum corneum.[5] A primary intrinsic alteration of the upper epidermis allows the entrance of pathogens and allergens and induces a subsequent immune response (the outside-inside theory).[1,6-9]

FLG is synthesized by granular keratinocytes as a large precursor called proFLG. ProFLG consists of a large repetitive central domain flanked by 2 unique N- and C-terminal domains. During the late steps of terminal differentiation, proFLG is cleaved. The generated basic FLG monomers aggregate the keratin cytoskeleton to form the corneocyte fibrous matrix. In the upper stratum corneum, FLG is completely proteolyzed into free amino acids that are essential for skin photoprotection and for acidification and hydration of the stratum corneum.[10-12] In turn, FLG deficiency has

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Abbreviations used
AD: Atopic dermatitis
FLG: Filaggrin
HRNR: Hornerin
OR: Odds ratio
qPCR: Quantitative PCR
TEWL: Transepidermal water loss

been associated with many clinical features of AD skin: a decrease in levels of stratum corneum free amino acids[13,14]; an increase in transepidermal water loss (TEWL), stratum corneum pH, and dryness[15,16]; and abnormal bacterial colonization.[17]

However, *FLG* loss-of-function mutations explain the AD-associated stratum corneum abnormalities in a maximum of one in 3 northern European patients,[18] and a very low frequency of *FLG* null alleles has been observed in patients with AD from the southern European[19] and Ethiopian[20] populations. In addition, some patients with AD have increased TEWL and percutaneous penetration irrespective of their *FLG* genotype.[21] These observations imply that in addition to *FLG* loss-of-function mutations, other factors that modulate epidermal barrier functions are involved in the pathogenesis of AD. Several proinflammatory cytokines, including IL-4, IL-13, IL-22, and IL-25, can reduce the expression of FLG through transcriptional regulation.[22-25] Also, mutations in a tight junction protein and in some protease/protease inhibitors involved in desquamation have been tentatively associated with AD susceptibility.[26-28]

In this study we focused on 2 other S100-fused type proteins: hornerin (HRNR) and FLG2. These proteins share many properties with FLG: a closely related structural organization, including a large central repetitive domain; a similar amino acid composition; an identical pattern of expression in the epidermis; and an analogous proteolytic processing.[29-33] In addition, HRNR is a component of cornified cell envelopes.[29] Altogether, this suggests that HRNR and FLG2 abnormalities might well be involved in AD-associated epidermal barrier defects. Reinforcing this idea, an AD predisposition factor distinct from *FLG* was suggested to be present in the 1q21 chromosomal region in which the *HRNR* and *FLG2* genes are located.[34] We compared the expression of FLG, HRNR, and FLG2 in the skin of healthy volunteers and in lesional and nonlesional skin of a large cohort of patients with AD. In addition, we investigated the effect of proinflammatory cytokines on expression of the 3 proteins.

METHODS

Study subjects

Seventy-three unrelated adults with a history of mild-to-severe AD were recruited and clinically characterized by an experienced dermatologist. Disease severity was determined by using the SCORAD and Nottingham Eczema Severity Score evaluations (see Table E1 in this article's Online Repository at www.jacionline.org). Seventy-three unrelated sex- and age-matched healthy subjects with no personal or familial history of AD, ichthyosis, asthma, or allergic rhinitis were recruited. All patients underwent four 3-mm punch biopsies, 2 on lesional and 2 on nonlesional skin sites. Two biopsy specimens were obtained from the control subjects at the corresponding sites. Blood was taken from all subjects for genotyping. Genomic DNA was prepared from whole blood by using standard methods. *FLG* genotyping was performed in patients and control subjects for the 4 *FLG* mutations most prevalent in the European population[3,4,11]: R501X, 2282del4, S3247X, and R2447X (see Table E2 in this article's Online Repository at www.jacionline.org). For further details, see the Methods section in this article's Online Repository at www.jacionline.org.

All experiments were performed according to the principles of the Declaration of Helsinki. Participants provided written informed consent before inclusion. The study was authorized by the General Board of the French Ministry of Health (Direction Générale de la Santé, DGS2008-0259, August 2008) and approved by the Comité de protection des Personnes Sud-Ouest et Outre Mer I (Ethics Committee).

Histologic, immunohistologic, and Western blot analyses of skin samples

Five-micrometer cryosections of skin biopsy specimens were used for either hematoxylin and eosin staining or indirect immunofluorescence analysis with the AHF_3 anti-FLG mAb[35] and with the AHP_2 anti-HRNR[29] and AIP_2 anti-FLG2[31] affinity-purified rabbit antibodies. Total epidermal proteins were separated on 10% acrylamide gels and immunodetected with either AHF_3, AHP_2, or a polyclonal goat antibody directed against the FLG2 spacer[32] by using an ECL kit (Pierce/Thermo Scientific, Rockford, Ill). The National Institutes of Health ImageJ software (Bethesda, Md) was used to quantify immunoreactive bands on Western blot films after scanning. Signals were normalized for total protein concentration (for details, see the Methods section in this article's Online Repository).

Effects of inflammatory cytokines on primary normal keratinocyte

Primary human keratinocytes were obtained from abdominal dermolipectomy of healthy subjects who had provided informed consent. They were cultured in DermaLife medium (CellSystems, Troisdorf, Germany), supplemented as recommended by the manufacturer, until they reached confluence. Keratinocytes were then differentiated for 4 days in DermaLife medium supplemented with 1.3 mmol/L $CaCl_2$. Recombinant IL-4, IL-25, IL-22 (CellSystems), and IL-13 (R&D Systems, Minneapolis, Minn) were added at 100 ng/mL to the keratinocyte culture medium for the entire differentiation period to investigate the effect of cytokines. For immunofluorescence analysis, cells were fixed in 4% formaldehyde buffered solution, permeabilized, and incubated with the primary antibodies. Total proteins were extracted for Western blot analysis, as described above. The previously described primary antibodies and the anti-HRNR antibody HPA031469 (1:500; Sigma-Aldrich, St Louis, Mo) were used. Immunodetection intensities were normalized against actin immunoreactivity. For RT–quantitative PCR (qPCR), total RNA was extracted with the RNeasy Plus Minikit (Qiagen France, Courtaboeuf, France), according to the manufacturer's instructions. Reverse transcription was performed by using Improm-II Reverse Transcriptase (Promega, Madison, Wis) with a combination of oligo(dT) and random hexamers. qPCR amplification was performed with the 7300 Real Time PCR System (Applied Biosystems, Foster City, Calif) by using the Sybr qPCR SuperMix W/ROX (Invitrogen Life Technologies, Carlsbad, Calif). Relative levels of gene expression among samples were determined by using the $\Delta\Delta$ cycle threshold method. Hypoxanthine-guanine phosphoribosyltransferase gene expression was used for normalization. For details, see the Methods section in this article's Online Repository.

Statistical analyses

MedCalc software 12.2.1 (MedCalc Software, Mariakerke, Belgium) was used for statistical calculations. Statistical differences between groups were determined with the Mann-Whitney test, Bonferroni correction was used to account for multiple testing, and adjusted P values are indicated. We analyzed more precisely paired nonlesional and lesional skin samples using the Wilcoxon nonparametric test. The Kruskal-Wallis test was used to compare the proFLG/FLG ratio between control, nonlesional, and lesional skin samples. For the correlation analyses, the Spearman rank correlation test was used. Differences were considered significant when the P (or $P_{corrected}$) value was less than .05. A 3-dimensional graph was made with Statistica software (Statsoft, Tulsa, Okla) by using a distance-weighted least-squares smoothing procedure.