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Skin barrier in atopic dermatitis

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1. ABSTRACT

The skin represents the largest organ of the body and provides a vital interface between the body and the environment. Hereditary and acquired alterations of structural proteins and lipids of the *stratum corneum* and epidermal tight junctions leading to a diminished skin barrier function are major causative factors for a number of skin diseases, in particular atopic dermatitis (AD). This review summarizes current knowledge on the role of the skin barrier in AD with regard to pathogenesis and treatment, on the relationship between skin barrier abnormalities and immune aberrations, and on potential therapies aimed at repair of the skin barrier. Furthermore recent advances in the genetics of AD will be addressed.

2. INTRODUCTION

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disorder with a wide spectrum of clinical presentations and combinations of symptoms. It affects up to 20 percent of children and 2-10 percent of adults and often predates the development of allergic airway diseases like rhinitis and asthma (1). Individuals with AD have genetically determined risk factors that affect both the skin barrier function and the immune response, and which interact with environmental factors. Skin barrier dysfunction, which can be inherited or acquired, is a major hallmark of AD, allowing for enhanced allergen and microbial penetration across the skin. The discovery that mutations in the gene encoding the *stratum corneum* (SC) structural protein filaggrin (*FLG*) are a remarkably strong risk factor for AD, underscores the importance of the skin barrier in AD. Among patients with moderate-to-severe AD, up to 46 percent to 57 percent carry 1 or more *FLG*-null mutations, and the population attributable risk fraction has been estimated at between 4.2 percent and 15.1 percent (2). Filaggrin is a major structural protein in the SC, crucial for the structural and biophysical integrity of the skin (3). However, a significant part of patients with AD does not carry any of the known FLG mutations thus other (epi)genetic factors important for the homeostasis of the skin barrier and the immune system are obviously important. Recent data from genome-wide association studies (GWAS) identified novel risk loci in genes responsible for epidermal barrier function and the adaptive and innate immune system (4). Though, known genetic risk factors only account for less than 10 percent of the heritability of AD (4). Apart from filaggrin, other epidermal proteins have been shown to play a role in altered barrier function of AD skin e.g. proteins of the cornified envelope and tight junctions. Furthermore, recent studies have shed more light on the alterations of the lipid

composition and structure in AD and their relevance for the skin barrier function.

This review will summarize recent insights in the role of SC proteins and lipids for the skin barrier in AD and genetic variations conferring increased risk for this common inflammatory disease.

3. GENETICS OF AD

The complex etiology of AD is based on a strong polygenic background and environmental factors that precipitate susceptibility into disease manifestation, the interaction of genetic and environmental factors (5). A total of six linkage studies for AD have been conducted in the past, which identified various putative disease susceptibility regions. However, the results from these genome screens largely differ from one another, and under a threshold of no more than 10 cM distance between linkage peaks replication can only be considered for one locus on chromosome 3p24 (6). With the exception of the *FLG* gene, which partly explains the linkage signal observed on chromosome 1q21 (7), no disease gene could be unambiguously assigned to the reported linkage regions so far.

More than 40 associated risk genes for AD have been reported by candidate gene association studies (8), but most of these studies lack a stringent replication and have to be interpreted with caution. Only a small number of reported susceptibility genes can be considered as established, and the majority of these validated risk genes are implicated in the context of atopic immune dysregulation and not as phenotype-specific factors for AD. A breakthrough in the genetics of AD was achieved with the identification of null mutations within the *FLG* gene through positional cloning. In 2006, Irwin McLean and his group showed that two variants (R501X, 2282del4) cause the monogenic keratinization disorder Ichthyosis vulgaris with a semidominant mode of inheritance (9). Subsequently they could demonstrate that these variants are also strong risk factors for AD (10-13). In the meantime, more than 40 additional *FLG* variants have been identified, all of which are functional null mutations causing a lack of biologically active filaggrin peptides in the skin (10). About 8 percent of the European population and 25 percent of AD patients carry *FLG* mutations, which increase the risk for AD more than 3-fold (11-12). The specific characteristics of the epidermal barrier defect caused by the lack of filaggrin and its exact mechanisms are not fully understood yet. Filaggrin peptides are crucial for cornification of keratinocytes by bundling keratin filaments, and they appear also important for the synthesis and secretion of lamellar bodies (14-15). Further, it has been shown that filaggrin deficiency is accompanied by decreased natural moisturizing substances (16) and decreased skin hydration and increased skin pH (17). In flaky tail mice having double-homozygous *FLG* and *matted* mutations, the penetration of allergens and antigens through the skin is enhanced (14), and *filaggrin* knockout mice show an enhanced hapten-induced contact allergy and ovalbumin-induced immune response (18). Clinically, it appears that might be a filaggrin-related yet no fully characterized endophenotype of AD comprising e.g. a generalized xerosis, an early onset of the disease, and an increased susceptibility towards allergic sensitization and concomitant asthma and/or rhinitis (3, 11).

These observations led to a change in pathogenetic concepts for AD in that a primary skin barrier defect sets the pace for the development of the disease, allergic sensitization and concomitant asthma in the context of AD (3). However, it should be noted that only approximately 20-25 percent of AD patients carry *FLG* mutations, and that 60 percent of *FLG* mutation carriers do not develop AD (19). That is to say that *FLG* mutations are neither necessary nor sufficient to develop AD, and that additional genetic and environmental factors are needed for disease development.

Using hypothesis-free genome-wide approaches, two large European studies identified four additional susceptibility loci, where common variants showed consistent association with AD on chromosome 11q13 (20), 11q13.1, 19p13.2 and 5q31.1 (4). The associated polymorphism on 11q13 is located in an intergenic region between the two protein coding genes *C11orf30* and *LRRC32*, which have been implicated in epithelial immunity and differentiation (EMSY/*C11orf30*), and the function of regulatory T-cells (GARP/*LRRC32*). The association signal on 11q13.1 points towards *OVOL1*, a member of a highly conserved gene family known to influence the development and differentiation of epithelial tissue and germ cells. The gene product interferes with terminal differentiation of keratinocytes by regulating the expression of Loricrin (*LOR*). The associated marker on chromosome 19p13.2 is flanked by the genes *ACTL9* and *ADAMTS10*. *ACTL9* encodes an actin-like hypothetical protein of unknown function, but actin proteins in general are known to be of importance for cytoskeletal functions, e.g. maintenance of epithelial cell shape and cellular movement. *ADAMTS10* belongs to a group of secreted zinc-dependent metalloendopeptidases which plays an important role in extracellular matrix organization, inflammation and cell migration. On chromosome 5q31.1, two independent signals from clusters of linked SNPs mapping to IL4/KIF3A and to IL13/RAD50 were observed.

Two further GWAS carried out in populations of Chinese Han (21) and Japanese (22) revealed a variety of further susceptibility loci on chromosome 5q22.1 (*TMEM232/SLC25A46*), 20q13.33 (*TNFRSF6B/ZGPAT*), and 2q12 (*IL1RL1/IL18R1/IL18RAP*), 3p21.33 (*GLB1*), 3q13.2 (*CCDC80*), 6p21.3 (*GPSM3, MHC*), 7p22 (*CARD11*), 10q21.2 (*ZNF365*), 11p15.4 (*OR10A3/NLRP10*) and 20q13 (*CYP24A1/PFDN4*), respectively. Considering the assumed heterogeneity of genetic risk variants between different ethnicities it should be noted that the associated variant on chromosome 20q13.33 could be validated in an independent German replication cohort (21). These findings from genome scans support the view that genetic factors involved in AD contribute to both the disturbed epidermal barrier function as well as the abnormal reactivity of the adaptive and innate immune system, although the magnitude of both dimensions might be different in different patient subgroups.

Given that for AD the phenotypic variance attributable to heritable factors has been estimated approximately 80 percent, the continuous extension of the

genetic risk map will continue to shape our understanding of its complex etiology.

4. IMMUNOLOGY OF AD

Immunologic modifications in AD are complex. A key feature is the expression of the high affinity receptor for IgE by different epidermal and dermal dendritic cell (DC) subtypes, such as Langerhans cells, inflammatory dendritic epidermal cells and inflammatory dermal cells (23). This enables the cells to take up IgE and via IgE allergens in order to process them, migrate to the lymph nodes and present them to T cells to prime T cell responses. While Th2 cytokines dominate in the acute phase, Th1, as well as Th17 and Th22 cytokines are detectable in chronic AD lesions (24). It has been demonstrated that unstimulated skin DCs are in principle capable to polarize any kind of T cell response (25). The local micromilieu and the nature of ligands stimulating DCs are decisive for the type of T cell immune response, which is finally primed.

Genetic modifications in the *IFNG, IFNGR1* as well as *IRF2* gene (26-27) as well as loser IFN-*alpha*R expression by DCs and consequently lower responsiveness of those DCs to IFN-*alpha* might contribute further to the lack of counterregulatory Th1 immune responses and dominance of Th2 immune responses in particular stages of AD (28).

Other factors such as Thymic stromal lymphopoetin (TSLP) released by keratinocytes have been demonstrated to stimulate DCs and to amplify their capacity to prime Th2 immune responses in AD (29).

A Th2 dominated micromilieu in the acute phase of AD is a hallmark of the disease and responsible for numerous modifications of innate and adaptive immune responses.

Lower expression of several antimicrobial peptides such as human beta defensins and cathelicidin might result from this Th2 prone micromilieu (30-31), since downregulation of those factors by IL-4 or IL-13 has been demonstrated in *in vitro* studies. The same is true for epidermal barrier proteins such as S100 protein or filaggrin (32-33), so that besides genetic factors, the character of the micromilieu might also play an important role in this context.

5. SKIN BARRIER FUNCTION IN AD

Skin is a highly specialized, complex, and efficient barrier to loss of water from the body ("inside-outside barrier") and ingress of exogenous compounds into the body ("outside-inside barrier"). The outermost layer of the epidermis, SC, forms the primary skin barrier. Although compounds have three potential diffusion routes across the SC (transcellular, intercellular and appendageal pathways), it has been widely accepted that intercellular pathway through lipid bilayer matrix, represents the principal route (34).

The extent and rate of dermal absorption are dependent on physico-chemical properties of a penetrant, exposure conditions and the condition of the skin barrier. Due to lipophilic nature of the SC, lipophilic penetrants partition more readily into the SC than hydrophilic penetrants (35). Once absorbed, a compound will diffuse across the SC by a rate which is mainly determined by its molecular size (35). It is generally considered that the molecular size cut-off for effective permeation across skin is approximately 500 Da (36). This 500 Da rule can, however be challenged in the case of impaired skin barrier. It is likely that reduced skin barrier which is a common feature of atopic skin will lead to a higher absorption and absorption of compounds that normally would not be able to cross a healthy, intact skin.

Skin barrier function is usually assessed by measuring trans-epidermal water loss (TEWL) or by determining percutaneous penetration of a model compound. Although TEWL reflects diffusion of a small hydrophilic molecule from the skin and might not be representative for dermal absorption of highly lipophilic and/or large size compounds (37), TEWL is extensively used in clinical studies as a general indicator of the skin barrier function. Several studies reported higher TEWL in AD patients in lesional (38-39), as well as, in clinically unaffected skin (17, 40-45). The magnitude of barrier impairment in unaffected skin was shown to correlate with disease severity and IgE levels (43, 46). Though, in several studies no elevated TEWL in the unaffected or completely healed atopic skin has been found (38, 44, 47-49), suggesting that decreased skin barrier function might not be inherent but rather a secondary event caused by inflammation of the skin (50). A possible reason for the reported inconsistencies in the barrier function of unaffected skin might be insufficient sensitivity of TEWL to detect subtle differences in barrier function between healthy and clinically unaffected atopic skin. Furthermore, it is still a point of debate whether TEWL which reflects inside-out barrier is a good predictor of the barrier to ingress of skin irritants and allergens which is clinically more relevant (51).

Whereas most of publications focused primarily on TEWL, the characterization of the epidermal barrier properties by measuring percutaneous penetration is scarce. In the study of Jensen *et al*., skin penetration of hydrophilic dye was substantially increased in lesional skin of AD patients compared to healthy subjects (52). Interestingly, dye penetration was significantly reduced after topical treatment with pimecrolimus and betamethasone, suggesting skin barrier repair although electron microscopy showed disruption of the SC lipid layers and lamellar body extrusion (52). Consistently, Hata *et al*., (53) showed higher permeability of uninvolved skin of AD patients for both, lipophilic and hydrophilic dyes and increased penetration of hydrophilic dye significantly correlated with severity of AD. These results are consistent with findings of Jakasa *et al*. (45, 54) showing higher permeability for sodium lauryl sulphate (SLS) and polyethylene glycols (PEGs) of different molecular weights in uninvolved skin of AD patients compared to healthy control subjects.

Discovery of loss-of-function mutations in the gene encoding epidermal protein filaggrin, which is reported to be the major predisposing factor for AD (55) brought up a question whether altered skin barrier would be limited only to carriers of *FLG* mutations. In the study of Jakasa *et al*. (56)

uninvolved skin of AD patients showed higher permeability for PEG molecule of 370 Da in comparison to healthy skin, irrespective of their *FLG* genotype. No significant differences in the skin barrier between filaggrin-AD and non-filaggrin AD were confirmed also in several studies measuring TEWL (17, 40, 42, 56-57), and so far only one study (57) reported higher TEWL in the carries of *FLG* mutation as compared to *FLG* wild type individuals.

As intercellular lipid bilayers are the principal diffusion route for most of compounds, enhanced skin permeability of AD skin has been linked to a different intercellular lipid composition and structure of the SC. Reduced ceramide (CER) content (38) and CER chain length (41), as well as decreased percentage of certain classes of CERs (17) have been found in both, lesional and nonlesional skin of AD patients. These changes correlated with reduced skin barrier as assessed by TEWL and with inflammation. Interestingly, altered lipid organization was not associated with *FLG* genotype but rather with the levels of filaggrin degradation products in the SC (41).

In conclusion, there is considerable evidence that AD patients have a defective epidermal permeability not only in lesional skin, but also in visibly unaffected skin areas. This has been shown on the basis of elevated TEWL and percutaneous penetration confirming a defective barrier in both directions. Barrier impairment seemed to be a common feature of AD irrespective of *FLG* mutations, but the degree of impairment is influenced by disease severity.

Future studies in well-defined subgroups are needed to give more insight in the relative contribution and relationship between *FLG* genotype, disease severity and SC lipid organization for the skin barrier function.

6. LIPIDS AND AD

The skin barrier is mainly located in the SC, which consists of corneocytes embedded in a lipid matrix arranged in stacked layers (58-59). These lipids are predominantly divided into three different classes: CER, cholesterol and free fatty acids (FA). The CER can be further divided into 12 subclasses (CER 1–12) (60), as well as according to chain length (61-62). The synthesis of lipids and the differentiation of keratinocytes are regulated by enzymes, pH and calcium gradient (63). CERs are crucial for the characteristic lipid bilayer organization, skin barrier function and therefore important for all transport trough the SC (64-65).

AD is the first disease where different groups in the 1990s reported major differences for CER subclasses as compared to healthy skin (66-67). They found lower levels of CER 1 and 3, as well as a lower CER/cholesterol ratio for non-lesional atopic skin (66-70). Recently, it has been shown by Ishikawa *et al*. that especially, the CER with very long-chain FAs are markedly reduced in SC with impaired skin barrier function and the average chain length of FAs of CERs was negatively correlated with TEWL in AD patients, suggesting that biosynthesis of the FA may be dysregulated in AD (38). The dominance of CER with shorter FAs (C < 22) over those with longer (> C24) FAs in the SC of AD has been confirmed in a hapten-induced AD mouse model (71). The altered profile of CER was assigned to the downregulation of elongases which mediate biosynthesis of very long FA. Janssens *et al*. (41) performed recently a comprehensive analysis of the SC CER composition and lipid organization in non-affected skin areas of AD patients and control subjects. This study provided further insights in the role of chain lengths for the lipid organization and skin barrier function. Consistently with the studies of Ishikawa (38) and Park (71), it has been demonstrated that the level of CER with an extremely short chain length is drastically increased in SC of AD patients, which was correlated with an aberrant lipid organization and a decreased skin barrier function. Furthermore, changes in SC lipid properties correlated with disease severity. The reduction in CER chain length found in the study of Janssens *et al*. (41) had a much stronger impact on the skin barrier function than did the changes in CER subclass levels: there was a clear positive correlation between TEWL increases and decreasing chain length. These findings suggest that the CER composition and chain length rather than the ratio between lipid classes play a major role in the increased TEWL in nonlesional skin in patients with AD (41).

With the discovery of the importance of the *FLG* mutations for AD the question arose whether SC lipids are influenced by *FLG* genotype. Breakdown products of filaggrin contribute to the acidic pH of the SC (17) which in turn regulates activity of various enzymes involved in the synthesis of SC CERs (72-75). However, recent studies, which have subdivided AD patients according to their *FLG* genotype, found no significant differences in the composition or organization of SC lipids (17, 41, 42). Though, in a study of Janssens *et al*. (41) the composition and organization of SC lipids was associated with the levels of filaggrin breakdown products although there was no association with *FLG* genotype (41). Possible explanation of the absence of the effect of *FLG* mutations on the SC lipids can be explained by other factors than *FLG* mutations which can influence the filaggrin expression such as *FLG* copy numbers (76) and inflammation (77-78). To get more insight into the interplay between inflammation, filaggrin expression and activity of key elongases on the profile and organization of SC lipids and skin barrier, further investigations in well defined sub-groups of patients and controls are needed.

7. INVOLVEMENT IN AD OF COMPONENTS OF THE CORNIFIED CELL ENVELOPES, INCLUDING MEMBERS OF THE S100-FUSED TYPE PROTEIN FAMILY DISTINCT FROM FILAGGRIN

Consistently with an impairment of the epidermal barrier as an essential factor in AD, an abnormal structure of the SC has been described, with decreased compaction/cohesion of the layer, altered aspect of the corneocyte cytoplasmic matrix, partial disruption of cornified cell envelopes, reduced/modified intercellular lipids, and increased TEWL (79). Since increased TEWL and percutaneous penetration are characteristics of the disease, irrespective of the patient *FLG* genotype (56), other genetic, environmental or humoral factors that modulate the epidermal barrier functions seem to be involved in the pathogenesis of AD. In line with this hypothesis, a GWAS has revealed an

association of AD with a genetic variant distinct from *FLG* but also located on chromosome 1q21 in the 2-Mb region known as the Epidermal Differentiation Complex (80). This region comprises sixty genes, most of them encoding structural and regulatory proteins that are of crucial importance for keratinocyte differentiation and SC properties: intermediate filament-associated proteins, calcium-binding proteins and components of the cornified cell envelopes (81). In particular, a single nucleotide polymorphism of the *HRNR* gene and an insertion in the *SPRR3* gene have been identified as AD susceptibility factors (80, 82).

HRNR, located near *FLG*, encodes hornerin (also known as S100A18), a member of the S100-fused type protein family. It is a basic (predicted isoelectric point = 10.05) protein of 2850 amino acids with a predicted molecular mass of 282 kDa. Hornerin shares many properties with profilaggrin: a closely related structural organization with an amino-terminal domain homologous to S100A calcium-binding proteins and a large basic central repetitive domain, a comparable amino-acid composition with high Ser, Gly, His and Gln content (as a whole they correspond to 77 percent and 59.2 percent of the total amino acids of hornerin and profilaggrin, respectively), a similar pattern of expression and localization in the epidermis restricted to the upper layers, and analogous proteolytic processing. Hornerin, like part of filaggrin, is incorporated into the cornified cell envelopes, most likely through the activity of transglutaminase 3. Hornerin function is probably to reinforce the envelopes and to contribute to the mechanical resistance of the SC. In addition, some hornerin-derived peptides display antimicrobial properties *in vitro* (81, 83). The expression of hornerin is strongly reduced in both non-lesional and lesional atopic skin (84). *HRNR* is a highly polymorphic gene, but no non-sense mutations have been identified yet. However, a single nucleotide polymorphism (rs877776; C/G substitution) located 7 kb downstream of the coding sequence has been reported as an AD susceptibility variant (80). Whether this polymorphism could modify either the transcription or the stability of *HRNR* mRNA is not known. Since a number of non-coding sequences with high keratinocyte- and differentiation-specific enhancer activity over a long distance have been identified throughout the locus (85), we can suspect this to be the case. Altogether, these data strongly suggest that abnormalities in hornerin expression contribute to alterations in the biomechanical properties of cornified cell envelopes and therefore to the epidermal barrier defects associated with AD. This hypothesis deserves to be tested experimentally.

The small proline rich proteins, encoded by the *SPRR* genes, form a class of ten keratinocyte-specific closely related proteins, the expression of which is strongly induced during differentiation. They all consist of a conserved amino-terminal domain, three to sixteen 9-8 amino acids long tandemly repeated units that are highly enriched in proline, and a conserved carboxy-terminal domain (81). In particular, SPRR3 (also called esophagin, cornifin-beta or 22-kDa pancornulin) is a 169 amino acids long protein (18.2 kDa). All small proline-rich proteins are cornified cell envelope components. Their head and tail regions are essential for cross-linking by transglutaminases. For example, in SPRR3, only Gln^{4}, Lys^{6}, Gln^{17} and Gln^{25}, and Glu^{158}, Lys^{161} and Lys^{163} are used by these enzymes (86). In the cornified cell envelope, the ratio of amounts of loricrin to small proline-rich protein varies from one body site to another. This is suspected to confer various mechanical properties on the structure (87 and references therein). In addition, the tightly regulated expression of *SPRR* genes seems to be part of an adaptive tissue response to environmental stress. In line with this assumption, some small proline-rich proteins have been reported to quench reactive oxygen species (88), and some *Sprr* genes, namely *Sprr2a* and *2h*, are upregulated in the epidermis of loricrin-deficient mice (89). In 2011, an association screening of the Epidermal Differentiation Complex identified a variant of *SPRR3* as a risk factor for dermatitis. The association has been replicated in a large cohort of 1,314 cases with early-onset dermatitis from central Europe and 1,322 controls, yielding an odds ratio of 1.3 (82). The AD-associated SPRR3 protein variant carries an insertion (CTKVPEPG-ins) of 8 amino acids in its central repetitive domain. Whether and how this extra-repeat could alter the epidermal barrier function is unknown.

In other recent genetic studies, mutations in two other genes encoding proteins important for the SC have been associated with AD: a protease involved in desquamation, named kallikrein 7, and the so-called LEKTI inhibitor of kallikreins (see the other chapters of this review for a discussion of the exact relevance of these observations for AD).

Non-sense mutations of *FLG* induce a reduction in the expression and even an absence of the corresponding protein. But filaggrin expression is also reduced in AD patients without *FLG* mutations. This may be due to the effect of pro-inflammatory cytokines. In fact, IL4, IL13, IL22 and IL25 reduce the levels of filaggrin, hornerin, involucrin and loricrin mRNAs and proteins in cultured keratinocytes (84, 90). Such treatments have been shown also to down-regulate the expression of a gene tightly related to *FLG* and located in the Epidermal Differentiation Complex near *FLG*, i.e. the *FLG2* gene (84). Like filaggrin and hornerin, filaggrin-2 is a member of the S100-fused type protein family. Its large central repetitive domain contains two types of 75-77 amino acids long repeats: nine A-type repeats highly homologous to hornerin subunits (50-77 percent identity), and fourteen B-type repeats closer to filaggrin monomers (28-39 percent identity) (81). In the epidermis, filaggrin-2 is mainly detected in the keratohyalin granules of the upper granular keratinocytes, located with profilaggrin, and in the intracorneocyte fibrous matrix of the lower corneocytes, located with filaggrin. Both proteins disappear concomitantly in the upper cornified layer (91). Filagrin-2 is synthesized as a large precursor with an apparent molecular weight of 250,000 in SDS-gels. This form, probably accumulated in the keratohyalin granules, is then proteolytically processed to fragments of 130 kDa and smaller. One of the proteases involved in the degradation of filaggrin and/or its precursor, i.e. calpain 1, is also implicated in the cleavage of filaggrin-2. Furthermore, the deimination of filaggrin-2 B-type repeat domain promotes its proteolysis by calpain 1 into numerous small peptides (91). The role of filaggrin-2 in the epidermis is still unclear. However, the above mentioned data suggest that filagrin-2 and filaggrin have similar functions in the epidermis. Thus filaggrin-2 may contribute to SC hydration and photoprotection properties. Since a recombinant fragment of mouse filagrin-2 has been

shown to interact with and bundle keratin filaments *in vitro*, filaggrin-2 may also contribute to the aggregation of intermediate filaments to form the intracellular corneocyte matrix. Finally, the high Gln content (14.9 percent) of the A-type repeats of filaggrin-2 together with the detection of two peptides derived from filaggrin-2 in purified cornified cell envelopes suggest that part of the protein is cross-linked to these structures. Comparative proteomic profiling of superficial extracts of the epidermis of AD patients has revealed that filaggrin-2 is detected at significantly lower levels in lesional than in non-lesional skin (92). Immunohistological and Western blotting analysis have demonstrated that filaggrin-2 is downregulated in lesional but also non-lesional atopic skin (84). Together, these data suggest that the barrier defects observed in the skin of AD patients is partly due to a reduction in filaggrin-2 expression.

High throughput analyses have identified broad defects in terminal differentiation of keratinocytes in AD. In particular, a reduction in the expression of numerous cornified envelope components, including loricrin and other proteins, such as LCE1, LCE2, cornifelin and sciellin, has been observed in a transcriptomic analysis. This is true for both the lesional and non-lesional skin of the AD patients (79, 93). The comparative proteomic profiling reported above has shown that components of corneodesmosomes, i.e. corneodesmosin, desmoglein-1 and desmocollin-1 (92), are reduced in lesional as compared to non-lesional sites. In addition, the mRNA level of cornulin in the skin is decreased in dermatitis, both in a Der-p2 induced mouse model and in AD patients (94). Cornulin is another filaggrin -related protein. Its function is not clearly known, even if some data suggest it is a cornified cell component. Therefore, further studies are needed to test whether the reduction in this particular protein contributes to the disease.

8. FILAGGRIN DEGRADATION PRODUCTS IN AD

Filaggrin plays a central role in the maintenance of the SC hydration and water retention as it is a predominant source of free amino acids and their derivatives contributing more than 50 percent to total content of natural moisturizing factor (NMF), a complex mixture of hygroscopic compounds (95-96). By regulating water content in the SC, NMF has important function in regulation of the activity of enzymes crucial for maintaining of epidermal barrier homeostasis. Furthermore, components of the NMFs contribute to the elasticity of the skin through interaction with keratin and keratin filaments (97). Dry skin, accompanied by reduced flexibility is a major hallmark of AD.

Protein filaggrin is formed by a cascade of chemical changes of its precursor profilaggrin, a large, histidine-rich and heavily phosphorylated protein with highly repetitive structure. At the interface of the *stratum granulosum* (SG)/SC profilaggrin is dephosphorylated and proteolytically processed into multiple filaggrin monomers (97-98) which bind to and aggregate keratin filaments into keratin fibrils within cytoskeleton of corneocytes contributing to mechanical strength and integrity of the SC (99). During the final stages of epidermal differentiation, filaggrin is deiminated by peptidylarginine deiminase into citrulline, changing the net charge of filaggrin molecule. This leads to disruption of the filaggrin-keratin aggregation (100-101) and subsequent degradation into free amino acids. Processing and degradation of filaggrin into amino acids is mediated by a number of proteases with a prominent role of caspase-14 (102). As shown by i*n vivo* Raman spectroscopy and biochemical analysis the levels of filaggrin degradation products are significantly reduced in caspase-14$^{-/-}$ mice compared with wild-type mice although profilaggrin was fully processed to filaggrin (103).

One of the most abundant amino acids in filaggrin is histidine which is further deaminated by histidase into *trans*-urocanic acid (*t*-UCA). *T*-UCA is a major UV absorbing chromophore in the SC. Upon exposure to UV, *t*-UCA is converted into *cis*-UCA which suppresses immune response (104-107). Caspase-14$^{-/-}$ mice deficient in UCA showed increased susceptibility to UV (103). UCA has also been suggested to contribute to acid mantle of the skin, although this view has been challenged based on studies with histidase- and filaggrin deficient mice showing no alterations in SC pH despite of reduced UCA levels (108). Though, individuals with *FLG* mutations showed elevated skin surface pH and furthermore pH values were inversely correlated with the NMF levels (17, 77, 109). Elevated pH might lead to altered activity of proteases and enzymes within the SC which are involved in biosynthesis of lipids, desquamation and cleavage of the precursors of IL-1 cytokines into their active form (110). NMF levels were inversely correlated with IL-1 cytokines concentrations in the uninvolved skin of patients with moderate-to-severe AD (77). These findings were supported by upregulated expression of IL-1b and IL-RA in filaggrin-deficient mice (flaky tail mice; *ft/ft*) suggesting that reduced filaggrin levels might cause a pre-existing or enhanced proinflammatory status in the skin (111) although causal relationship between pH and IL-1 cytokines still has to be elucidated.

Another important product of filaggrin degradation is pyrrolidone-5-carboxylic acid (PCA) which is derived from glutamine and glutamic acid. PCA is highly hygroscopic compound and the most abundant single constituent of NMF (contributing for more than 10 percent) often regarded as a predictor of total NMF level in the SC (95).

Although the importance of NMF in the skin hydration and water retention and its relevance in filaggrin processing has been known for some time (95-96), the discovery of *FLG* loss-of-function mutations contribute to better understanding of these relationships. *In vivo* study employing Raman confocal microscopy showed that the carriers of *FLG* null mutations have reduced NMF levels on different body locations and SC depth as compared to non-carriers (16). This was confirmed also by biochemical analysis of PCA and UCA in the SC tape stripped samples of individuals of different *FLG* genotype (40,112). In a large cohort of AD patients O`Regan *et al*. showed that the levels of NMF were related to *FLG* mutations (98). The association between NMF levels and the number of *FLG* mutations was sufficiently strong to discriminate three endophenotypes within AD (98-99). In addition to *FLG* loss-of-function mutations, Brown *et al*. (76) showed recently that also intragenic copy number variations (CNV) in *FLG* are related to the NMF levels. The frequencies of CNV have been identified in Irish children as a risk factor for AD with a dose-dependent effect.

In addition to genetic variations, reduced filaggrin expression can be acquired, likely due to the T_H2 cytokine milieu in AD (78, 111). Filaggrin expression was lower in patients with AD regardless of *FLG* genotype (78) and furthermore Th2 cytokines downregulated *FLG* expression in differentiated keratinocytes. This is consistent with reduced levels of NMFs found in AD patients wild-type for *FLG* mutations as compared to wild type healthy individuals (40-41, 113). Severity of the disease was shown to significantly influence NMF levels in the SC, although to a minor extent compared to *FLG* genotype (40). Interestingly, it has been shown recently that the levels of NMF in the SC are associated with altered composition and structure of SC lipids (41, 65), although a relationship between lipid changes and *FLG* genotype could not be demonstrated. This points on the importance of acquired deficiency of filaggrin on the skin barrier.

In conclusion, although it is obvious that filaggrin degradation products interfere with key processes and structures in the SC, their roles in the modulation of the skin barrier function and aberrant immune responses in AD still need to be elucidated.

9. TIGHT JUNCTIONS IN AD

Tight Junctions (TJs) are complex multi-protein structures well known from simple epithelia to connect neighboring cells to form a paracellular barrier. Their components can be classified into transmembrane and cytoplasmic plaque proteins. The transmembrane proteins comprise the families of claudins, TAMPs (tight junction-associated marvel proteins) and JAMs (junctional adhesion molecules). The claudins (27 members in mammals) are the main determinants of the tightness and selectivity of the paracellular barrier. For instance, depending on the claudin composition, a tight junction can be permeable or tight for anions, cations or water. TAMPs embrace, among others, occludin and tricellulin. Occludin is important for the regulation of tight junctions. Tricellulin is a protein predominantly localized at tricellular tight junctions (tTJs) and has recently been shown to be involved in the passage of macromolecules through tTJs. Members of the JAM family are important for the formation of tight junctions, but have also been shown to be involved in transmigration of immune cells and to be misemployed as receptors for virus entry. The TJ cytoplasmic plaque proteins comprise a variety of proteins involved in scaffolding (e.g. MUPP1, ZO-1-3), cell polarity (e.g. aPKC/Par3/Par6), vesicle transport (e.g. Rab proteins), and signalling (e.g. c-jun) (for reviews see (114-115).

A large variety of TJ proteins or their respective messenger RNAs have been identified in human and/or mouse epidermis, e.g. claudin-1, claudin-3 (mouse), claudin-4, claudin-7, claudin-8, claudin-12, claudin-17, claudin-23, occludin, tricellulin, JAM-A, ZO-1, ZO-2, cingulin, MUPP-1, aPKC, Par3 and Par6 (for review see (116). TJs form a barrier in the skin of mouse and man for a 557 Da tracer from inside to outside in the SG of the epidermis (e.g. 117-118). After impairment of the SC barrier by occlusion of murine skin with a PBS moistened filter paper, barrier function of TJs was also observed for 45 kDa FITC-ovalbumin brought in contact with the skin surface (outside-in; (119)). Thus, TJs provide a second barrier for larger molecules in the skin, in addition to the SC. TJs are also likely to form a barrier to ions in the epidermis, because firstly, electron microscopic studies show that there is a barrier for La3+ in the SG (e.g. 119-120) and secondly, measurement of transepithelial resistance (TER) in adult human skin after tape-stripping, which means after removal of the strong ion barrier formed by the SC, still shows a substantial value (121-122). However, whether this barrier also exists without SC disturbance is not clear yet, because it was shown that SC barrier impairment induces a quick response of upregulation of the tightening TJ protein claudin-4 (123) and some authors suggested that the TJ ion barrier in mammalian epidermis is only relevant during development (124).

Since the detection of the correlation of filaggrin-mutation and atopic dermatitis (AD) there is accumulating evidence that an impairment of skin barrier may result in increased levels of antigens entering the skin and starting the inflammation typical for AD. To enter the skin, antigens first have to conquer the SC. Subsequently, they can be taken up by Langerhans-cells which protrude their dendrites through tight junctions into the area between SG and SC (119). Obviously, when TJs are impaired, it is likely that uptake of antigens is considerably increased.

Indeed, in non lesional skin of patients with AD, downregulation of claudin-1 and claudin-23 was found. In addition, CLDN1 haplotype-tagging SNPs revealed associations with AD in 2 North American populations (125). Given the fact that downregulation of Cldn-1 was shown to be involved in loss of barrier for macromolecules in the size of allergens (126) this could explain increased accessibility of the epidermis of AD patients for allergens through TJs. However, still allergens have to conquer the *stratum corneum* first. In light of this, it is highly interesting that it was reported that downregulation (human keratinocytes) or loss of Cldn-1 (Cldn-1 knock-out mice) results in alteration of SC proteins and SC barrier function (123, 126-127), suggesting a role of TJs as "master regulators" for the SC. Thus, downregulation of Cldn-1 may drive increased uptake of allergens by leading to impaired SC and TJ barriers. Interestingly, downregulation of filaggrin also influences tight junction proteins, suggesting that there is a mutual influence of SC and TJ barriers (109, 128). However, whether in this case TJs are more permeable for molecules of the size of allergens still has to be shown.

Besides the impairment of TJs in AD by genetic alteration of claudin-1, TJs can also be influenced by external stimuli present in AD skin. TJ-function was shown to be upregulated (short term) or downregulated (long-term) by IL-1beta and TNF*alpha* (129), proinflammatory cytokines whose levels have been shown to be elevated in non-lesional skin of AD patients, especially in those with filaggrin mutations (77, 130). For IL-4, a typical cytokine of AD, surprisingly an increase of TJ barrier function to ions (TER) was described in cultured keratinocytes (125), however, this effect might depend on the local environment, as we observed a downregulation of TER in different culture conditions (unpublished observation). Further, activation of TLR2 was shown to enhance TJ barrier function to ions and solutes (122, 131). This is of special interest, because, TLR2 is down-

regulated in AD (122, 132). Thus, these molecules/receptors are very likely to modulate the individual tightness of TJs in the epidermis of non-lesional skin of AD patients, but of course also in lesional skin.

Several TJ proteins in the epidermis are not restricted to the SG, where typical TJ structures have been identified and where the TJ barrier to the 557 Da tracer and 45 kDA FITC-ovalbumin is localized, but are found in all (claudin-1, MUPP-1, JAM-A) or several (e.g. claudin-4, claudin-7, ZO-1) layers of the epidermis. In addition, also in the SG TJ proteins are not always restricted to typical TJs (for reviews see (116, 133-135)). This clearly argues for further roles of TJ proteins in the epidermis besides their barrier function. Indeed, for claudin-1 involvement in cell proliferation (125) and for occludin in apoptosis and cell adhesion (136) of keratinocytes was shown. Therefore, alteration of TJ proteins might also play additional roles in AD, independent from impaired barrier function.

10. THERAPEUTIC OPTIONS INVOLVING SKIN BARRIER REPAIR IN AD

Therapeutic intervention in AD should be aimed at both restoring skin barrier function and reducing inflammation in the entire integument in general and in AD lesions in particular, as impaired skin barrier function is present in lesional as well as clinically uninvolved skin (137). Emollients have been shown to restore skin barrier function as measured by decreased TEWL and increased skin capacitance (138-139) and their frequent and generous use is an essential part of the treatment regimen of AD (140). A pilot study in which neonates at high risk of developing AD were treated with an oil-in-water, petrolatum-based cream starting within the first week of life suggested a potential preventive effect of emollients (141). Emollients are best used immediately after bathing, as bathing leads to cleansing and hydration of skin, while the emollient prevents evaporation of water. It is generally believed that the working mechanism of lipid containing emollients is by forming an occlusive layer over skin. However, it seems that these lipids actually may penetrate into the SC and accelerate the rate of barrier recovery (138). The working mechanisms of emollients are diverse. One effect by which emollients improve barrier function is by reduction of itch and therefore scratching, as scratching facilitates the release of proinflammatory mediators that induce itching, thereby breaking the vicious itch-scratch circle. Emollients also increase skin hydration and SC elasticity, making skin less susceptible to cracking and barrier disruption (142). Humectants may be added to emollients as they are believed to have increased hydrating effects on skin (142). However, care must be taken as these ingredients, as well as other components such as fragrances, preservatives and lanolins can have adverse effects such as contact dermatitis leading to deterioration of AD.

Coal tar has been used for decades in the treatment of AD, but it does not have a regular place in treatment guidelines for AD (140). Until recently, the action mechanism of coal tar had not been elucidated. It has now been shown that coal tar activates the aryl hydrocarbon receptor (AHR), resulting in accelerated epidermal differentiation and increased expression of the major skin barrier proteins filaggrin, hornerin, loricrin and involucrin (143), leading to increased skin barrier repair. Coal tar also decreased histopathological features of AD such as spongiosis. Despite its side effects such as unpleasant odour, folliculitis and phototoxicity, these observations justify a more active role for coal tar in the treatment regimen of AD. Furthermore, induction of AHR activation may open new therapeutic possibilities for barrier function repair in AD.

Corticosteroids have been the mainstay of treatment for AD, in the acute as well as the chronic phase of the disease. In short term however, corticosteroids may have a negative impact on skin barrier function. One study showed that application of a potent corticosteroid for three days lead to decreased production and secretion of epidermal lamellar bodies leading to decreased extracellular lamellar bilayers, and the SC cohesion and integrity was impaired by decreased corneodesmosomes in the lower SC (144). Similar observations were made with the usage of calcineurin inhibitors, such as pimecrolimus and tacrolimus. Application of these agents twice daily for 5 days leads to decreased lamellar body secretion and decreased lipid synthesis (145). Interestingly, in both studies the negative effect on skin barrier could be restored by application of emollients containing an equimolar ratio of ceramides, free fatty acids and cholesterol. However in the long term, both betamethasone and pimecrolimus lead to improved skin barrier function (146), probably due to the decrease of skin inflammation, as it is known that the inflammatory infiltrate in AD can downregulate expression of genes in the epidermal differentiation complex that are essential for skin barrier homeostasis (147).

Phototherapy, another well known treatment modality for AD shows a similar pattern of acute injury to the skin barrier while improving AD in the long term. Twelve weeks of 3 weekly NB-UVB treatments improved clinical scoring of AD and showed a normalization of skin barrier proteins such as filaggrin, loricrin and involucrin (148). However, studies in mouse models using increasing doses of UVB showed disruption of skin barrier function as measured by an increase in TEWL peaking at 96 hours post-exposure. This negative effect on the skin barrier was markedly decreased in athymic mice, but reappeared when mice were transplanted with a T-cell-enriched mixture of immune cells (149). These observations made in these two different treatment modalities for AD emphasize once more the intricate relationship between barrier function and inflammation.

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Abreviations: AD: atopic dermatitis, aPKC: atypical protein kinase C, CER: ceramide(s), CLDN1: claudin 1, DC: dendritic cells, Der-p2: (dermatophagoides pteronyssinus) allergen-like protein, FA: free fatty acids, FITC-ovalbumin: fluorescein isothiocyanate-ovalbumin, FLG: filaggrin, filament aggregating protein, FLG: gene encoding filaggrin, HRNR: hornerin, IFNalpha: interferon alpha, IFNG interferon gamma, IFNGR1: interferon gamma receptor 1, IgE: immunoglobulin E, IL: interleukin, IRF2: interferon regulatory factor 2, JAM: junctional adhesion molecules, LCE1: Late cornified envelope protein 1, LCE2: Late cornified envelope protein 2, LEKTI: lympho-epithelial Kazal-type-related inhibitor, MUPP1: multi-PDZ domain protein 1, NMF: natural moisturizing factor, Par3: polarity protein 3, Par6: polarity protein 6, SC: stratum corneum, SDS-gel: sodium dodecyl sulfate-gel, SG: stratum granulosum, SNP: single nucleotide polymorphism, SPRR3: small proline-rich protein 3, Sprr2a: small proline-rich protein 2a, TAMP: tight junction-associated marvel protein, TER: transepithelial resistance, TEWL: trans-epidermal water loss, Th cell: T helper cell, TJ: tight Junction, tTJ: tricellular tight junction, TLR2: toll-like receptor 2, TSLP: thymic stromal lymphopoetin

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