Lowering relative humidity level increases epidermal protein deimination and drives human filaggrin breakdown

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Abstract

Background—Deimination (also known as citrullination), the conversion of arginine in a protein to citrulline, is catalyzed by a family of enzymes called peptidyl-arginine deiminases (PADs). Three PADs are expressed in the epidermis, one of their targets being filaggrin. Filaggrin plays a central role in atopic dermatitis and is a key protein for the epidermal barrier. It aggregates keratins and is cross-linked to cornified envelopes. Following its deimination, it is totally degraded to release free amino acids, contributing to the natural moisturizing factor (NMF). The mechanisms controlling this multistep catabolism in human are unknown.

Objective—To test whether external humidity plays a role, and investigate the molecular mechanisms involved.

Methods—Specimens of reconstructed human epidermis (RHEs) produced in humid or dry conditions (>95% or 30–50% relative humidity) were compared.

Results—RHEs produced in the dry condition presented structural changes, including a thicker *stratum corneum* and a larger amount of keratohyalin granules. The transepidermal water loss and the *stratum corneum* pH were decreased whereas the quantity of NMF was greater. This highly suggested that filaggrin proteolysis was up-regulated. The expression/activity of the proteases involved in filaggrin breakdown did not increase while PAD1 expression and the deimination rate of proteins, including filaggrin, were drastically enhanced. Partial inhibition of PADs with Cl-amidine reversed the effect of dryness on filaggrin breakdown.

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Conclusion—These results demonstrate the importance of external humidity in the control of human filaggrin metabolism, and suggest that deimination plays a major role in this regulation.

Keywords

atopic dermatitis; citrullination; skin; posttranslational modification; peptidyl-arginine deiminase; proteolysis

1. Introduction

Atopic dermatitis (AD; OMIM #603165) is a chronic inflammatory skin disease affecting up to 20% of children and around 3% of adults in industrialized countries. The pathophysiology of AD involves genetic and environmental factors. Most of the susceptibility genes encode proteins involved in the immune response and epidermal barrier formation [1–3]. Loss-of-function mutations in the gene *FLG* encoding filaggrin are the strongest known risk factor for AD [4–6]. *FLG* mutations appear to define a subgroup of AD patients with more severe clinical signs, such as reduced skin hydration, increased transepidermal water loss (TEWL), reduced levels of components of the natural moisturizing factor (NMF) and higher skin surface pH [7–10]. Moreover, filaggrin down-regulation has been reported in adult patients regardless of *FLG* mutations status, its expression being influenced by inflammatory cytokines [11–13]. Therefore, filaggrin deficiency appears to have a central place in AD pathogenesis.

Human filaggrin is an essential epidermal protein synthesized as a large precursor (400 kDa) named profilaggrin and stored in keratohyalin granules. Profilaggrin consists of a large central repetitive domain comprising 10–12 filaggrin repeats, flanked by two single N- and C-terminal domains. During the *stratum granulosum* to *stratum corneum* transition, profilaggrin is proteolytically processed to release basic filaggrin monomers (~37 kDa). These monomers associate with keratin filaments and facilitate their aggregation leading to the corneocyte matrix formation [14]. In addition, some monomers are cross-linked to the cornified envelope by transglutaminases [15]. In the lower *stratum corneum*, filaggrin is deiminated by peptidyl-arginine deiminases 1 and/or 3 (PAD1 and PAD3). This post-translational modification (transformation of arginyl residues into citrullyl) induces a decrease in the number of filaggrin positive charge leading to filaggrin acidification, and thus promotes its detachment from the keratin filaments. Subsequently, filaggrin is fully proteolyzed into free amino acids [16–18].

Filaggrin degradation is accomplished by bleomycin hydrolase, caspase 14, calpain 1 [19–22] and probably other proteases. Some of the amino acids generated are further modified. In particular, glutamine spontaneously transforms into pyrrolidone-5-carboxylic acid (PCA), a highly hygroscopic molecule, and histidine is modified by histidase to form trans-urocanic acid (UCA), a major UVB-absorbing chromophore [23–25]. The amino acids and their derivatives produced from filaggrin degradation participate in the formation of the NMF, and contribute to skin photoprotection and to *stratum corneum* hydration and acidification (reviewed in [26]). The low pH of the *stratum corneum* in turn prevents skin colonization by

pathogenic microorganisms, is important for the activity of enzymes involved in lipid synthesis and controls desquamation [27, 28].

Despite the importance of filaggrin in both epidermal homeostasis and AD pathogenesis, the molecular mechanisms controlling its degradation are still not well understood. In rodents, the proteolysis of filaggrin appears to be modulated by environmental humidity [29]. To know whether the humidity controls filaggrin breakdown in humans and to investigate the mechanisms involved, specimens of tridimensional reconstructed human epidermis (RHEs) were produced under either >95% relative humidity (RH) (humid condition) or 30–50% RH (dry condition). We compared the *stratum corneum* morphology and functional properties. Then we focused on filaggrin and on the expression/activity of the enzymes involved in its metabolism.

2. Materials and methods

2.1. RHE production in humid and dry conditions

Primary normal human keratinocytes were isolated after abdominal dermolipectomy of four healthy female (34–48 years old) subjects who had given their informed consent. Four keratinocyte banks were thus constituted. *FLG* was sequenced using next-generation deep sequencing, and no loss-of-function mutations were observed. RHEs were produced as previously described [30,31], except that after exposure to the air–liquid interface they were cultivated in either humid (>95% RH) or dry (30–50% RH) conditions at 37°C and 5% CO_2. Low humidity was induced by removal of the water pan from the incubator. Humidity rate was continuously monitored using the 176H1 data logger (Testo, Lenzkirch, Germany). To avoid water condensation, RHEs produced in the dry condition were cultivated without culture plate covers which were replaced by gas-permeable films (Breathe-Easy; Diversified Biotech, Dedham, MA). When RHEs were maintained in the dry condition, the culture medium was renewed each day to avoid changes in osmolarity since a higher medium evaporation occurred. The medium was renewed every 2 days in the humid condition. RHEs were harvested 10 days after air-liquid interface exposure.

2.2. Transmission electron microscopy analysis

RHEs, produced with keratinocytes from 3 different donors, were processed as previously described [32] and observed with an HT7700 electron microscope (Hitachi, Tokyo, Japan). Quantifications were performed using 2 RHEs/condition/donor. The numbers of corneocyte layers and of granular and living keratinocyte layers were quantified on 3 independent areas for each epidermis (2500x and 300x magnification pictures, respectively). The density of keratohyalin granules (area of granules/total area of cytoplasm) and their mean size were measured for each epidermis on 6 pictures (2500x) of the *stratum granulosum* just behind the *stratum corneum*, using ImageJ software (National Institutes of Health, Bethesda, MD). Briefly, for all pictures, positive areas were picked using the "wand tool" with an 8-connected mode and a fixed tolerance.

2.3. Transepidermal water loss measurement

Transepidermal water loss (TEWL) of RHEs produced with keratinocytes from 3 different donors (3 RHEs/condition/donor) was measured using the tewameter TM300 (Courage & Khazaka, Cologne, Germany). For further information see Supplementary Methods.

2.4. Protein extractions

Different protein extracts were produced: (i) total proteins were extracted in Laemmli buffer 5X [175 mM Tris-HCl pH 6.8, 7.5% SDS, 25% glycerol, 12.5% β-mercaptoethanol, and 0.4% bromophenol blue] (2 pooled RHEs/condition/donor; 4 different donors), (ii) detergent-soluble proteins were extracted in the presence of Nonidet-P40 (NP-40) [40 mM Tris–HCl pH 7.5, 0.5% Nonidet-P40, 10 mM EDTA, 0.25 mM PMSF, 1/100 (v/v) mammalian protease inhibitor cocktail] to get the so-called TE-NP40 extracts (3 pooled RHEs/condition/donor; 3 donors), and (iii) deiminated filaggrin-enriched extracts (3 pooled RHEs/condition/donor; 3 donors) were prepared as previously described [33]. Briefly, TE-NP40 extracts were precipitated with absolute ethanol. After centrifugation, pellets were dried at 80°C for 20 min and resuspended in water. After homogenization, all extracts were cleared by centrifugation for 15 min at 15,000 rpm.

2.5. Quantification of UCA and PCA

At day 10, RHEs were homogenized in PBS-Triton X100 0.1%, and UCA and PCA were analyzed by HPLC on a C18 reverse-phase column (3 RHEs/condition/donor; 3 different donors). Their amount was expressed in μg/mg of proteins.

2.6. pH measurement

As recommended by the manufacturer's instructions, 2 μL of ultra-pure water was topically applied to RHEs (3 RHEs/condition/donor; 3 different donors) and surface pH was measured using the HI-99181a skin pH meter (HANNA instruments, Woonsocket, RI).

2.7. In situ transglutaminase activity assay

In situ transglutaminase assay was performed as described previously [34, 35]. For further information see Supplementary Methods. For each RHE, the area of fluorescence was measured on 3 images with ImageJ software using a fixed threshold and mean ± standard deviation was calculated.

2.8. PAD inhibition

To inhibit PAD activity, Cl-amidine [36] was added into the culture medium to reach a concentration of 800 μM, at day 9 of the culture period. Each RHE specimen was harvested at day 10.

2.9. Statistical analysis

All data are presented as mean ± standard deviation. Statistical differences were determined with bilateral paired Student's t-tests for paired samples. For unpaired samples, we used unpaired Student's t-tests if the equality of sample variances was verified by the Fisher's test

with an α-risk of 0.05, and Welch's *t*-tests if not. Differences were considered significant when the *p*-value (*p*) was less than 0.05.

3. Results

3.1. Low environmental humidity induces stratum corneum thickening and improves barrier function in RHEs

Primary keratinocytes from normal human skin were used to produce RHEs as described in details in the methods' section. As a prerequisite, DNA was extracted from keratinocytes, and *FLG* gene was sequenced. No loss-of-function mutations were detected (data not shown). RHEs were produced in either humid (> 95% RH) or dry (30–50% RH) conditions for 10 days after air-liquid interface exposure. We first verified that the dry condition did not alter cell viability (Supplementary Fig. S1a). The morphological consequences of humidity lowering were then analyzed by hematoxylin-eosin staining and transmission electron microscopy. RHEs produced in the dry condition (dry RHEs) showed a thicker *stratum corneum* than RHEs cultivated in a humid environment (humid RHEs) (Fig. 1a and b), with a 1.8 fold increase in the number of corneocyte layers (Supplementary Fig. S2a). They presented a 1.5 fold thicker *stratum granulosum* while the thickness of total living keratinocyte layers was not modified. The density and size of keratohyalin granules were increased by factors of 1.6 and 1.8, respectively (Supplementary Fig. S2b and c).

To investigate the effect of the dry environment on the *stratum corneum* permeability, two complementary tests were performed: a lucifer yellow assay to evaluate the outside-in permeability and the TEWL measurement to assess the inside-out barrier. No differences in the penetration of the fluorescent dye were observed between RHEs produced in humid and dry conditions, a negligible quantity being detected in the medium whatever the culture conditions (Supplementary Fig. S1b). However, dry RHEs showed a decreased TEWL (7.7 g H_2O/hxm^2 vs 12.3 for humid RHEs; Fig. 1c).

3.2. Low environmental humidity modulates keratinocyte differentiation in RHEs

To compare keratinocyte proliferation in RHEs, Ki67 labelling was performed. The number of Ki67 positive nuclei detected in the basal layer was similar in both culture conditions (Supplementary Fig. S1c). Moreover keratin 14 expression, analyzed by RT-qPCR (Supplementary Fig. S3a) and western blotting (Fig. 2a), was not modified. Thus, keratinocyte proliferation was not changed by environmental humidity variations.

The expression of various differentiation markers was then analyzed. At the mRNA level (Supplementary Fig. S3a), no significant differences were observed in the expression of keratin 10, loricrin or transglutaminases, except a ~2 fold increase of transglutaminase 5. A significant increase was also noted for corneodesmosin and involucrin. At the protein level (Fig. 2a and b), detections of keratin 10 and transglutaminases 1 and 3 were not modified, but changes were observed for the others. In dry RHEs, higher amounts of involucrin (1.60 $\pm$ 0.12 arbitrary units vs 1.00 in humid RHEs; n = 3; *p* = 0.013), of transglutaminase 5 active form at 53 kDa (2.16 $\pm$ 0.42; n = 4; *p* = 0.011) and of fragments (40 kDa) of corneodesmosin (1.67 $\pm$ 0.22; n = 4; *p* = 0.009) were detected, whereas the detection of

loricrin was significantly decreased (0.45 ± 0.10; n = 3; $p = 0.012$). Transglutaminase activity was compared by an *in situ* enzymatic assay (Fig. 2c). The fluorescent signal resulting from the crosslink of Alexa-Fluor-555–cadaverine at the cell periphery in the *stratum granulosum* was of similar intensity whatever the condition but more cell layers were labelled in dry RHEs, the area of detection increasing 1.80 ± 0.53 fold ($p = 0.001$).

3.3. Low environmental humidity stimulates filaggrin breakdown and increases the production of UCA and PCA

To explore the impact of environmental dryness on filaggrin metabolism, its expression was studied by RT-qPCR, and by western blotting and indirect immunofluorescence performed with AHF3 monoclonal antibody directed to both profilaggrin and filaggrin monomers. At the mRNA level, *FLG* expression was increased in the dry condition (1.58 ± 0.38 fold; n = 6; $p = 0.02$) (Supplementary Fig. S3b). Profilaggrin immunodetection tended to increase in dry RHEs (1.43 ± 0.30 fold; n = 4; $p = 0.06$) whereas that of filaggrin monomers significantly decreased (0.64 ± 0.06 fold; n = 4; $p = 0.006$) (Fig. 3a). Furthermore, labelling patterns obtained by indirect immunofluorescence were different (Fig. 3b and Supplementary Fig. S4). In humid RHEs, a granular labelling of the granular keratinocytes – probably corresponding to profilaggrin stored in the keratohyalin granules – and a diffuse intense staining of the lower *stratum corneum* – probably corresponding to filaggrin monomers – were observed. In dry RHEs, the *stratum granulosum* staining clearly increased while the *stratum corneum* labelling decreased drastically.

The decreased immunodetection of filaggrin monomers and the reduced immunolabelling of the *stratum corneum* in the dry condition could be due either to a lower affinity of AHF3 to the deiminated filaggrin, as previously observed [17], or to an enhanced degradation of filaggrin, or both. To test if filaggrin degradation was increased, PCA and UCA contents were analyzed (Fig. 3c). The concentrations of both amino acid derivatives were significantly enhanced in the dry condition (2.71 and 7.62 fold, respectively). Furthermore, the concentration of serine, a very abundant (25.3%) amino acid in filaggrin, was also shown to increase in the dry condition (36.23 ± 4.70 *versus* $10.29 \pm 0.91 \mu g/mg$; 3.52 ± 0.55 fold; n = 9; $p = 3.7 \times 10^{-7}$). This showed that filaggrin breakdown was enhanced. In link with this observation, NMF being known to partly acidify the *stratum corneum*, the pH of RHE surface was measured. The dry condition induced a statistically significant lowering of pH from 6.7 to 5.9 (Fig. 3d).

3.4. In dry RHEs, peptidyl-arginine deiminase expression and activity were increased but those of proteases involved in filaggrin breakdown were not

In order to further understand the molecular mechanisms involved in the increase of filaggrin breakdown under dry vs humid conditions, the expression of three proteases known to target filaggrin monomers was compared. RT-qPCR revealed no differences in their mRNA amounts (Supplementary Fig. S3c). Western blotting showed an unexpected decreased expression of bleomycin hydrolase in dry RHEs (0.50 ± 0.07 versus 1.00) whereas calpain 1 and caspase 14 (both the zymogen (~28 kDa) and active (~17 kDa) forms) expression was similar (Fig. 4a). An *in vitro* assay performed on RHE extracts showed that calpain activity was not different between dry and humid conditions (Fig. 4b). We also

assessed the expression of other proteases proposed to be implicated in the control of filaggrin proteolysis, namely kallikrein 5, skin aspartic protease, matriptase and elastase 2 [37–40]. At the mRNA level, no significant variation of their expression was measured. However, the amounts of skin aspartic protease, matriptase and elastase 2 detected by western blotting were divided by two in dry RHEs whereas that of kallikrein 5 was not modified (Supplementary Fig. S5). Finally, when analyzing the expression of histidase (responsible for UCA production), we detected three different variants of the enzyme but no differences in their expression (Supplementary Fig. S6).

Since no protease overexpression seemed to explain the increased filaggrin degradation in the dry condition, we looked for the expression and activity of PADs, and for filaggrin deimination. *PADI4* and *PADI6* transcripts were not detected in RHEs by RT-qPCR, as was to be expected since they are not expressed in human epidermis. Unlike human epidermis, RHEs showed no *PADI2* expression under the experimental conditions used [41–43]. *PADI1* mRNA level was strongly increased (13.06 fold) in dry RHEs whereas *PADI3* level was not changed (Fig. 4c). The increased expression of PAD1 was also significant at the protein level (3.89 ± 1.38 fold; $n = 4$; $p = 0.025$; Fig. 4d). The protein deimination rate was analyzed using the anti-modified citrulline antibody (AMC), as previously described [43, 44], and was shown to be increased in dry RHEs, both by indirect immunofluorescence (5.46 fold; Fig. 4e) and by western blotting (7.68 ± 3.95 fold; $n = 4$; $p = 0.043$; Fig. 4f). To study the deimination of filaggrin more specifically, extracts enriched in the neutral to acidic low-salt soluble forms of the protein (essentially the deiminated forms), were prepared from dry and humid RHEs, as previously described for human epidermis [33]. Protein staining confirmed the high degree of filaggrin purity (data not shown). Western blotting with AMC antibody of an equal volume of each extract indicated that filaggrin deimination was increased by a factor of 13.4 ± 4.9 in the dry condition ($n = 3$; $p = 0.048$; Fig. 4g).

To sum up, in the dry condition filaggrin deimination and degradation were enhanced, PAD1 expression and PAD activity were increased whereas the proteases known to target filaggrin were not. Altogether, these results suggest that filaggrin deimination rate may control its terminal breakdown.

3.5. Deimination is involved in the regulation of filaggrin proteolysis

To test the above hypothesis and try to reverse the effect of dryness on filaggrin deimination and degradation, dry RHEs were treated with Cl-amidine, a specific pan PAD inhibitor [36,45], for 24 hours before their harvest at day 10. Cl-amidine treatment induced a slight decrease of global deimination assessed by western blotting of total proteins with AMC (data not shown). This is not surprising because stable deiminated proteins such as keratins had probably accumulated in the *stratum corneum* for several days, deimination being an irreversible post-translational modification. However, the effect on filaggrin was evident as shown by western blotting of the deiminated filaggrin-enriched extracts (0.07 ± 0.04 vs 1.00 arbitrary units; $n = 3$; $p = 0.0006$; Fig. 5a). In turn, the amount of filaggrin monomers in the total protein extracts tended to increase (1.25 ± 0.44 fold; $n = 3$; $p = 0.4$) and to return to the level observed in humid RHEs (Fig. 5b), while the amount of filaggrin monomers in TE-NP40 extracts (corresponding to low ionic strength-soluble forms) was down-regulated from

100 to 20 ± 7% (n = 3; p = 0.0006; Fig. 5c). This shows that partial inhibition of PAD activity induced accumulation of filaggrin monomers and reduced their solubility. However, Cl-amidine treatment did not change the expression of caspase 14, calpain 1 and bleomycin hydrolase, as tested by western blotting (data not shown).

4. Discussion

We showed that lowering the RH in the incubator to 30–50% during the production of RHEs profoundly altered the properties of the epidermis at the structural, functional and biochemical levels (Fig. 6). In particular, the *stratum corneum* of RHEs at low humidity level was thicker, with an increased number of corneocyte layers, and its TEWL decreased. In parallel, the amount of PCA, a highly hygroscopic NMF component, was higher. This suggests that the epidermis adapted to its environment so as to retain water in the cornified layer. This is in agreement with Sun *et al.*'s recent report of normalization of lipid lamellar bilayer formation in the *stratum corneum* of RHEs grown at 50% RH, associated with an increased expression of glucosylceramide synthase [46]. Hyperkeratosis, decreased TEWL and increased secretion of lamellar bodies have also been reported in hairless mice maintained in a very dry environment (RH < 10%) as compared to humid one (> 80%) [47]. In addition, the detected amounts of loricrin and involucrin, two main components of cornified envelopes, were inversely modified, whereas their encoding mRNA levels did not change. We also observed an increased number of cells with transglutaminase activity, and overexpression of transglutaminase 5, one of the enzymes necessary for envelope formation. This suggests that the difference of loricrin and involucrin solubility was due to differences in their cross-linking rather than their synthesis. Therefore the composition of the envelopes may vary. In agreement, Sun *et al.* reported modification in the thickness of these structures in RHEs grown at 50% RH [46].

We demonstrated that the multi-step proteolysis of human filaggrin was increased in the low humidity condition, which was likely to produce more hygroscopic molecules. This indicates that filaggrin degradation is dependent on the environmental water content. Similar observations have been made in rodents. For example, during late foetal development of rats, filaggrin accumulates through the entire thickness of the *stratum corneum* and, immediately after birth, normal filaggrin proteolysis occurs in the outer *stratum corneum*. If the newborn rats are maintained in 100% humidity, mimicking the *in utero* milieu, the activation of filaggrin degradation is blocked. Similarly, application of occlusive patches to adult rats to maintain 100% humidity at the skin surface prevents the normal proteolysis of filaggrin [29].

In our conditions, deimination appeared as a major regulator of filaggrin breakdown (Fig. 6). Expression and activity of proteases known to be involved in the process was not found to be modified whereas the expression of PAD1, known to act on filaggrin monomers, and PAD activity were highly enhanced in dry RHEs. In agreement, the deimination of filaggrin is up-regulated two hours after birth of mice [48], when filaggrin degradation would probably occur. An increase of deimination thus seems to be sufficient to drive filaggrin degradation. This is consistent with the fact that deimination of rat filaggrin has previously been shown to induce its detachment from keratin filaments [49], and that human filaggrin is more efficiently proteolyzed *in vitro* by calpain 1 when it is deiminated [20, 50]. Moreover, partial

inhibition of PADs for 24 hours with the PAD inhibitor Cl-amidine (this work) tends to reverse the effect of culture humidity lowering on filaggrin metabolism (association with intermediate filaments and degradation), suggesting that deimination is necessary for filaggrin degradation to occur.

In line with these observations, it has been shown that genes encoding filaggrin, PAD1 and PAD3 are not present in the genome of fishes and amphibian [51, 52], animals living in extremely humid environment.

We previously described deimination as a multilock controlled process [43]. Here we show that, in normal keratinocytes, environmental dryness unlocks this process. The mechanisms involved remain to be deciphered, but the data reported by Sun *et al.* give a clue. Calcium concentration is probably involved as dry RHEs expressed larger amounts of osmolyte transporters and displayed a higher calcium concentration and a marked calcium increase at the granular layer level, with a calcium gradient similar to native epidermis [46]. On the other hand, PADs are known to be calcium-dependent, and in native epidermis the peak of calcium at the granular/lower cornified layers is suspected to activate the enzymes (deiminated proteins are only detected in the *stratum corneum*).

Do our data have any other relevance to native healthy human epidermis? In extremely preterm neonates, the lack of an effective skin barrier leads to high TEWL with a high risk of dehydration. A progressive decrease of ambient humidity (from 75% to 50% RH) after the first post-natal week has been shown to accelerate barrier development in the premature infants [53]. Workers exposed to low RH have a lower TEWL (reviewed in [54]). In both cases, an adaptive mechanism similar to the one described in this study is probably involved. Whether filaggrin degradation is affected is not known but can be suspected.

Do our data have any relevance for the understanding of AD? Climatic variations impact the disease, as recently discussed [54], but the mechanisms involved are incompletely understood. For example, a negative correlation between indoor RH and disease severity has been reported [55]; children with AD and *FLG* mutations have a higher severity score and their lesions are more often located in air-exposed skin areas [56]; *FLG* mutations are a weaker risk factor for AD in subtropical climates [57], and children with AD experienced a reduction in the disease severity after one month spent in such a humid climate [58]. In a large-scale study of the influences of US climate on childhood eczema prevalence, a lower prevalence has been found in areas with higher outdoor RH [59]. All these observations are consistent with our results showing that more filaggrin and more filaggrin proteolysis is required in a dryer environment. In fact, we can suspect that the epidermis of AD patients, when the patients change from a humid to a dryer environment, cannot adapt because it is unable to increase filaggrin synthesis/degradation, whatever the reason (*FLG* loss-of-function mutations, effects of Th2-type inflammatory cytokines, others). Therefore the epidermal barrier is more altered, and the disease worsens. On the opposite, when the patients move from a dry to a more humid environment, their epidermis needs less filaggrin to be effective; therefore their symptoms regress. Altogether, these data convince us that eczema predisposed children should be encouraged to increase their indoor humidity. Our data also suggest that PAD expression/activity could be of importance in AD, although no

genetic variations in *PADI* genes have been associated with the disease susceptibility until now. PADs could therefore constitute therapeutic skin targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

AD	atopic dermatitis
NMF	natural moisturizing factor
PAD	peptidyl-arginine deiminase
PCA	pyrrolidone-5-carboxylic acid
RH	relative humidity
RHE	reconstructed human epidermis
TEWL	transepidermal water loss
UCA	urocanic acid

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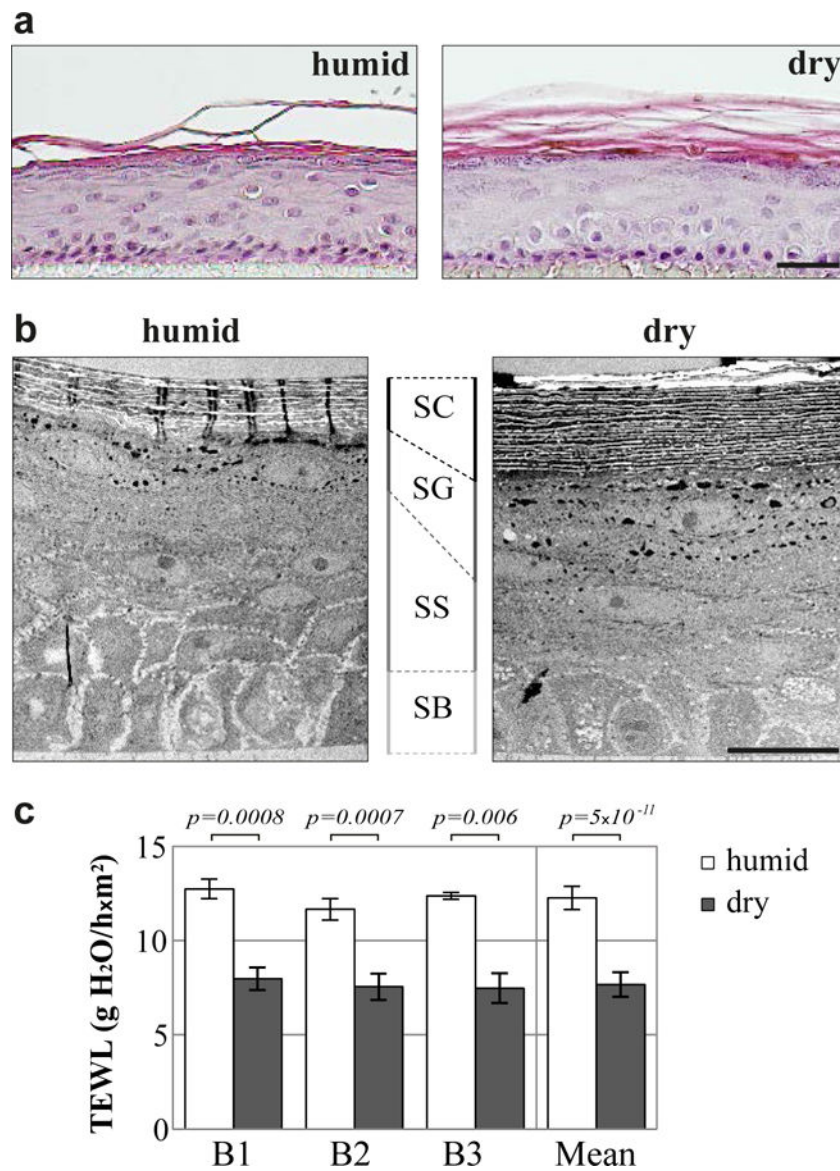
Fig. 1. Thicker *stratum corneum* and reduced transepidermal water loss in dry RHEs
(a and b) Hematoxylin-eosin staining (**a**) and transmission electron micrographs (**b**) of humid and dry RHEs produced with keratinocytes from 3 different donors, only one being illustrated. (**c**) Transepidermal water loss (TEWL) measured for humid and dry RHEs produced with keratinocytes from 3 different donors (B1–B3). Scale bars = 20 μm. SC: *stratum corneum*, SG: *stratum granulosum*, SS: *stratum spinosum*, SB: *stratum basale*.
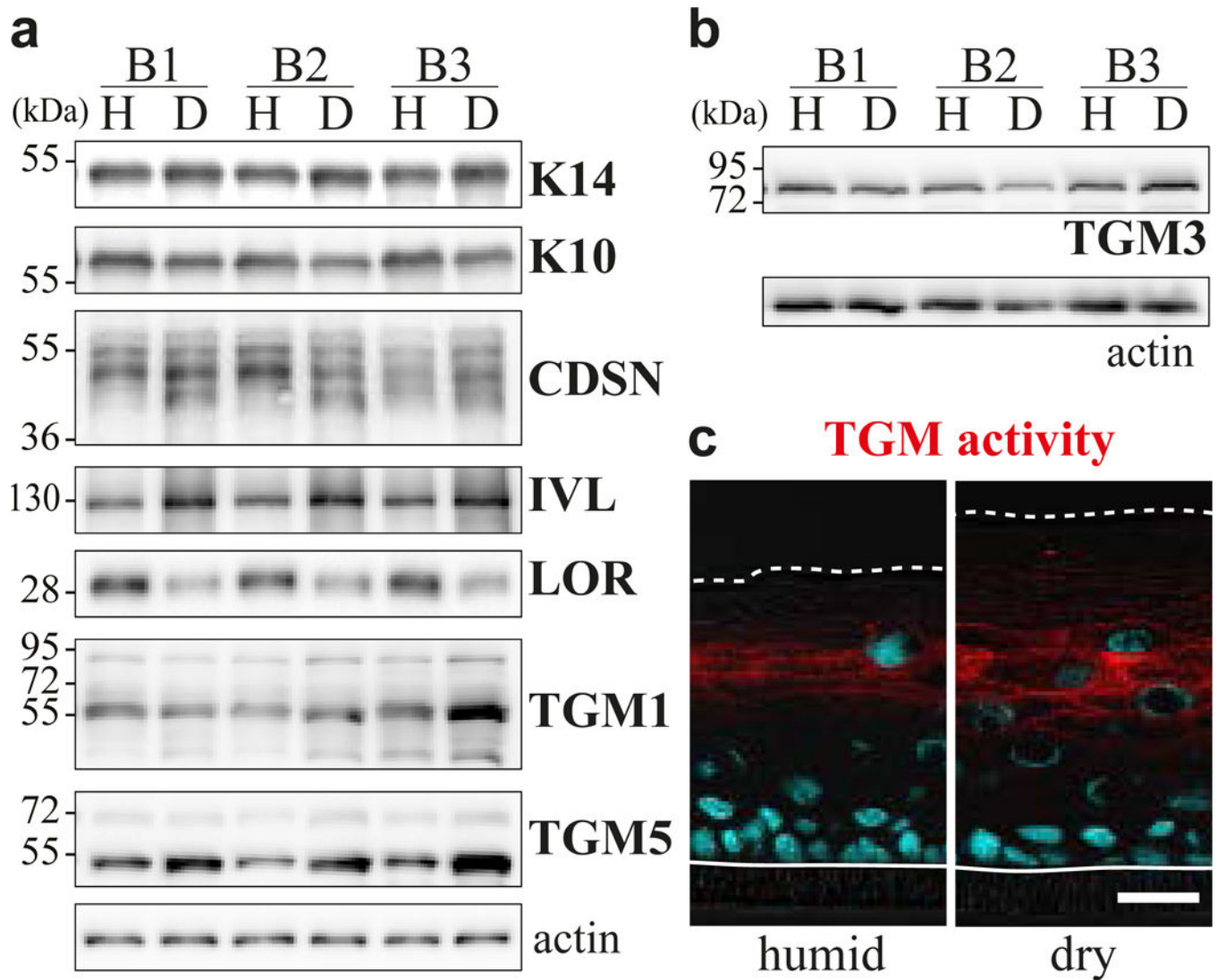
Fig. 2. Modulation of the expression of differentiation markers in dry RHEs

(a and b) Expression of actin (loading control), keratin14 (K14) and various differentiation markers analyzed by western blotting of either total **(a)** or TE-NP40 extracted **(b)** proteins of humid (H) and dry (D) RHEs produced with keratinocytes from 3 different donors (B1-B3). (K10, keratin 10; CDSN, corneodesmosin; IVL, involucrin; LOR, loricrin) **(c)** *In situ* transglutaminase (TGM) activity assay performed on cryosections of humid and dry RHEs produced with keratinocytes from 3 different donors, only one being illustrated. Scale bar = 20 μm.
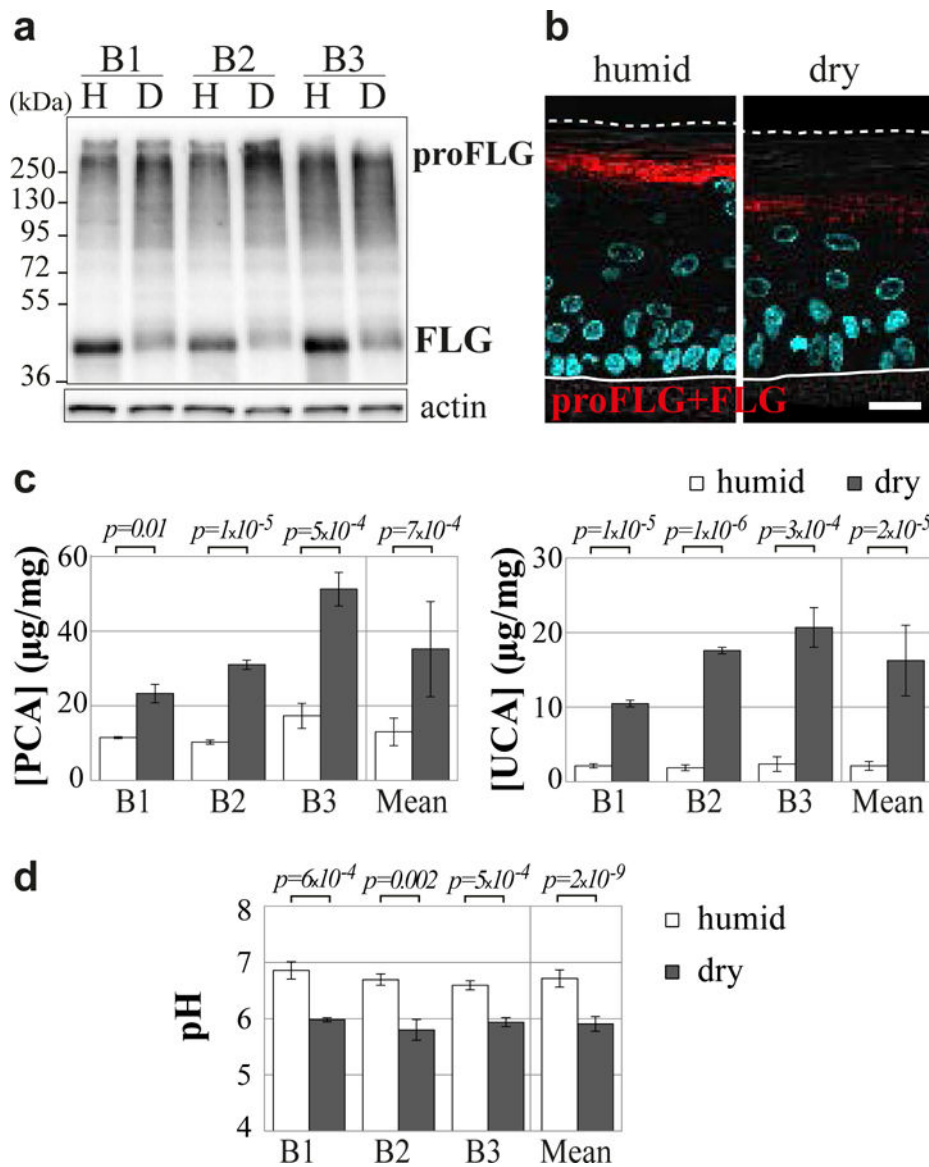
Fig. 3. Increased filaggrin breakdown in dry RHEs

(a and b) Humid (H) and dry (D) RHEs were produced with keratinocytes from 3 different donors (B1-B3) and analyzed. Immunodetection of profilaggrin (proFLG) and filaggrin (FLG) in total protein extracts **(a)** and on fixed-tissue sections **(b)**. Differential interference contrast images are superimposed; nuclei are stained in blue by DAPI. SC: *stratum corneum*, SG: *stratum granulosum*. Scale bar = 20 μm. **(c)** Amounts of pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA) in RHE lysates are expressed in μg/mg of proteins. **(d)** Surface pH.
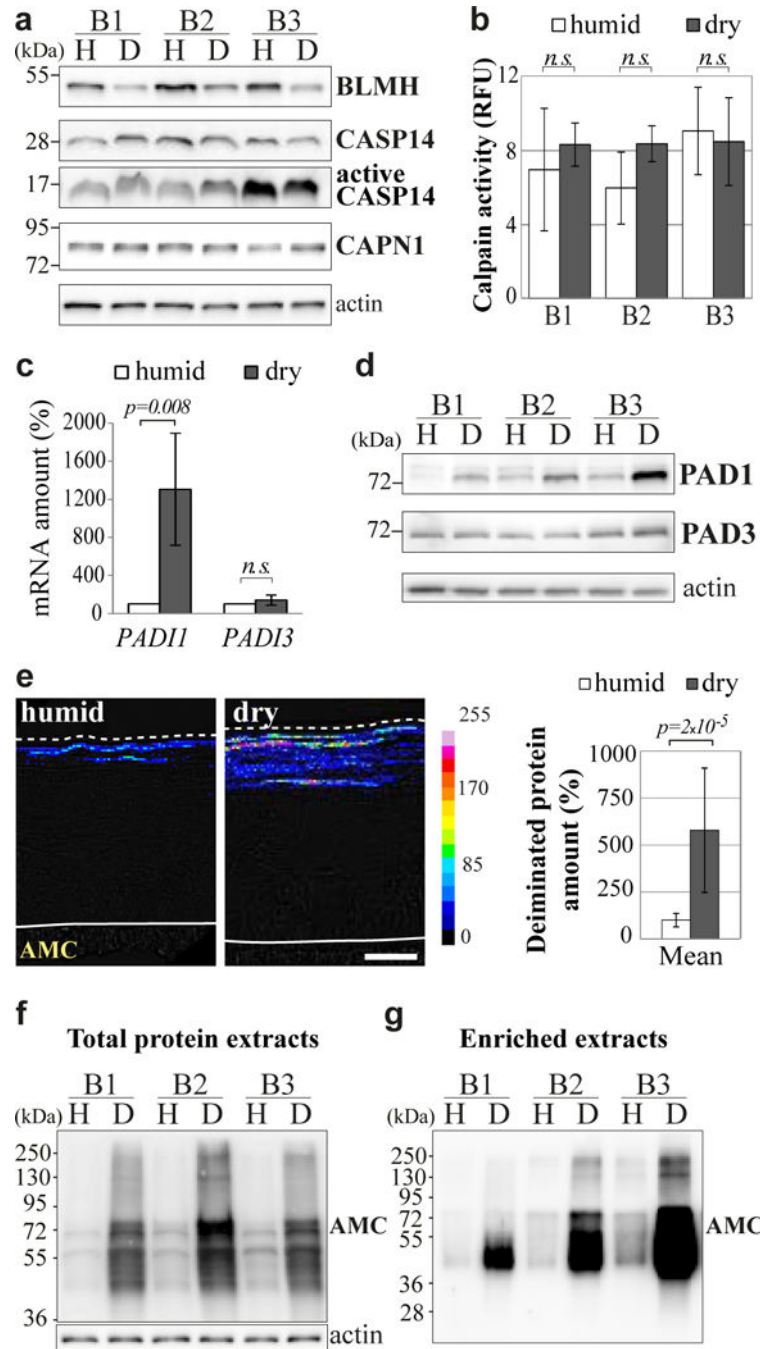
Fig. 4. Increased filaggrin deimination and peptidyl-arginine deiminase expression in the dry condition

(a) Expression of bleomycin hydrolase (BLMH), procaspase-14 (CASP14), active caspase-14 and calpain-1 (CAPN1) analyzed by western blotting of total proteins of humid (H) and dry (D) RHEs produced with keratinocytes from 3 different donors (B1-B3). **(b)** Calpain activity measured in lysates of humid and dry RHEs. Results are expressed as relative fluorescence unit (RFU). *n.s. = non-significant.* **(c)** Expression of *PADI* genes analyzed by RT-qPCR (4 different donors). **(d)** Expression of actin, PAD1 and PAD3

analyzed by western blotting of TE-NP40 extract of the RHEs. **(e)** Immunodetection of deiminated proteins with AMC antibody on sections of RHEs produced with keratinocytes from 3 different donors. A representative result is shown on the left. Quantification of the signal intensities is shown on the right. Scale bar = 20 μm. **(f and g)** Equal amounts of total proteins **(f)** and equal volumes of deiminated filaggrin-enriched extracts (**g**) were immunodetected with AMC. Note that deiminated filaggrin migrates in the form of a smear between 40 to 72 kDa (fully deiminated), as previously described [43].
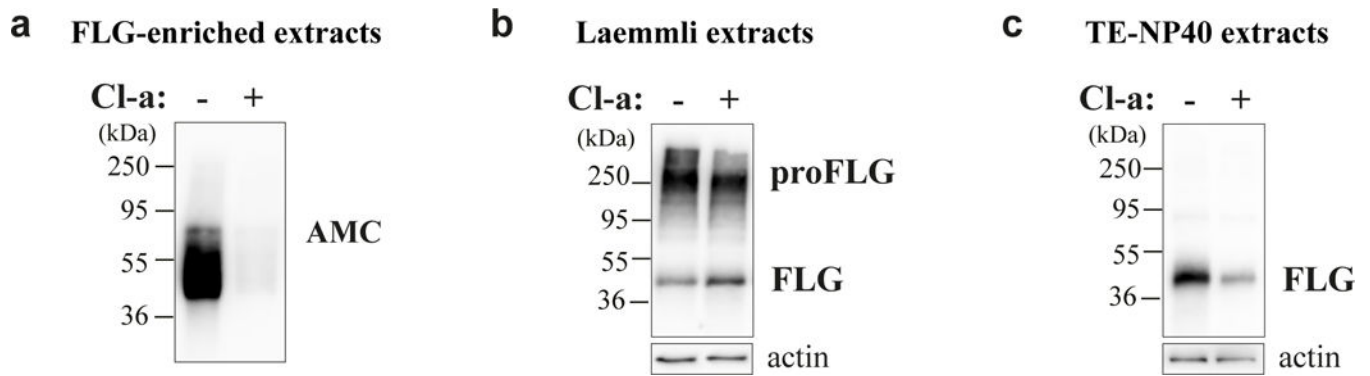
Fig. 5. Partial inhibition of deimination by Cl-amidine reverses the effect of dryness on filaggrin metabolism

(**a–c**) Dry RHEs produced with keratinocytes from 3 different donors were treated for 24 hours (+) with Cl-amidine (Cl-a) or were untreated (−), the results being illustrated for only one donor. (**a**) Equal volumes of deiminated enriched-filaggrin extracts were immunodetected with AMC antibody to detect the deiminated protein. (**b**) Total protein extracts were immunodetected with AHF3 antibody to detect both profilaggrin (proFLG) and filaggrin (FLG). (**c**) TE-NP40 extracts were immunoblotted with AHF3 to detect the low ionic strength-soluble form of FLG.
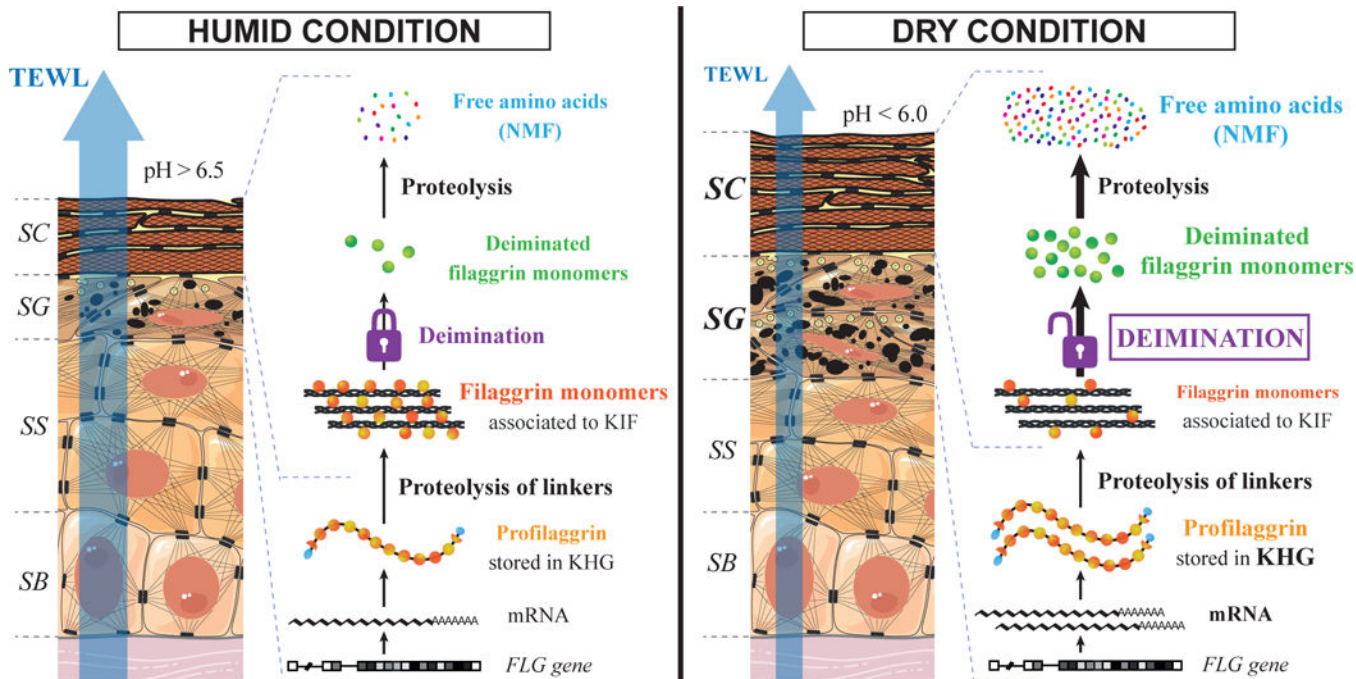
Fig. 6. Schematic representation of the effect of the dry condition on RHEs and filaggrin metabolism

For details, see the text. *SC: stratum corneum*, *SG: stratum granulosum*, *SS: stratum spinosum*, *SB: stratum basale*, *FLG: filaggrin*, KHG: keratohyalin granules, KIF: keratin intermediate filaments, NMF: natural moisturizing factor, TEWL: transepidermal water loss.