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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 peptidylarginine 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.
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.

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

Abbreviations: AD, atopic dermatitis; NMF, natural moisturizing factor; PAD, peptidylarginine deiminase; PCA, pyrrolidone-5-carboxylic acid; RH, relative humidity; RHE, reconstructed human epidermis; TEWL, transepidermal water loss; UCA, urocanic acid.

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[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 peptidylarginine 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 (2500× and 300× 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 (2500×) 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

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