Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin

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CHAPTER 7

Summary and future perspectives

A defective permeability barrier exists in both lesional and non-lesional skin areas of AD skin. The impaired skin barrier in AD is characterized by increased trans-epidermal water loss (TEWL), increased permeability and reduced hydration in the upper layer of the skin, the stratum corneum (SC) where the skin barrier is located (1-4). The SC comprises of a multilayered structure of the flattened corneocytes surrounded by an intercellular lipid matrix. The lipids serve as a boundary between the hydrophilic corneocytes and the lipophilic lipid matrix. The SC organization is often described as a "brick" and "mortar" structure in which the corneocytes are the bricks and the lipids the mortar (5).

The lipid matrix mainly consists of ceramides (CERs), cholesterol (CHOL) and free fatty acids (FFAs) that are present at approximately equimolar ratios (6-8). The intercellular SC lipid matrix is a major penetration pathway of most molecules/compounds through the SC. This is the reason why its organization and composition is important for an intact barrier (9, 10). The SC lipids are organized in lamellae stacks with repeat distances of approximately 13nm and 6nm namely: the long periodicity phase (LPP) and short periodicity phase (SPP) respectively (11-13). Within the lipid lamellae, the lipids adopt a well-organized structure, the so called lateral organization. In human SC an orthorhombic packing (dense) is mainly present, while a small fraction of lipids assembles in a hexagonal (less dense) packing (14-18).

In SC of AD patients several changes in the lipid composition have been reported. Some alterations in the lipid composition include: reduction in SC lipid levels in relation to protein content, reduced relative CER content (CER/CHOL ratio and CER levels), shorter mean lipid chain length in lesional skin, increased level of unsaturated fatty acids and decreased level of hydroxy fatty acids. Several of these changes correlated with an increased TEWL(19-25).

AD being a complex chronic relapsing inflammatory skin disease has several interacting pathogenic pathways. These are manifested by fillagrin (*FLG*) mutations, disrupted epidermal barrier, microbe skin colonization and activation of $T_H1/T_H2/T_H17/T_H22$ inflammatory pathways (26-28). AD is also associated with an increased risk of developing other allergic diseases such as asthma, allergic rhinitis and food allergies which is termed as 'atopic march' (29). Among the known pathogenic factors suggested for AD, the disrupted epidermal barrier in combination with the abnormal immune response is considered as very significant to the development of AD (2, 30, 31).

In AD a TH_1 and T_H2 balance exists. Acute AD is regulated by T-helper 2 (T_H2) cells that produce cytokines such as IL-4, IL-13 and IL-5. In chronic AD this switch ($T_H2 \rightarrow T_H1$) results into higher expression of T_H1 cytokines such as IL-12 and IFN-γ (32). In relating inflammation in AD to the skin barrier, both T_H1/T_H2 cytokines have been shown to modify mRNA and protein levels of lipid synthesizing enzymes (CER and FFA) which result in altered levels of the corresponding lipids (33, 34).

Mouse models have been very valuable in elucidating the pathogenesis and molecular mechanisms involved in AD. Although these are excellent models to study biochemical pathways, there are limits in the translation to AD in humans due to differences in skin architecture between mice and humans (35, 36). As an alternative, validated and robust three dimensional (3D) human skin equivalents (HSEs) are a useful tool in exploring the pathogenesis of AD and validating new therapeutic molecules.

Aim

The aim of this thesis is to develop reproducible HSEs that mimic key characteristics of AD *in vitro*. Additionally we also focused on the effect of inflammation in the development of AD barrier characteristics. This was achieved by investigating the following research subjects:

1) Linking lipid synthesis enzyme expression in AD with SC lipid composition and organization
   a. Is the expression of CER and FFA biosynthesis enzymes altered in AD skin compared with native human skin?
   b. Do these changes in enzyme expression (CER and FFA biosynthesis enzymes) affect SC lipid composition and organization in AD skin?
2) Does inflammation play a role in the changes in epidermal morphogenesis, differentiation and lipid properties observed in lesional AD skin?
3) Can epidermal characteristics of AD skin be maintained in AD-HSEs derived from AD skin biopsies *in vitro*?
4) Is it possible to develop a model for skin barrier repair that mimics several barrier characteristics observed in AD skin?

Inflammation, lipid biosynthesis enzyme expression and SC lipid properties in AD

Using cell and tissue culture, the direct effect of inflammatory cytokines on epidermal morphology and differentiation proteins has been studied (37-40). However, much still remains unknown about the mechanism and direct effect of various chemokines and cytokines upregulated in AD skin on lipid biosynthesis and thus lipid composition and organization in SC. The lipid abnormalities in AD skin have been suggested to result from changes in the expression or activity of enzymes involved in SC lipid synthesis. In the research described in this thesis a number of enzymes involved in CER and FFA synthesis have been examined. These include ß-glucocerebrosidase (GBA), acid-sphingomyelinase (aSmase), CER synthase 3 (CerS3), stearoyl CoA desaturase (SCD), Elongase1 (ELOVL1) and ELOVL6.

GBA and aSmase catalyze the last step in CER subclass synthesis from glucosylceramides and sphingomyelin, respectively into CERs (41). ELOVL1 elongates FFAs with carbon chain length of C20-C26 and ELOVL4 elongates those longer than C26 (42-45). CerS3 acylates the ultra-long FFA chains (≥C26) to the sphingoid backbone during biosynthesis of CERs (34). In addition, ELOVL6 is primarily responsible for the elongation of FFAs with a chain length of C16 and C18 and SCD functions to produce unsaturated FFAs from saturated FFAs (46).

The studies in **chapter 2** focus on the expression of lipid biosynthesis enzymes (these include SCD, ELOVL1,ELOVL6, CerS3, GBA and aSmase) as underlying factors that may contribute to the change in barrier properties in AD skin. Besides the protein expression of enzymes in the epidermis, the SC lipid composition was also assessed in the patient cohort. Lesional as well as non-lesional AD skin was examined and compared to a control group. The expression of SCD is increased in AD lesional skin compared to the control

and non-lesional AD skin. This is associated with the significant increase in the level of unsaturated FFAs in AD lesional skin. Similarly, the pattern of ELOVL1 expression also differs in AD lesional skin compared to control (healthy) skin and non-lesional skin and is accompanied by reduction in FFAs C22-C28 observed in AD lesional skin. A similar trend in the level of FFAs ≥C26 produced by ELOVL4 was also observed. These very long FFAs were significantly reduced in AD lesional skin compared to control subjects. We observed no changes in ELOVL6 expression in AD skin.

The expression of aSmase, CerS3 and GBA (that are involved in CER synthesis) in AD lesional skin was changed in comparison to non-lesional skin and control subjects. The relative levels of lipids associated with these enzymes also showed significant changes, such as increased CER AS and NS (aSmase) and decreased esterified ω-hydroxy CERs which are derived from CerS3 activity.

The AD features described in **chapter 2** together with previous studies describing other epidermal AD characteristics provided the benchmark required to characterize an AD-HSE. Using the Leiden epidermal model (LEM) in **chapter 3**, the direct effect of upregulated cytokines in AD on epidermal morphogenesis, differentiation and SC lipid properties was investigated. Results show that the studied inflammatory cytokines upregulated in AD skin (TNF-α, IL-4, IL-13 and IL-31) affect epidermal morphogenesis, proliferation, differentiation, and SC lipid properties singly or in synergy. TNF-α and T_H2 cytokines reduced the expression of keratin 10 (K10), filaggrin and loricrin in LEMs. These cytokines also had an effect on stimulating keratinocytes to secrete thymic stromal lymphopoietin (TSLP) and increased basal cell proliferation. Regarding the SC lipid composition, TNF-α alone or in combination with T_H2 cytokines reduces the expression of ELOVL1 in LEMs corresponding with the progressive reduction in saturated long chain FFAs (≥ C20:0). The CER composition was also influenced by IL-31 and TNF-α as these cytokines reduce the ratio of ester linked ω-hydroxy (EO) CER to non-EO CER subclasses and the expression of CerS3 in LEMs. These changes in CER composition are reflected in the repeat distance (d) of the long periodicity phase (LPP) as TNF-α alone or in combination with T_H2 cytokines reduce significantly the repeat distance of the LPP. It can be concluded from this study that i) inflammation could be an influencing factor in the development of several important features of AD skin barrier ii) supplementation of LEMs with cytokines (individually or in synergy) may serve as an excellent candidate for the testing of prospective drugs and formulations for the treatment of AD.

Modelling *AD in vitro using s*kin explants from AD patients

AD skin biopsies can serve as an alternative way to develop AD-HSEs than using isolated primary keratinocytes in developing 3D HSEs as described in **chapter 3**. This involves placing full-thickness 4mm skin punch biopsies onto a fibroblast populated collagen matrix *i.e.* dermal equivalent, forming the so-called 1st generation explant human skin equivalent (Ex-HSE). This technique can be applicable for analyses that require larger amounts of epidermal tissue, which cannot be harvested from patients with skin diseases e.g. quantitative protein analysis or when analyzing the lipid barrier properties. A 1st generation Ex-HSEs can may be even further expanded by growing a 2nd generation and even a 3rd generation Ex-HSE. The 2nd generation can be produced by harvesting a biopsy from the 1st generation Ex-HSE and implanting this onto

a fresh dermal substrate. This approach can be repeated using a biopsy from the 2nd generation Ex-HSE to establish a 3rd generation Ex-HSE. The results from **chapter 4** reveal that 2nd and 3rd generation Ex-HSEs display similar epidermal morphology and expression of K10, loricrin and filaggrin as in native human skin and 1st generation Ex-HSEs. The 2nd and 3rd generation Ex-HSEs also show many similarities with 1st generation Ex-HSEs and native human skin in lipid properties *e.g.* presence of all lipid classes, similar fatty acid chain length distribution and lamellar lipid organization. However, some differences arise in the lipid properties, especially when comparing the 1st generation with the native human skin. These include: increased level of hexagonal lateral packing and an increased level of EO CERs. The expansion of skin biopsies to the 2nd and 3rd generation Ex-HSEs could be a promising method to expand valuable epidermal tissue to produce HSEs with similar morphological and differentiation parameters in the native epidermis. However, the reproducibly does not extend to the SC lipid properties in Ex-HSEs.

Using this technique of developing Ex-HSEs, AD Ex-HSEs were generated using non-lesional AD skin biopsies from wildtype or patients with homozygous *FLG* mutations. The AD Ex-HSEs were analyzed to examine whether the AD epidermal features can also be maintained *in vitro* and if *FLG* mutations have an effect on epidermal morphogenesis. The results described in **chapter 5** show that the expression of epidermal differentiation proteins in the original AD biopsies (filaggrin, involucrin, kallikrein 5 and Lekti) was maintained in the AD Ex-HSE and this was unaffected by *FLG* mutations. Although the expression of loricrin was also maintained in Ex-HSEs, the expression of loricrin was reduced in AD biopsies with homozygous *FLG* mutations and their corresponding Ex-HSEs. The study reveals that AD-HSEs can be derived from small AD biopsies which maintain most *in vivo* characteristics of AD skin including the genotypic and phenotypic features of FLG. This may be useful in providing additional material in understanding the role of *FLG* in AD or *FLG* replacement therapies and evaluation of prospective therapies directed toward filaggrin replacement in AD patients.

*An ex vivo mod*el for skin barrier repair

Currently, no suitable *in vitro* human skin models are available to study skin barrier repair. An impaired barrier induced by tape stripping cannot be performed with HSEs, due to the poor epidermal/dermal adhesion (28). The study in **chapter 6** introduces an *ex vivo* human skin model to study skin barrier repair mechanisms using a cyanoacrylate stripping technique to remove SC from *ex vivo* human skin. The regrowth of the SC *in vitro* (at 37°C and 32°C), epidermal morphogenesis, differentiation, SC lipid composition and organization was investigated. The results show that the stripped skin cultured for 8 days at 37°C or 32°C has an actively proliferating and differentiating epidermis resulting in the regeneration of SC *in vitro*. The SC lipids generated by the stripped explants adopt mainly a hexagonal lateral organization as opposed to an abundant orthorhombic organization in native human SC. The relative CER levels are also increased in the regrown SC *in vitro*. The above mentioned SC lipid features of the cultured stripped skin bear similarities with AD (23, 24, 47) and provide a system where formulations can be tested to enhance skin barrier repair. Furthermore, the cyanoacrylate stripped skin explants provides a potential model to investigate the effect of culture medium composition and environmental conditions during culture on the restoration of the skin barrier.

Conclusion

The studies in this thesis describes the barrier defects in AD skin and various techniques to develop AD-HSEs which can be used to better understand the role of several factors in the pathogenesis of AD skin. We show that Inflammation plays a pivotal role in the development of epidermal and SC features of AD skin and that AD epidermal features can be maintained *in vitro* when AD skin biopsies are used to generate Ex-HSEs. These AD-HSEs can also serve as a tool to screen potential therapeutics for AD and skin barrier repair. However, limitations exist in the complexity and full representation of all possible factors known to influence the development of AD e.g. *FLG* mutations, other aspects of inflammatory microenvironment, microbe colonization etc. Several possibilities exist to overcome these limitations and are discussed below.

Perspectives

Understanding in more detail the barrier defect in AD skin

To obtain more information on the barrier defects in AD, studying the activity and expression of CER and FFA synthesis enzymes (e.g. CerS3, GBA, ELOVL1, ELOVL4 etc.) is of particular interest. These enzymes have been shown to be related to the various changes in lipid composition in AD skin. A quantitative analysis of the expression and activity of these enzymes can be correlated with the lipid composition. For example, a bivariate analysis of the activity of SCD-1 and the level of mono-unsaturated FFA for FFAs and bivariate analysis of the expression of GBA in relation to the glucosylCER and CER levels in AD skin. This could provide more insight into the origin of the changes in the lipid composition observed in AD skin.

The bound lipids in the SC i.e. ω-hydroxy (EO) CERs and ω-hydroxy FFAs are thought to be a relevant factor in the skin barrier function as they form a lipid monolayer around corneocytes by an ester linkage of the ω-hydroxy group to involucrin (48-50). Filaggrin is a known component of the cornified envelope and since these lipids are chemically linked to the cornified envelope, analysis of the level and chain length of bound lipids may be important in understanding the relationship between the barrier defect in AD skin and filaggrin.

Additionally, inflammation has been shown to be a key contributing factor to the changes in expression of these enzymes. *in vitro* studies which examine the effect of other upregulated cytokines (individually or in synergy) in AD on CER and FFA synthesis enzymes can provide more insight into the interplay between inflammation and SC lipid synthesis in AD skin.

Apart from analyzing the factors that contribute to the barrier of the skin in AD e.g. SC lipids, it is also important to measure the overall quality of the barrier in developed AD-HSEs in relation to control HSEs and native human skin. Studies analyzing TEWL in HSEs *in vitro* and permeation studies using model drugs are further endpoints that can be used to characterize AD-HSEs.

AD-HSEs can potentially be used to screen formulations that can contribute to barrier repair in AD skin. CER and FFA synthesis enzymes are known to be regulated by various factors including hormones, temperature, pH, presence of other lipids etc. (51, 52). Formulations containing components which can directly promote the activity of CER and FFA synthesis enzymes or create an environment to stimulate these enzymes could be a promising approach to investigate as a treatment for AD.

Improving the SC lipid properties of human skin equivalents

HSEs have been very useful in broadening our understanding of the pathogenesis in AD. However, limitations exist in their uses to mimic the barrier properties of human skin *in vitro*. Studies from **chapter 4** and 5 reveal that Ex-HSEs do not maintain the exact lipid composition and organization in the original biopsy. HSEs show a more hexagonal lipid organization, less FFA and increased mono-unsaturated FFAs (MUFAs) (53, 54). *in vitro* studies have shown that an increased level of MUFAs can contribute to a more hexagonal

lipid organization (55). The expression of SCD-1 which synthesize MUFAs is increased in HSEs. Therefore inhibition of SCD-1 may result in a reduced level of mono-unsaturated FFAs and lead to a more orthorhombic lateral organization in HSEs. Thyroid hormone, leptin, glucagon, thiazolidinediones are known to repress the expression of SCD-1 and may be introduced in an optimized amount into the culture medium to regulate SCD-1 expression(51).

A temperature gradient exists in the skin between the hypodermis and the SC as the skin surface temperature is 32°C. Since enzyme activity and expression is known to be also temperature dependent, the gradient in temperature may be necessary for the proper function of enzymes involved in epidermal differentiation and lipid synthesis. *in vitro* culture procedures which can provide such a gradient at the time point where the HSEs are just fully differentiated may support the development of HSEs that fully resemble human skin barrier properties. This could be achieved by culturing HSEs at 37°C from the point of seeding of keratinocytes to air exposure. After 7 days of air exposure where a thin layer of SC has been formed, the HSEs can be cultured in conditions were the culture medium is maintained at 37°C and the surrounding temperature set at room temperature in order to mimic conditions *in vivo*. Microfluidic systems can also be applied in the culture of HSEs as they can provide a small and complex environment which mimics the *in vivo* environment for cells.

The humidity during the culture of HSEs (95% humidity) is much higher than what native human skin is exposed to. This may hinder the formation of an intact barrier in HSEs in comparison to human skin and optimization studies that investigate the effect of various levels of reduced humidity are useful to perform. Other factors that may influence the barrier include exposure to daylight and development of the dermal microenvironment that includes immune regulatory cells as seen *in vivo*. The dermal immune regulatory cells may assist in reversing the activated state of the epidermis in HSEs which is shown by expression of activation associated markers such as Keratin 6, 16 and 17 (53). The introduction of a combination of these factors mentioned above in the development of HSEs may lead to HSEs with similar barrier properties as native human skin.

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