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CHAPTER 5

Explant cultures of atopic dermatitis biopsies maintain their epidermal characteristics in vitro

Abstract

Atopic dermatitis (AD) is a common inflammatory skin disorder characterised by various epidermal alterations. Filaggrin (FLG) mutations are a major predisposing factor for AD and much research has been focused on the FLG protein. Human skin equivalents (HSEs) might be useful tools for increasing our understanding of FLG in AD and to provide a tool for the screening of new therapies aimed at FLG replacement.

Our aim is to establish an explant HSE (Ex-HSE) for AD by using non-lesional skin from AD patients wild-type for FLG or harbouring homozygous FLG mutations. These Ex-HSEs were evaluated as to whether they maintained their in vivo characteristics in vitro and whether FLG mutations affected the expression of various differentiation markers. FLG mutations did not affect the outgrowth from the biopsy for the establishment of Ex-HSEs. FLG expression was present in healthy skin and that of AD patients without FLG mutations and in their Ex-HSEs but was barely present in biopsies from patients with FLG mutations and their corresponding Ex-HSEs. AD Ex-HSEs and AD biopsies shared many similarities, i.e., proliferation and the expression of keratin 10 and loricrin, irrespective of FLG mutations. Neither KLK5 nor Lekti expression was affected by FLG mutations but was altered in the respective Ex-HSEs. Thus, Ex-HSEs established from biopsies taken from AD patients maintain their FLG genotype-phenotype in vitro and the expression of most proteins in vivo and in vitro remains similar. Our method is therefore promising as an alternative to genetic engineering approaches in the study of the role of FLG in AD.
Introduction

Atopic dermatitis (AD) is one of the most frequently occurring inflammatory skin diseases and is characterized by various epidermal alterations, including disrupted skin barrier function. Currently, AD affects 15-30% of Caucasian children and 2-10% of adults (1). Clinically, the skin from AD patients is characterised by dry, red and pruritic skin with possible chronic or relapsing eczematous lesions. Since the discovery of filagrin (FLG) gene mutations as a major predisposing factor for AD, much research has been focused on this protein (FLG) in the context of AD (2).

The role of FLG in the formation of the cornified envelope and as a precursor for the natural moisturising factor (NMF) is well established (3-5). However, the relationship between the presence of FLG mutations and the skin barrier defects in AD is currently unclear. In previous studies, reduced FLG protein expression attributable to mutations in vivo or FLG knockdown (FLG-KD) in vitro appears not to affect various skin barrier properties (6-9).

In order to study AD pathogenesis and the role of FLG in AD development, various model systems have been used, including murine models and in vitro three-dimensional human skin equivalents (HSEs). The murine models include the flaky tail (ft) mouse, which contains a homozygous frameshift mutation in the FLG gene. These ft mice are FLG-deficient and are therefore frequently used as a model for studying the role of FLG in the skin barrier (10-13). However, recent studies have shown that additional mutations in the Tmem79/matt gene are the cause of the barrier defects in ft mice (14, 15).

Because of the morphological and functional differences between mouse and man, in vitro skin equivalents might serve as a useful alternative (16). The establishment of HSEs in vitro involves FLG-KD in keratinocytes to reduce FLG protein expression, thereby mimicking the FLG mutations as seen in vivo in AD patients (9, 17, 18). Other approaches include the supplementation of cytokines to mimic lesional AD skin (19, 20). Whereas these studies have used manipulated keratinocytes, either by genetic engineering or through cytokine supplementation, our aim in the current study is to establish an explant HSE (Ex-HSE) by using the primary keratinocyte outgrowth from non-lesional AD biopsies. Such an HSE will represent more closely AD compared with genetically engineered keratinocytes. This approach should recapitulate features of the original AD biopsies. Earlier studies used this explant approach to establish an HSE for recessive epidermolysis bullosa simplex (REBS) or squamous cell carcinoma (SCC) and these have showed that specific characteristics of these skin disorders, e.g., disturbed differentiation in the SCC skin equivalents persist in vitro (21, 22). Based on these findings, we reason that AD biopsies that are wildtype for FLG (FLG+/+) or that harbour a homozygous FLG mutation (FLG-/-) can be used to establish HSEs. Here, we present such an HSE, which has been characterised for the expression of various epidermal markers for epidermal differentiation, proliferation and desquamation. The results presented in this study indicate that FLG mutations result in reduced FLG protein expression in vivo and in vitro and in the in vitro maintenance of the expression of various epidermal markers from the original AD biopsy.
Materials and methods

Healthy volunteers and AD patients
The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of the Leiden University Medical Centre. All subjects (healthy volunteers and AD patients) were recruited by public advertisement and gave their written informed consent. Three healthy Caucasian volunteers without a history of dermatological disorders and six Caucasian AD patients (age range: 20-42; 2 male and 7 female) were included. Of the AD patients, three were wildtype (AD FLG+/+) and three had a homozygous FLG mutation (AD FLG-/-); the FLG genotyping analysis is described below. No dermatological products were applied onto the forearms for at least 1 week prior to the study. From each individual/patient, two biopsies from non-lesional skin from the inner forearm were taken; one biopsy was used to evaluate the original status and the other biopsy was used to establish the outgrowth skin model.

FLG genotyping
Heparinised blood was drawn from healthy volunteers and AD patients. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation. Dry cell pellets were stored at -80°C until DNA isolation. From the PBMCs, DNA was isolated by using the DNeasy Blood and Tissue kit according to the manufacturer’s instructions (Qiagen, Venlo, The Netherlands). All individuals were genotyped for the four most prevalent mutations found in European Caucasians (2282del4, R501X, S3247X and R2447X), which cover approximately 93% of the currently known FLG mutations that have been detected in Western Europe (23), according to the method described earlier (24). After determination of the FLG genotype of the participants, three wildtype healthy volunteers, three wildtype AD patients and three AD patients with a homozygous FLG mutation were selected for the study.

Fibroblast culture
For the isolation of human dermal fibroblasts, dermis was obtained by overnight incubation of freshly obtained skin in dispase II solution (Roche Diagnostics, Almere, The Netherlands). To isolate the fibroblasts, the dermis was incubated for 2 h in a solution containing collagenase II (Invitrogen, Breda, The Netherlands) and dispase II (ratio 1:3 and 3 ml/g dermis) at 37°C for 2 h. The cells were filtered by using a 70-μm cell strainer (BD Biosciences, Breda, The Netherlands) and cultured in fibroblast medium at 37°C and 5% CO2 until subconfluency. Fibroblast medium consisted of Dulbecco’s modified Eagle’s medium (DMEM; Gibco/Invitrogen) supplemented with 5% fetal bovine serum (FBS; HyClone/Greiner, Nurtingen, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Culture medium was refreshed every 3 days. Fibroblast at passages two to five from a single donor were used for the experiments.

Explant human skin equivalents
Fresh punch biopsies (4 mm) from the inner forearm from healthy volunteers and from AD patients were trimmed and rinsed three times in sterile phosphate-buffered saline (PBS). Thereafter, one biopsy was immediately fixed in 4% paraformaldehyde and one biopsy was placed on dermal equivalents that were
constructed as described earlier (25-27). In short, rat-tail collagen was seeded with \(1.25 \times 10^5\) fibroblasts/ml and incubated for at least 1 day and maximally 7 days under submerged conditions.

All explants were cultured at the air-liquid interface under serum-free conditions for 3 weeks. The medium used during this period was composed of DMEM and Ham’s F12 medium (3:1 ratio) supplemented with 0.5 μm hydrocortisone, 1μM isoproterenol, 0.1 μM insulin (Sigma-Aldrich, Zwijndrecht, The Netherlands), 100 U/ml penicillin and 100 μg/ml streptomycin, 53 μM selenious acid, 10 mM l-serine, 10 μM l-carnitine, 7 μg/ml ß-dextrin, 1 μM DL-α-tocopherol-acetate, 100 μg/ml ascorbic acid phosphate and a lipid supplement containing 2.4 x 10^5 M bovine serum albumin (Sigma-Aldrich), 25 μM palmitic acid, 30 μM linoleic acid and 7 μM arachidonic acid.

**Immunohistochemistry**

After the culture period, Ex-HSEs were fixed in 4% paraformaldehyde, dehydrated and paraffin-embedded. The morphology of the biopsies and skin equivalents was examined in 5-μm-thick sections by using haematoxylin and eosin (HE) staining.

Immunohistochemical analysis was performed in the central part of the outgrowth to detect markers specific for keratinocyte proliferation, basement membrane formation and epidermal differentiation. For immunohistochemical analysis, 5-μm-thick sections were cut, deparaffinised and rehydrated, followed by heat-mediated antigen retrieval. After the blocking of non-specific binding with PBS containing 1% BSA and 2% normal human serum (NHS; Sanquin, Leiden, The Netherlands), primary antibodies were incubated overnight at 4°C. The used primary antibodies were raised against: Ki67 (1:100; clone MIB1, DAKO, Glostrup, Germany), Collagen type IV (1:75; clone MAB1430, Chemicon, Melbourne, Victoria, Australia), keratin 10 (K10, 1:100; clone DE-K10, Labvision/Neomarkers, Fremont, Calif., USA), loricrin (1:1000; clone AF62, Covance, Rotterdam, The Netherlands), kallikrein-related peptidase 5 (KLK5, 1:400; polyclonal, Santa Cruz, Heidelberg, Germany), lympho-epithelial Kazal-type-related inhibitor (Lekti, 1:20; clone 1C11G6, Invitrogen, Breda, The Netherlands). Following incubation with the appropriate secondary antibody, the streptavidin-biotin-peroxidase system (GE Healthcare, Buckinghamshire, UK) was used according to the manufacturer’s instructions with 3-amino-9-ethylcarbazole (AEC) for visualisation. The sections were briefly counterstained with haematoxylin and sealed with Kaiser’s glycerin. For the collagen type IV staining, a protease treatment with a 0.025% protease solution (Sigma, Zwijndrecht, The Netherlands) was performed prior to incubation with the primary antibody.

**Immunofluorescence**

For immunofluorescence analysis, 5-μm-thick paraffin sections were cut, deparaffinised and rehydrated, followed by heat-mediated antigen retrieval. Hereafter, non-specific binding was blocked by using PBS containing 1% BSA and 2% NHS followed by overnight incubation at 4°C with the primary antibody for FLG (1:1000; Covance, Rotterdam, The Netherlands). Subsequently, sections were incubated with the appropriate secondary antibody (conjugated to Rhodamine Red, 1:300; Jackson ImmunoResearch, Amsterdam,
The sections were mounted with Vectashield containing 4,6-diamidino-2-phenylindole (DAPI) for visualisation of the nuclei (Vector Laboratories, Amsterdam, The Netherlands).

**Proliferation index**

The proliferation index was determined by an independent researcher who counted the number of Ki67-positive nuclei in a total number of 100 basal cells (x 100 %) at three locations per slide for three different donors and their corresponding skin models. Data represent means + standard error of the mean (SEM).

**Statistical analysis**

Statistical analysis was conducted by using GraphPad Prism, version 5.04. The statistical differences between biopsy groups, between Ex-HSE groups and between the biopsies and their corresponding Ex-HSEs were analysed by using two-way analysis of variance.
Results

Explant cultures of AD biopsies with \textit{FLG} mutations display normal epidermal morphogenesis

To evaluate whether AD biopsies could be used to establish Ex-HSE and whether \textit{FLG} loss-of-function mutations affected epidermal regeneration, 4-mm biopsies from non-lesional AD skin were placed onto a fibroblast-populated collagen matrix. The AD patients from whom this skin was taken were either wildtype (AD FLG+/+) or had homozygous \textit{FLG} mutations (R501X or 2282del4, AD FLG-/-).

After culture for 21 days at the air-liquid interface, keratinocytes grew from the biopsies in a lateral fashion and covered over 90% of the collagen matrix (figure 1a). This occurred irrespective of the presence of \textit{FLG} mutations. The biopsies from healthy volunteers and AD patients displayed the presence of all epidermal layers, namely the stratum basale, stratum spinosum, stratum granulosum (figure 1b-d). We observe reduced granular structures in the location of the stratum granulosum in AD (-/-) biopsies compared to healthy biopsies and AD (+/+) biopsies. The Ex-HSEs established with biopsies from healthy volunteers (healthy Ex-HSE), AD patients without \textit{FLG} mutations (AD Ex-HSE [+/+]) or with \textit{FLG} mutations (AD Ex-HSE [-/-]) displayed the presence of all the epidermal layers. However, similar to AD (-/-) biopsies, AD Ex-HSE (-/-) also showed reduced granular structures in the location of the stratum granulosum (figure 1e-g). In addition, the number of viable cell layers was comparable between healthy Ex-HSE, AD Ex-HSE (+/+) and AD Ex-HSE (-/-). \textit{FLG} expression was present in the granular layer of the epidermis (which could be identified by from healthy volunteers and AD FLG+/+ biopsies, whereas the AD FLG-/+ biopsies showed an almost complete absence of FLG, as demonstrated by immunofluorescence staining (figure 1h-j). More importantly, whereas the healthy Ex-HSE and the AD Ex-HSE (+/+l) showed pronounced \textit{FLG} protein expression in the granular layer, the AD Ex-HSE (-/-) displayed an almost complete absence of \textit{FLG} protein expression, similar to the original biopsies from these patients (figure 1k-m).
Figure 1: Establishment of explant human skin equivalents (Ex-HSEs) by using biopsies from atopic dermatitis (AD) patients and healthy volunteers. a Example of an AD Ex-HSE established by placing an AD biopsy onto a fibroblast-populated collagen matrix. Biopsies from both healthy and AD patients (FLG+/+ [wild-type for filaggrin] and FLG-/-) gave similar results, i.e., coverage of at least 90% of the collagen matrix (dotted line boundaries of the outgrowth, solid line original biopsy). b-g Cross-sections of biopsies and their corresponding Ex-HSEs. All epidermal layers were present in all biopsies and their corresponding Ex-HSEs, irrespective the presence of FLG mutations, as shown by haematoxylin and eosin staining. h-m Immunofluorescent staining for FLG showing reduced FLG expression in the granular layer of AD FLG (-/-) and AD Ex-HSE (-/-). Scale bar 50 μm
AD Ex-HSEs maintain expression of differentiation markers

After evaluation of the morphology and FLG protein expression, the Ex-HSEs were further characterised for their expression of various epidermal markers. The presence of the basement membrane was assessed by collagen type IV staining. Biopsies from healthy volunteers and AD FLG+/+ and AD FLG-/- patients showed continuous collagen type IV expression at the dermal-epidermal junction indicating the presence of a normal basement membrane (figure 2a-a’’). Early differentiation was assessed by keratin 10 staining. Keratin 10 was expressed in all suprabasal cell layers of the epidermis from biopsies from healthy volunteers and AD patients, irrespective of FLG mutations (figure 2b-b’’). Terminal differentiation was assessed by staining for involucrin and loricrin. Involucrin expression was present in the granular layer and in the upper spinous layers of all biopsies, irrespective of FLG mutations (figure 2c-c’’). However, whereas the biopsies from healthy volunteers and AD FLG+/+ patients displayed continuous loricrin expression in the granular layer of at least two cell layers thick, loricrin expression was present in one cell layer and strongly reduced in the granular layer in the biopsies from the AD FLG-/- patients (arrows, figure 2d-d’’).

All Ex-HSEs showed the formation of a basement membrane as assessed by a collagen type IV staining, similar to their respective original biopsies (figure 2e-e’’). In all Ex-HSEs, keratin 10 expression was present in all suprabasal layers indicating that early differentiation was similar in the Ex-HSEs compared with the original biopsies. Involucrin expression was present in almost all suprabasal layers of all Ex-HSEs, irrespective of the presence of FLG mutations. This earlier expression of involucrin was different in the Ex-HSEs compared with the original biopsies. Expression of loricrin in the Ex-HSEs was present in the granular layer of healthy Ex-HSE and AD Ex-HSE (+/+), similar to their original biopsies. However, the AD Ex-HSE (-/-) displayed a reduced loricrin expression in the granular layer (arrows, figure 2h-h’’) with the thickness of the granular layer staining being 1-2 cell layers thick in contrast to that of the healthy and AD FLG +/+ HSEs with 3-4 cell layers thickness.

To evaluate whether proliferation was affected in the AD Ex-HSEs and/or is influenced by FLG mutations, staining for the proliferation marker Ki67 was performed. The proliferation index in biopsies from healthy volunteers (24.7 ± 0.7), AD FLG+/+ patients (23.7 ± 4.9, p=0.76) and AD FLG-/- patients (21.7 ± 6.2, p=0.49) was similar, whereas the Ex-HSEs showed a proliferation index of 27.5 ± 0.5 (healthy Ex-HSE), 23.7 ± 1.7. p=0.07 (AD Ex-HSE (+/+)) and 28.3 ± 3.5, p=0.71 (AD Ex-HSE (-/-); figure 2i).
Figure 2: Expression of epidermal markers is maintained in AD Ex-HSEs. a-h” Immunohistochemical staining for collagen type IV, keratin 10, involucrin and loricrin in biopsies from healthy volunteers and AD patients (a-d”) and their corresponding Ex-HSEs (e-h”). Collagen type IV expression was continuously expressed at the dermal-epidermal junction in all specimens. Keratin 10 expression was present in all suprabasal cell layers of the biopsies and their corresponding Ex-HSEs. Involucrin
was expressed in the granular layer of the biopsies but the expression was increased in their corresponding Ex-HSEs. Loricrin expression was present in the granular layer of healthy volunteers and in AD FLG+/+ biopsies and their corresponding Ex-HSEs but the expression was reduced in the AD FLG-/- biopsies and in the AD Ex-HSE (-/-); arrows indicate residual loricrin expression. Scale bar 50 μm. i Graph showing that the proliferation index of the initial biopsies (white bars) and Ex-HSEs (black bars) was similar and not affected by the presence of FLG mutations. Data represent means + SEM of three independent biopsies or Ex-HSEs for each condition.

**AD Ex-HSEs show alterations in expression of desquamation-related enzymes**

To evaluate whether the expression of enzymes involved in the desquamation process were affected in AD biopsies and in Ex-HSEs, immunohistochemical analyses for KLK5 and Lekti were performed. In the biopsies, both enzymes were expressed in the granular layer of the epidermis and were unaffected by the presence of FLG mutations (figure 3a-a”, b-b”). In the Ex-HSEs, KLK5 expression was continuously present in the granular layer of the healthy Ex-HSE and AD Ex-HSE (+/+). However, in the AD Ex-HSEs (-/-), KLK 5 expression was reduced and patchy (figure 3c”). In one case, KLK5 expression was barely present in the granular layer of the AD Ex-HSE (-/-) (data not shown). Compared with the biopsies, Lekti showed a different expression pattern in all Ex-HSEs. In the biopsies, Lekti was expressed in the uppermost two granular layers, whereas in all Ex-HSEs, the expression was present in the granular layer and in the upper layer of the stratum spinosum (figure 3d-d”).
Figure 3: Expression of desquamation-related enzymes in Ex-HSEs. Cross-sections of the initial biopsies (a-a'', b-b'') and Ex-HSE cultures (c-c'', d-d''). Immunohistochemical staining for kallikrein-related peptidase 5 (KLK5) and lympho-epithelial Kazal-type-related inhibitor (Lekti) in biopsies from healthy volunteers and AD patients (a-a'', b-b'') and their corresponding Ex-HSEs (c-c'', d-d''). Both KLK5 and Lekti are expressed in the granular layer of the biopsies, irrespective of FLG mutations. In the healthy Ex-HSEs and AD Ex-HSEs (+/+), KLK5 expression is present in the granular layer but is reduced in the AD Ex-HSE (-/-) culture. All Ex-HSEs show Lekti expression in the granular layer and in the upper spinous layers. Scale bar 50 μm
Discussion

The objective of the current study was to establish an *in vitro* EX-HSE for AD. Previous studies used FLG-KD in keratinocytes or cytokine supplementation to mimic AD *in vitro*. In the current study, we used biopsies from non-lesional skin from AD patients, wildtype for FLG (FLG+/+) or harbouring a homozygous FLG mutation (FLG-/-; R501X or 2282del4). These biopsies were placed onto a fibroblast-populated collagen matrix. Our main purpose was to evaluate whether we could maintain characteristics from the original biopsy *in vitro* and to evaluate whether FLG mutations played a role in the epidermal morphogenesis of the Ex-HSEs. FLG mutations did not affect the ability to form an Ex-HSE. Earlier studies showed that reduced profilaggrin expression eliminated the granular cell layer by abolishing keratohyalin granules and FLG monomers from the epidermis (28). In the current study, AD biopsies and the generated Ex-HSEs displayed all epidermal layers, including the granular layer. More importantly, the presence of FLG mutations resulted in reduced FLG protein expression in the biopsies and in the outgrowth areas of the corresponding Ex-HSEs.

FLG-KD skin models have previously been described (9, 17). However, no skin models have been presented in which primary FLG-deficient cells from AD patients have been used for their establishment. In addition, to the best of our knowledge, no studies have so far been conducted in which the effect of FLG mutations on the expression of various epidermal proteins *in vivo* and *in vitro* has been evaluated within the same patient.

The role of FLG monomers in aggregating keratin filaments, as a component of cornified cell envelope and as a source of free amino acids for the NMF has been well established (29). Furthermore, FLG has been found to be associated with in particular keratins 1 and 10 (4). In the current study, we show that the expression of keratin 10 is similar in all biopsies and Ex-HSEs, irrespective of FLG mutations. These findings are similar to those in which HSEs were generated after FLG-KD in keratinocytes; in these FLG-KD HSEs, no effect on keratin 10 expression was observed (9, 17).

Since the FLG gene is located in the epidermal differentiation complex, mutations in FLG might affect the expression of other genes and proteins located in this complex, e.g., involucrin and loricrin. The expression of involucrin in the biopsies is not affected by the presence of FLG mutations. However, in all Ex-HSE, involucrin is expressed earlier, irrespective of FLG mutations. These observations are in agreement with earlier studies that characterised various *in vitro* HSEs (27). Loricrin expression is reduced in AD FLG-/- biopsies and in their corresponding Ex-HSEs; this implies that the reduction of FLG protein expression is not compensated by loricrin. Lentiviral-mediated knockdown of profilaggrin in primary keratinocytes has been shown to result in a hyperproliferative epidermis in HSEs (30). However, in the Ex-HSEs established with FLG-/- biopsies, we did not observe an effect of FLG mutations on epidermal proliferation. The observation that FLG mutations, i.e., R501X and 2282del4, do not affect proliferation indicates that such mutations do not affect the presence of (a partly) functional profilaggrin. Other studies have demonstrated the presence of a truncated profilaggrin in the epidermis of human ichthyosis vulgaris patients and in the flaky tail mouse (10, 24, 31, 32).
The processing of profilaggrin is controlled by a complex network of serine proteases, including KLK5 and Lekti(33-36). Although we did not observe an effect of FLG mutations on KLK5 expression in vivo, reduced KLK5 expression was observed in the AD Ex-HSEs (-/-). Since desquamation is absent in HSEs, the underlying mechanisms and the consequences of this reduced KLK5 in the AD Ex-HSEs (-/-) are difficult to address. Lekti loss-of-function mutations are the underlying cause of Netherton syndrome, a rare skin disorder that shares many similarities with AD. Lack of Lekti has been shown to result in premature profilaggrin processing (37), attributable to uncontrolled activity of proteases during the formation of the cornified envelope. However, here we show that the lack of FLG does not affect Lekti expression. The earlier expression of Lekti in the stratum spinosum of Ex-HSEs might result in increased Lekti activation and the subsequent increased inhibition of KLK5 activity, as observed in various HSEs (40). Such a change in Lekti expression in HSEs might therefore (partially) explain the absence of desquamation in vitro.

In conclusion, we present a novel AD-HSE that is established with biopsies from non-lesional AD skin wild-type for FLG or harbouring a homozygous FLG mutation. We have shown that most of the in vivo AD characteristics, including the FLG genotype-phenotype, are maintained in vitro. Therefore, we believe that this approach is a promising alternative to FLG-KD in keratinocytes in order to be able to mimic this in vivo feature of AD in vitro and to improve the study and understanding of the role of FLG in AD. FLG-KD is an effective but rather artificial method for reducing FLG expression in skin models, as opposed to using primary FLG-deficient cells. Furthermore, this Ex-HSE model might aid in the development and evaluation of therapies aimed at FLG protein replacement (38, 39).
References


