Rapid publication

Cytokine modulation of atopic dermatitis filaggrin skin expression

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Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease that is characterized by a defective skin barrier function. Recent studies have reported mutations of the skin barrier gene encoding filaggrin in a subset of patients with AD.
Objective: We investigated whether reduced filaggrin expression was found in patients with AD who were not carriers of known filaggrin mutations and whether filaggrin expression was modulated by the atopic inflammatory response.
Methods: Filaggrin expression was measured in skin biopsies and cultured keratinocytes using real-time RT-PCR and immunohistochemistry. Filaggrin loss-of-function mutations were screened in a total of 69 subjects.
Results: Compared with normal skin, filaggrin expression was significantly reduced ($P < .05$) in acute AD skin, with further reduction seen in acute lesions from 3 European American subjects with AD who were heterozygous for the 2282del4 mutation. This was confirmed by using immunohistochemistry. AD skin is characterized by the overexpression of IL-4 and IL-13. Keratinocytes differentiated in the presence of IL-4 and IL-13 exhibited significantly reduced filaggrin gene expression (0.04 ± 0.01 ng filaggrin/ng glyceraldehyde 3-phosphate dehydrogenase; $P < .05$) compared with media alone (0.16 ± 0.03).
Conclusion: Patients with AD have an acquired defect in filaggrin expression that can be modulated by the atopic inflammatory response.
Clinical implications: The atopic immune response contributes to the skin barrier defect in AD; therefore, neutralization of IL-4 and IL-13 could improve skin barrier integrity.
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Key words: *Atopic dermatitis, skin barrier, filaggrin*

Atopic dermatitis (AD) is a chronic inflammatory skin disorder that affects approximately 17% of children and leads to significant disruption in quality of life.[1,2] There has been considerable interest in the disease mechanism that drives the so-called *atopic march*, reflecting a progression from AD to asthma.[3] Recent studies have focused on the impaired epidermal barrier identified in patients with AD.[4] Using a murine model of AD, it has been shown that skin barrier dysfunction not only enhances allergen sensitization but also leads to systemic allergic responses such as increased IgE levels and airway hyperreactivity.[5] These observations support the concept that absorption of allergens through the skin of patients with AD may predispose to the atopic march.

Filament aggregating protein (filaggrin) is a key protein that plays an important role in formation of the cornified cell envelope (CCE), which is critical for an effective skin barrier. Filaggrin binds to and is responsible for the aggregation of keratins (K1/10), which induce the cytoskeleton to collapse and result in formation of corneocytes. These corneocytes are then heavily cross-linked by the action of transglutaminases and make up the CCE.[6] Recent genetic studies have shown that loss-of-function (null) mutations in the gene encoding filaggrin (*FLG*) result in impaired skin barrier function and are strongly associated with the development of asthma in patients with AD.[7-9] Of note, the filaggrin null mutation is observed in less than 1/3 of the general AD population.[7-9] Because most patients with AD have a defective skin barrier, this suggests there must be additional mechanisms that modulate filaggrin expression and barrier integrity.

Atopic dermatitis skin is characterized by the overexpression of IL-4 and IL-13, T_H2 cytokines known to

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Abbreviations used
AD: Atopic dermatitis
CCE: Cornified cell envelope
dNTP: Deoxynucleotide triphosphate
FLG: Filaggrin
TBST: TRIS-buffered saline with Tween

induce atopic responses and downregulate skin innate immune response genes.[10-12] Therefore, we investigated whether filaggrin deficiency in AD may be acquired as the result of T_H2 cytokine modulation, and to what extent the 2 well characterized *FLG* mutations (R501X and 2282del4) contribute to altered filaggrin expression in a North American population of AD.

METHODS

Study subjects

Subjects included 39 healthy persons (27 European American, 11 African American, 1 Asian American) with no history of skin disease (mean age, 36.8 ± 2.1 years), 30 patients (17 European American, 12 African American, 1 Hispanic) with moderate AD (mean age, 36.2 ± 1.8 years; 20% to 60% skin involvement), and 6 subjects with lichen planus (mean age, 51.3 ± 7.3 years). Subjects were recruited as part of the National Institutes of Allergy and Infectious Diseases–funded Atopic Dermatitis and Vaccinia Network. None of the subjects had received systemic corticosteroids or cyclosporine previously, and none had received topical corticosteroid or calcineurin inhibitors for a period of at least 1 week before enrollment. The study was approved by the institutional review board at the National Jewish Medical and Research Center in Denver and University of Rochester Medical Center. All subjects gave written informed consent before participation in these studies.

Two-millimeter punch biopsies were collected from acute erythematous AD lesions (defined as less than 3 days after onset according to Hamid et al.[13] and uninvolved skin of the same patients with AD (n = 16), lichen planus lesions (n = 6), and normal healthy skin (n = 15). The skin samples were submerged immediately in either Tri-Reagent (Molecular Research Center, Inc, Cincinnati, Ohio) or 10% buffered formalin for real-time RT-PCR and immunohistochemical studies, respectively.

Genotypic analysis of filaggrin

Blood samples were collected from all 69 subjects for extraction of DNA for genotyping using PaxGene Blood DNA tubes (PreAnalytix GmbH, Hombrechtikon, Switzerland), and genomic DNA was extracted from peripheral blood samples by using M48 Biorobot (Qiagen, Inc, Valencia, Calif).

R501X mutation analysis. Genotyping for R501X was performed with the ABI 3730 Detect System (Applied Biosystems, Foster City, Calif). Briefly, a 178-bp fragment was amplified by PCR with a pair of primers: R051X-F, 5′-GACCAGCACTGGAGGAAGAC; and R501X-R, 5′-ATGGGAACCTGAGTGTCCAG. PCR was performed in a total volume of 20 μL containing 1 × PCR buffer (including 1.5 mmol/L $MgCI_2$), 12 pmol primers, 2 mmol/L deoxynucleotide triphosphate (dNTP), 20 ng genomic DNA, and 1.0 U DNA polymerase (Qiagen, Inc). Amplification was performed as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, 60°C for 1 minute; and 72°C for 5 minutes. All PCR products were purified with QIAquick PCR purification Kit (Qiagen, Inc) and bidirectionally sequenced on an ABI 3730 automated sequencer with Big-Dye terminator cycle sequencing reagents (Applied Biosystems).

2282del4 Mutation analysis. Genotyping for the 2282del4 was performed with an Applied Biosystems 3700 DNA Sequencer with the addition of a size standard and data analysis using Genotyper (Applied Biosystems), an allele calling software program. In brief, a PCR fragment amplifying 152 bp of genomic DNA was amplified with primers 5′-6-FAM-CCAGTGGTAGTCAGGCCACT and 5′-AAAGACCCTGAACGTCGAGA. PCR was performed in a total volume of 20 μL containing 1 × PCR buffer (including 1.5 mmol/L $MgCI_2$), 12 pmol primers, 2 mmol/L dNTP, 20 ng genomic DNA, and 1.0 U DNA polymerase (Qiagen, Inc). Amplification was performed as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, 60°C for 1 minute; and 72°C for 5 minutes. Fragments were diluted 1:20 and sized according to the manufacturer's recommended protocol (Applied Biosystems). The wild-type allele was 152 bp, and the 2282del4 allele was 148 bp.

Quantitative real-time RT-PCR

Total RNA was isolated from 2-mm skin biopsy samples by chloroform:phenol extraction and isopropanol precipitation according to the manufacturer's guidelines (Molecular Research Center, Inc). RNeasy Mini Kits (Qiagen, Inc) were used according to the manufacturer's protocol to isolate RNA from cell cultures and to purify RNA from skin biopsies further. One microgram of RNA was reverse-transcribed in a 20-μL reaction containing Random Primers (500 μg/mL; Invitrogen, Carlsbad, Calif), dNTP (10 mmol/L; Invitrogen), 5X First Strand Buffer (Invitrogen), DTT (0.1 mol/L; Invitrogen), Superscript III enzyme (200 U/μL; Invitrogen) and RNase inhibitor (10 U/μL; Invitrogen). Real-time PCR was performed and analyzed by the dual-labeled fluorogenic probe method by using an ABI Prism 7300 sequence detector (Applied Biosystems). Primers and probes for human GAPDH and filaggrin were purchased from Applied Biosystems. Amplification reactions were performed in MicroAmp optical tubes (Applied Biosystems) in a 25-μL volume as previously described.[14] Relative expression levels were calculated by the relative standard curve method as outlined in the manufacturer's technical bulletin. A standard curve was generated using the fluorescent data from the 10-fold serial dilutions of total RNA of the highest expression sample. This was then used to calculate the relative amounts of target mRNA in test samples. Quantities of all targets in test samples were normalized to the corresponding GAPDH levels in cultured keratinocytes and skin biopsies.

Filaggrin immunohistochemical staining

Paraffin-embedded tissues were cut at 5 μm and placed on frosted microscope slides. Slides were deparaffinized by using a series of xylene and ethanol washes and then stained using the Cell and Tissue Staining Kit (R&D Systems, Minneapolis, Minn). Endogenous peroxidase was blocked by incubating slides with 3% H_2O_2 for 5 minutes, and tissue sections were then blocked with serum blocking reagent G (R&D Systems) for 15 minutes. Slides were stained overnight with a monoclonal mouse antihuman filaggrin (Abcam, Cambridge, Mass) at a 1:500 dilution. The secondary antibody and 3,3′-diaminobenzidine stain were added according to the manufacturer's protocol (R&D Systems). The skin sections were counterstained with hematoxylin. Antibody specificity was confirmed by using purified nonimmune mouse IgG (Southern Biotechnology, Birmingham, Ala). All slides were coded before analysis and read blindly to ensure patient anonymity. The intensity of the immunostaining was graded with the use of microscopy on a scale from 0 to 5, with 0 indicating no staining and 5 the most intense staining.

TABLE I. Number of filaggrin mutation genotypes in European American subjects with AD and healthy controls

Mutation	Genotype	AD	Control
2284del4	AA	14	25
	Aa	3 (0.18)	2 (0.07)
R501X	RR	17	27
	RX	0	0
No. of cases		17	27

Immuno-dot-blot for filaggrin

Immunoblot was performed with a Bio-Dot Microfiltration Apparatus from Bio-Rad (Hercules, Calif). Protein was isolated from primary keratinocytes by acetone precipitation and washed by using 0.3 mol/L guanidine hydrochloride in 95% ethanol with 2.5% glycerol according to the manufacturer's guidelines (Molecular Research Center, Inc). Twenty-five micrograms of total protein was loaded in a final volume of 100 μL TRIS-buffered saline with 0.5% Tween 20 (TBST) and allowed to bind to the nitrocellulose membrane (Bio-Rad) by gravity filtration. The membrane was then blocked in 5% milk in TBST for 30 minutes at room temperature. After blocking, the membrane was washed once with TBST to remove residual blocking solution and then incubated overnight at 4°C with antifilaggrin (Abcam) at 4 μg/mL in TBST. The blot was washed 3 times with TBST for 5 minutes and then incubated with antimouse horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, United Kingdom) at a dilution of 1:5000 in TBST for 1 hour at room temperature. Blots were washed 3 times for 5 minutes each with TBST and then developed with ECL Western Blotting Detection Reagents (Amersham Biosciences) according to the manufacturer's protocol. The intensity of filaggrin protein was determined by digital imaged analysis using the National Institutes of Health image 1.61 software programs (National Institutes of Health, Bethesda, Md). Specificity of the antibody was determined by replacing the primary antibody with a mouse IgG_1 isotype control (Southern Biotechnology). A standard curve was generated for filaggrin protein by using recombinant human filaggrin protein (Abcam).

Keratinocyte cell culture

Primary human keratinocytes (Cascade Biologics, Portland, Ore) were grown in serum-free keratinocyte growth medium (EpiLife; Cascade Biologics), supplemented with 1% human keratinocytes growth supplement V2 (Cascade Biologics), 0.06 mmol/L $CaCl_2$, and 1% penicillin and streptomycin.

To investigate the effects of cytokines on filaggrin expression, keratinocytes were differentiated for 5 days in 1.3 mmol/L $CaCl_2$, and cytokines were added for the entire differentiation period or the final 24 hours. IL-4 (50 ng/mL) and IL-13 (50 ng/mL; R&D Systems) were purchased from R&D Systems (Minneapolis, Minn). Total RNA was then isolated for analysis of filaggrin gene expression by real-time RT-PCR.

Statistical analysis

Statistical analysis of gene expression and immunohistochemical staining was conducted by using Graph Pad Prism, version 4.03 (San Diego, Calif). Statistical differences between groups were determined by using an unpaired *t* test with significant differences conferred when $P < .05$. In cases in which multiple groups were compared with a control, data were analyzed by a 1-way ANOVA, and significant differences were determined by a Tukey-Kramer test.[15] For associations of genetic mutations with disease status, data were analyzed in STATA 8.2 (StataCorp LP, College Station, Tex) using the Fisher exact test. Analyses of the European American and African American subjects were conducted separately; subjects from the other ethnic groups were considered for allele frequency only. Departures from Hardy-Weinberg equilibrium proportions were tested among cases and controls separately by using SIB-PAIR 1.00 (David Duffy, Queensland, Australia).

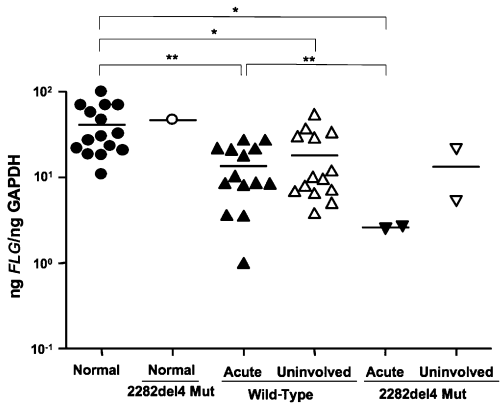

FIG 1. Filaggrin deficiency in AD skin. RNA was isolated from the skin of normal subjects (n = 15) and patients with AD (n = 16) with or without the 2282del4 mutation. Filaggrin gene expression was evaluated using real-time RT-PCR. $*P < .05$; $**P < .01$.

RESULTS

Characterization of filaggrin expression

Recent studies have shown that the loss-of-function mutations R501X and 2282del4 are associated with AD in European Americans.[7-9] Therefore, we genotyped these mutations in 39 healthy subjects (27 European American, 11 African American, 1 Asian American) and 30 subjects with mild to moderate AD (17 European American, 12 African American, 1 Hispanic). None of the subjects genotyped had the R501X mutation. The frequency of the 2282del4 loss-of-function mutation was similar to recent reports of frequencies among subjects with and without AD;[7-9] however, all 5 subjects with the 2282del4 in our study were heterozygotes. Counts of European Caucasian AD and healthy subjects by genotype are presented in Table I, and given the heterozygote status of all subjects with the variant, allele frequencies for 2282del4 among the European American AD and healthy subjects were 8.8% and 3.7%, respectively. The marker 2282del4 was in Hardy-Weinberg equilibrium at a level of $P > .1$ for both cases and controls. Using the Fisher exact test, we found no evidence for association between presence of 2282del4 allele and AD among European Americans ($P = .36$). All of the 23 African American subjects genotyped had wild-type genotypes for both mutations, providing further evidence that these mutations may be less common in this racial group.[7]

To determine whether the 2282del4 mutation affected *FLG* expression, we compared filaggrin gene expression in a subset of European Americans subjects using real-time RT-PCR. As illustrated in Fig 1, *FLG* expression

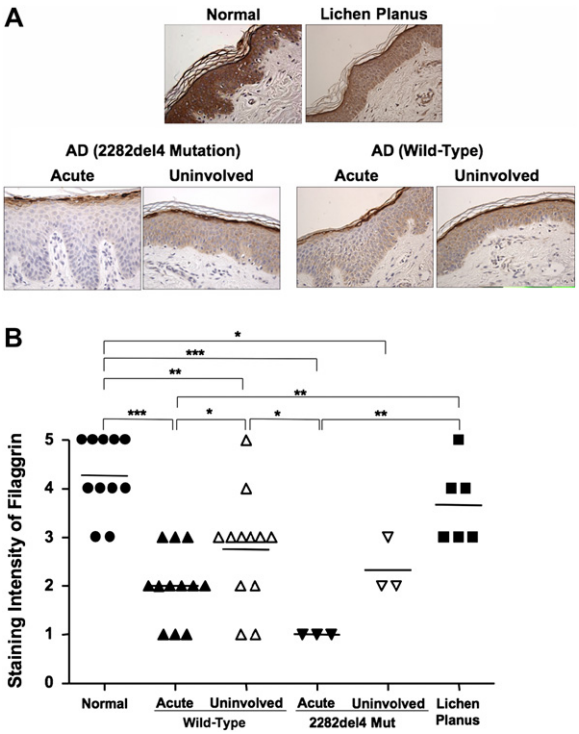

FIG 2. Decreased filaggrin staining in AD skin. **A,** Representative paraffin embedded skin biopsies from normal subjects (n = 11), patients with AD (n = 12), patients with AD with the 2282del4 mutation (n = 3), and patients with lichen planus (n = 6) stained for filaggrin are shown. Images were collected at 40× magnification, and the *scale bar* represents 50 μm. **B,** The intensity of the staining was graded visually on a scale from 0 (no staining) to 5 (the most intense staining). *$P < .05$; **$P < .01$; ***$P < .001$.

was decreased in lesional and uninvolved AD skin compared with the skin of normal healthy subjects without the mutation (40.77 ± 6.75 ng *FLG*/ng GAPDH; $P < .05$). Whereas similar levels of *FLG* were observed in the uninvolved skin of patients with AD with (13.4 ± 11.4) and without (13.36 ± 8.06) the 2282del4 mutation; *FLG* expression was higher in lesional skin from patients with AD without the mutation (13.54 ± 2.41) compared with the 2 patients who were heterozygous for the 2282del4 mutation (2.59 ± 0.07). This difference was determined to be significant ($P < .01$) using an unpaired *t* test with Welch correction because of differences in variance. Further investigation on a subject by subject basis revealed higher levels of filaggrin expression in the uninvolved compared with lesional skin of 8 out of 14 patients with AD without the mutation. The presence of a single 2282del4 mutation in a normal healthy subject did not significantly affect *FLG* expression (46.56 ng *FLG*/ng GAPDH). One normal healthy control and 1 patient with AD with the 2282del4 mutation were excluded from analysis because of a failure to amplify their GAPDH gene.

Immunohistochemical expression of filaggrin

On the basis of our observations, we further evaluated the epidermal expression of filaggrin in healthy skin from normal subjects, uninvolved and lesional skin from patients with AD, and lesional skin from patients with lichen planus. Lichen planus was chosen as a positive control because, like AD, it is a T-cell–mediated skin disease; however, its primary response is T_H1 rather than the T_H2 observed in AD.[16] In addition, there currently is no known link between lichen planus and a null filaggrin mutation. Differences in filaggrin staining intensity are illustrated in Fig 2, *A*. Filaggrin staining was more intense in skin from normal healthy subjects and patients with lichen planus compared with lesional and uninvolved skin from patients with AD. Filaggrin staining was also significantly greater in uninvolved AD skin compared with lesional AD skin from the same subjects ($P < .05$). This suggests that filaggrin mutations do not account for the decrease in filaggrin expression. In addition, filaggrin protein expression was higher in the uninvolved compared with lesional skin of 8 out of 12 patients with AD without the mutation. Lesional skin from patients with AD with the 2282del4 mutation exhibited the least intense staining for filaggrin. The composite data for filaggrin immunostaining in all samples are shown in Fig 2, *B*.

Modulation of filaggrin expression

Filaggrin is involved in the terminal differentiation of keratinocytes to form the CCE.[6] Therefore, we initially investigated whether differentiation of keratinocytes induced filaggrin expression. Keratinocytes were differentiated in 1.3 mmol/L $CaCl_2$ as previously described.[17] Fig 3 demonstrates that differentiation in increased calcium (1.3 mmol/L) significantly induces filaggrin gene expression (mean, 0.86 ± 0.12) compared with 0.06 mmol/L (mean, 0.27 ± 0.04; $P < .001$). Because acute AD skin is characterized by the overexpression of IL-4 and IL-13,[13] and filaggrin expression is increased in uninvolved AD skin compared with acute AD skin, we investigated whether IL-4 and IL-13 modulates filaggrin expression during keratinocyte differentiation. IFN-γ, which is minimally expressed in acute AD, was used as a T_H1 cytokine control . Keratinocytes were differentiated in the presence and absence of IL-4 and IL-13 or IFN-γ for 5 days. Differentiation of keratinocytes in the presence of IL-4 and IL-13 significantly ($P < .001$) reduced filaggrin expression (0.18 ± 0.02; Fig 3, *A*). In contrast, differentiation in the presence of IFN-γ augmented filaggrin expression (3.31 ± 0.63; $P < .001$). On further investigation, we also found that incubation of differentiated keratinocytes with IL-4 and IL-13 for 24 hours was able to downregulate filaggrin expression (0.137 ± 0.023; $P < .05$) compared with media alone (0.606 ± 0.028; data not shown).

Immuno-dot-blot analysis was used to confirm our observations on the protein level (Fig 3, *B*). We confirmed that IL-4 and IL-13 also downregulates filaggrin protein expression (0.11 ± 0.03 μg filaggrin protein; $P < .001$) compared with media alone (2.42 ± 0.27; Fig 3, *C*). As was seen in the gene expression, IFN-γ augmented filaggrin protein expression (3.04 ± 0.04; $P < .001$; Fig 3, *C*).

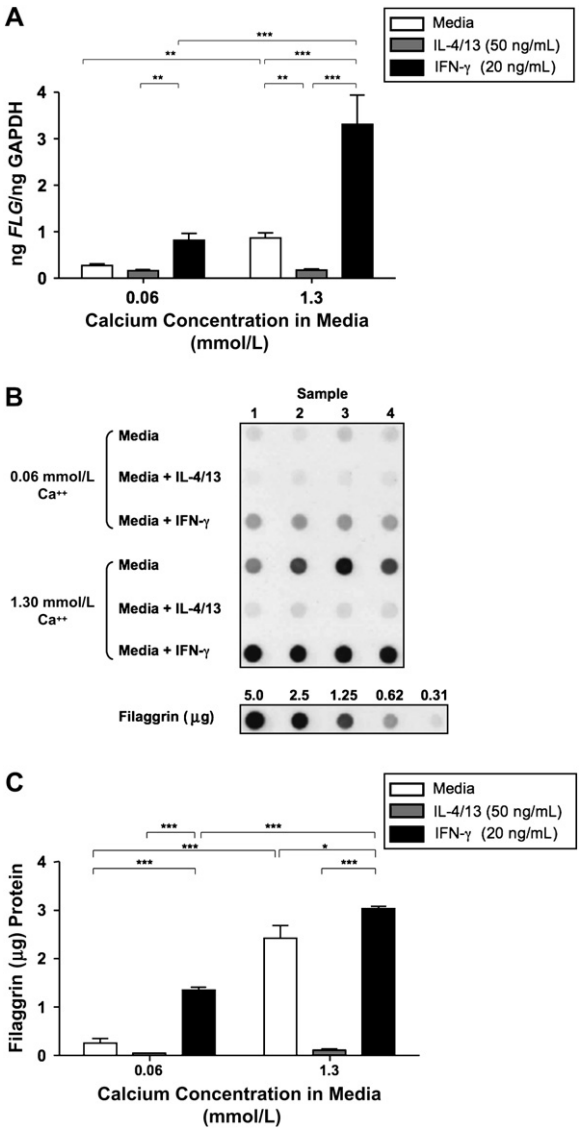

FIG 3. T_H2 cytokines downregulate filaggrin gene expression. Primary human keratinocytes were cultured in 0.06 or 1.3 mmol/L $CaCl_2$ in the presence and absence of IL-4 and IL-13 or IFN-γ for 5 days. RNA was collected from the cells, and the levels of filaggrin were evaluated by real-time RT-PCR **(A)** or immuno-dot-blot **(B and C)**. $*P < .05$; $**P < .01$; $***P < .001$ between exposure groups.

DISCUSSION

The epidermal barrier serves as the first line of defense against invading pathogens and allergens. In AD skin, this barrier is significantly compromised and is associated with increased allergen sensitization, trans-epidermal water loss, and viral and bacterial skin infections.[18,19] This has been shown to augment the atopic inflammatory response,[5,20] thereby further exacerbating the disease state and potentially modulating the effectiveness of the epidermal barrier. The epidermal differentiation complex contains a number of proteins involved in barrier function including filaggrin. Our current study evaluated filaggrin expression in the skin of healthy subjects and patients with AD to determine whether there is a relationship between filaggrin expression and the atopic immune response.

Several recent studies have now demonstrated an association between null mutations in the filaggrin gene and AD.[7-9] This observation is important in our understanding of the significance of skin barrier function in AD; however, this mutation has been observed in less than 1/3 of general populations of patients with AD of European decent.[7-9] In addition, this mutation was heterozygous in most cases. Genetic variants of filaggrin were absent in patients with AD of African American and Asian descent, suggesting that the evolutionary pressure that led to the development of these mutations in subjects of European American descent differs from those observed in other racial groups. [7]

In our current study, we observed a heterozygous 2282del4 mutation in 8.8% of European American patients with AD and 3.7% of our normal healthy subjects. Interestingly, the acute lesional skin of patients with AD with the mutation exhibited lower levels of filaggrin expression compared with the uninvolved skin in the same patient. This suggests that the heterozygous mutation does not completely abolish filaggrin expression. In fact, a homozygous mutation is necessary to eliminate filaggrin completely.[7] Combined with the knowledge that skin barrier disruption is seen in all patients with AD, we investigated other factors that may contribute to the deficiency in filaggrin.

Studies by Gustafsson et al[21] and Illi et al[22] demonstrate that most patients with AD outgrow their skin disease. This suggests that the filaggrin deficiency may be a result of the atopic skin immune response in many patients with AD. In addition, our laboratory has previously demonstrated that deficiencies in antimicrobial peptides, important components of the innate immune response, are acquired rather than constitutive.[14,23] Further studies demonstrated that this acquired defect was a result of the T_H2 cytokine milieu within AD skin. In addition, patients with AD respond well to topical treatments such as tacrolimus and pimecrolimus that suppress the inflammatory reaction in AD skin.[1,19] This further suggests that the defect in AD skin barrier function can be acquired.[24]

Acute AD skin is characterized by the overexpression of the T_H2 cytokines IL-4 and IL-13. Combined with the observation that filaggrin expression is increased in uninvolved AD skin compared with acute AD skin, we therefore investigated whether these cytokines altered the expression of filaggrin. During the differentiation process, profilaggrin is cleaved into multiple 37-kd filaggrin subunits that are critical for the formation of the intact CCE. Therefore we differentiated normal primary keratinocytes in the presence and absence of IL-4 and IL-13 for 5 days to determine whether filaggrin gene expression would be modulated. Differentiation alone induced filaggrin; however, this was significantly inhibited in the presence of IL-4 and IL-13. In additional experiments, we demonstrate that this suppression could also occur in keratinocytes that

have been differentiated first and then treated with IL-4 and IL-13 for the final 24 hours. This strongly suggests that the filaggrin deficiency in many patients with AD is acquired because of the T_H2 cytokine milieu.

Our current study not only demonstrates that filaggrin gene expression and protein are decreased in the skin of patients with AD but also indicates that this deficiency is caused, in part, by the overexpression of T_H2 cytokines that downregulate filaggrin expression during the differentiation process. Our data suggest that many patients with AD acquire the filaggrin deficiency and subsequent barrier disruption as the result of the local inflammatory immune response. This may contribute to decreased filaggrin gene expression in patients with wild-type as well as heterozygous null mutations in FLG. Further studies are needed to determine whether early, effective treatment of AD may impede the development of asthma in such patients by repairing their skin barrier defect.

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