Molecular Pathogenesis of Genetic and Inherited Diseases

Premature Terminal Differentiation and a Reduction in Specific Proteases Associated with Loss of ABCA12 in Harlequin Ichthyosis

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One of the primary functions of skin is to form a defensive barrier against external infections and water loss. Disrupted barrier function underlies the most severe and often lethal form of recessive congenital ichthyosis, harlequin ichthyosis (HI). HI is associated with mutations in the gene that encodes the ABC transporter protein, ABCA12. We have investigated the morphological and biochemical alterations associated with abnormal epidermal differentiation and barrier formation in HI epidermis. An in vitro model of HI skin using human keratinocytes retrovirally transduced with shRNA targeting ABCA12 in a three-dimensional, organotypic co-culture (OTCC) system has also been developed. A robust reduction in ABCA12 expression had a dramatic effect on keratinocyte differentiation and morphology comparable with that observed in HI skin, including a thicker epidermis and abnormal lipid content with a reduction in nonpolar lipids. As seen in HI epidermis, proteins that are normally expressed in late differentiation were highly dysregulated in the ABCA12-ablated OTCC system. These proteins were expressed in the stratum basale and also in the stratum spinosum, indicative of a premature terminal differentiation phenotype. Expression of the proteases kallikrein 5 and cathepsin D was dramatically reduced in both HI epidermis and the OTCC model. These data suggest that ABCA12 is a key molecule in regulating keratinocyte differentiation and transporting specific proteases associated with desquamation. (Am J Pathol 2009, 174:970–978; DOI: 10.2353/ajpath.2009.080860)
ceramide into the LGs and then the ABCA12-positive LGs secrete the lipid into the extracellular space to form the intercellular lipid layer. Recent evidence has found that peroxisome proliferators-activated receptor (PPAR)-γ, PPAR-β, and PPAR-δ activators stimulate the expression of ABCA12 mRNA in cultured human keratinocytes. It was also found that liver X receptor (LXR) activators also increased ABCA12 mRNA expression but to a lesser extent than the PPAR activators. The PPAR and LXR activators have been shown to stimulate downstream effects in keratinocytes related to differentiation including increased LG secretion and lipid synthesis.

The histology of HI skin is striking. The most obvious abnormality is the sheer thickness of the stratum corneum (SC) and indeed the entire epidermis. With closer histological inspection, the nuclei become flattened early on in differentiation but are retained in the cornified layer (parakeratosis) and hyperkeratosis and hypergranulosis have also been noted as hallmarks of HI skin. In addition to the increased epidermal thickness, an abnormal localization of epidermal lipids has been described.

A mouse knockout for ABCA12 has been described as having a postnatal lethal phenotype (line NIH-01279; neonatal lethal Mouse Genome Informatics). Recently, the Akiyama group has generated and characterized an ABCA12-null mouse. The phenotype resembles that of newborn HI skin plus reveals alveolar collapse as an additional phenotype linked to the nonviability in these mice. The expression of ABCA12 in normal fetal skin development and the grafting of HI keratinocytes on immunodeficient mice have also been described. To complement these studies and because of the lethality of the ABCA12-null mice, we have generated and characterized an in vitro human model of HI skin using shRNA targeting ABCA12 in a normal keratinocyte cell line to study the role of ABCA12 in human epidermis.

Materials and Methods

Cell Lines

The immortalized keratinocyte cell line NEB1 was cultured in 3:1 Dulbecco’s modified Eagle’s medium-F12 medium, supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 10 × 10⁻¹⁰ mol/L cholera toxin, 5 μg/ml transferrin, 2 × 10⁻¹¹ mol/L liothyronine, and 50 μg/ml penicillin-G and 50 μg/ml streptomycin sulfate. Primary keratinocytes extracted from face lift skin were grown in co-culture with mouse 3T3 fibroblast cells in the same media.

Retroviral Transduction

The pSUPERIOR-retro-puro shRNA system (Oligoengine, Seattle, WA) was used to suppress the expression of ABCA12. Four pairs of oligos were designed as follows (sense sequence only shown): shRNA1: 5’-GATCCCCGAACGTCCAGGAAATAAGCTTCAAGAGAGCTATTTCCGGAGTTCTTATTTA-3’, shRNA2: 5’-GATCCCGGAACGTCCAGGAAATAAGCTTCAAGAGAGCTATTTCCGGAGTTCTTATTTA-3’, shRNA3: 5’-GATCCCGGAACGTCCAGGAAATAAGCTTCAAGAGAGCTATTTCCGGAGTTCTTATTTA-3’, shRNA4: 5’-GATCCCGGAACGTCCAGGAAATAAGCTTCAAGAGAGCTATTTCCGGAGTTCTTATTTA-3’. Subcloning of the oligos, transfection of the packaging cell line, production of retrovirus, and transduction of target cells were performed as per the manufacturer’s instructions. shRNA1 was selected for subsequent experiments described in this article.

Skin Biopsies and Organotypic Co-Culture (OTCC)

Normal skin was obtained from redundant skin and a skin biopsy from a Bangladeshi male HI patient, age 14, homozygous for the ABCA12 mutation 6378delGC, was cryo-mounted before frozen sectioning. Consent and ethical approval was obtained for these studies. OTCC was performed as described previously.

Tissue Staining

Each tissue was cut in half such that one half was snap-frozen while embedded in Cryo-M-Bed (Bright) and was stored at −80°C. The other half was fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut at 5 to 6 μm in thickness. Hematoxylin and eosin (H&E) staining was performed following standard protocol.

Epidermal Thickness Measurements

Nine frozen sections were cut from different positions of each OTCC and three measurements of each section were taken at random using the measuring tool with the MetaMorph software (Molecular Devices, Sunnyvale, CA) on an Eclipse microscope (Nikon, Tokyo, Japan). Statistical analysis was performed using an unpaired two-tailed t-test as described previously.

Nile Red Lipid Analysis

A stock solution containing 0.05% Nile Red in acetone was diluted to 2.5 μg/ml with 75:25 glycerol:water, followed by brisk vortexing. A drop of the glycerol-dye solution was applied to each tissue section and immediately covered with a coverslip. Slides were then viewed using a Nikon Eclipse TE2000-S fluorescence microscope. Pictures were taken separately of the green and red fluorescence and then subsequently merged.

Antibodies

The ABCA12 antibody was generated by Harlen (Oxford, UK), using the 15-amino acid ABCA12 (NM_173076) motif present at residues 2504 to 2519 (QLHFPKTYLK-
DQHLS) plus a cysteine for conjugation. The rabbit polyclonal antibody obtained was then G-protein-purified before use, and used at a dilution of 1:500 for immunofluorescence. All other antibodies were used at 1:200 dilution. The LEKT1 antibody was a gift from Dr. W.L.Di (Institute of Child Health, UCL, London, UK). The involucrin (clone SY5) and K2e (clone LHK2E) antibodies were obtained from CRUK (Cancer Research UK, London, UK). The mouse anti-transglutaminase 1 (Biogenesis, Poole, UK), mouse anti-filaggrin (Biomeda, Foster City, CA), rabbit anti-KLK5 and goat anti-KLK7 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-cathepsin D (Abcam, Cambridge, UK) are available commercially.

Immunohistochemical Staining

Frozen sections were air-dried for 30 minutes and permeabilized in 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 15 minutes. Sections were washed in PBS, blocked in 0.2% gelatin from cold water fish skin for 15 minutes, and incubated with primary antibody for 1 hour. After three PBS washes, fluorescent secondary antibody (donkey anti-rabbit or donkey anti-mouse Alexa Fluor 488; Molecular Probes, Eugene, OR) was added at a 1/1000 dilution and incubated for 1 hour in the dark. Sections were washed three times in PBS, incubated with 4,6-diamidino-2-phenylindole (DAPI) (0.125 µg/ml) for 5 minutes, and washed three more times. Sections were mounted with immunomount reagent (Thermo Shandon, Waltham, MA) and viewed under a Nikon Eclipse TE2000-S microscope.

Confocal Immunofluorescence

Immunocytochemistry was performed using a standard protocol. Briefly, NEB1 cells that had been seeded onto glass coverslips were fixed in 4% paraformaldehyde for 30 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. Nonspecific binding was blocked with 3% bovine serum albumin for 15 minutes before the cells were incubated in primary antibody solution for 1 hour. After washing, the cells were incubated with the relevant Alexa Fluor secondary antibodies (Molecular Probes) before being washed and mounted in Shandon Immumount (Thermo Electron Corporation, Waltham, MA) containing 10 µg/ml of DAPI. Cells were imaged using a Zeiss 510 confocal microscope (Zeiss, Thornwood, NY).

Results

ABCA12 Expression Is Absent in HI Skin

Immunofluorescence demonstrated ABCA12 was expressed throughout the normal interfollicular epidermis with prominent expression in the stratum granulosum (Figure 1A). Immunohistochemistry (IHC) with the ABCA12 antibody showed ABCA12 expression was significantly reduced/absent in the skin of this HI patient (Figure 1B).

Figure 1. A and B: IHC with the ABCA12 antibody (green). A: In normal skin the strongest staining appears in the stratum granulosum. B: ABCA12 expression was significantly reduced/absent in the skin of this HI patient. DAPI-stained nuclei are shown in blue and the dermal-epidermal junctions are marked by a dotted white line.

Figure 2. IHC of normal and HI patient skin biopsies with markers of late epidermal differentiation revealed an abnormal expression pattern in HI epidermis. In normal skin, the expression of involucrin (A) was observed primarily in the granular layer with keratin 2e (K2e, B) expressed variably throughout the suprabasal layers. In contrast, in HI skin both involucrin (E) and K2e (F) were expressed throughout the entire epidermis. The TGase 1 (C) and filaggrin (D) were expressed primarily in the SC in normal skin, however, in HI skin, TGase 1 (G) was expressed throughout the entire epidermis including the basal layer. H: Filaggrin was expressed throughout the upper spinous layer but was significantly reduced/absent from the SC. DAPI- or propidium iodide-stained nuclei are shown in blue and red, respectively, and the dermal-epidermal junctions are marked by a dotted white line.
Abnormal Epidermal Differentiation and Protease Transport in HI Skin

IHC analysis with antibodies raised against proteins expressed in late epidermal differentiation including involucrin, keratin 2e (K2e), transglutaminase 1 (TGase1), and filaggrin revealed an abnormal expression pattern in HI epidermis. Involucrin and K2e are primarily expressed in the granular suprabasal layers of normal control skin (Figure 2, A and B). In contrast, both K2e and involucrin were expressed throughout the entire suprabasal epidermis of the HI skin biopsy, with K2e expression also in the basal layer (Figure 2, E and F). The localization of both TGase1 and filaggrin was primarily in the SC in normal skin (Figure 2, C and D). This localization was consistent with the respective roles of these proteins in the epidermis. TGase1 is involved in the interprotein cross-linking of the cornified envelope. Filaggrin is a product of proteolytic cleavage of profilaggrin and is thought to aggregate intermediate filaments during terminal differentiation. However, in HI skin TGase1 localization was membranous and was expressed throughout the entire epidermis including the basal layer (Figure 2G). The pattern of filaggrin expression was also abnormal in HI skin; it was absent from the SC but present throughout the upper spinous layer supporting a previously reported error in filaggrin processing in HI skin (Figure 2H). The filaggrin antibody also detects profilaggrin.

The expression of a number of LG-associated components was analyzed in HI and normal skin by immunofluorescence including lympho-epithelial Kazal-type-related inhibitor 1 (LEKT1), kallikrein (KLK) 7, KLK5, and cathepsin D (CTSD). LEKT1 was expressed throughout the epidermis but most strongly in the granular and upper spinous layers (Figure 3A) where it is associated with the LG network. However, some fainter expression of KLK7 was additionally identified in lower keratinocyte layers in HI skin. KLK5 expression in normal skin was observed in the stratum granulosum and upper spinous layers but was significantly reduced/absent from HI skin biopsy (Figure 3, C and G). CTSD was present in the stratum granulosum and upper spinous layers of normal skin but was also significantly reduced/absent from HI skin (Figure 3, D and H).

Nonpolar Lipids Reduced in HI Skin

Nile Red analysis of lipids was performed to compare the lipid profile between the HI skin biopsy and normal control skin (Figure 4, A and B). Nile Red fluoresces green in the presence of nonpolar lipids (such as ceramides) and red in the presence of polar lipids (such as phospholipids and sphingomyelin). Pictures were taken to detect both red and green fluorescence separately and subsequently merged. In the SC of normal skin, a bright yellow-gold color is observed indicating the presence of both polar and nonpolar lipids. However, in HI skin the vast majority of lipid was polar because only red fluorescence was detected with no obvi-
uous yellow-gold color seen after the pictures were merged. The nonpolar ceramides comprise the bulk of the lipid contained within the lipid bilayers of the SC and are formed from the hydrolysis of glucosylceramide (GlcCer) transported via the LG system. There is evidence to suggest ABCA12 is involved in the transport of GlcCer into the LG system. Human keratinocytes lacking functional ABCA12 show an abnormal intracellular localization of GlcCer that is resolved after functional gene transfer.

**Development of an in Vitro Skin Model for HI**

To ascertain if the abnormal epidermal differentiation and LG transport identified in HI skin was attributable specifically to lack of functional ABCA12, three-dimensional OTCCs using NEB1 cell line keratinocytes retrovirally transduced with a pSUPERIOR.retro.puro vector containing an ABCA12 shRNA construct. After shRNA retroviral transduction of NEB1 keratinocytes, cells were fixed onto coverslips for immunocytochemical analysis of ABCA12 expression. Robust ABCA12 knockdown was achieved (Figure 5A). OTCC using this shRNA construct was created on three separate occasions along with vector-only control (VOC) and a scrambled control (ScramC) (Figure 5, B and C). These cultures were allowed to grow at the air-liquid interface for 10 days.

IHC with the ABCA12 antibody showed the protein was strongly expressed throughout the VOC (Figure 5E) and ScramC (Figure 5F) epidermis but was significantly reduced in the ABCA12 shRNA knockdown OTCC (Figure 5D). H&E staining for the knockdown OTCC (G) showed a thicker and more disorganized epidermis compared with the control OTCCs (H and I). Dermal-epidermal junctions are marked by a dotted white line.

**Abnormal Epidermal Differentiation and Protease Transport Replicated in ABCA12 Knockdown OTCCs**

Frozen sections from the OTCCs were stained with antibodies against involucrin, K2e, and TGase1. As was
observed using the HI skin biopsy (Figure 2E), in the ABCA12 knockdown OTCC involucrin was expressed throughout the entire epidermis even in the basal layer (Figure 7D). This was in contrast to the VOC (Figure 7A), where involucrin was expressed only in the uppermost keratinocytes. K2e is expressed mainly in the stratum granulosum in the VOC OTCC (Figure 7B) but was expressed primarily throughout the epidermis of ABCA12 knockdown samples (Figure 7E). TGase1 is normally expressed in the upper layers of the epidermis as observed in control OTCCs (Figure 7C). In ABCA12 knockdown samples, TGase1 expression was observed throughout the epidermis (Figure 7F).

LG components were also analyzed by immunofluorescence in the frozen OTCC sections and mirrored the observations seen in HI skin. LEKT1 showed a similar pattern of expression between ABCA12 knockdown samples and controls (Figure 8, A and E). The protein was expressed throughout the epidermis but most strongly in the upper epidermis. Like LEKT1, the expression of KLK7 was similar between ABCA12 knockdown and control OTCCs (Figure 8, B and F). Expression of KLK7 is mostly confined to the spinous and granular layers of co-cultures. KLK5 was located throughout the epidermis of control samples (Figure 8C) but was significantly reduced/absent in all ABCA12 knockdown co-cultures analyzed (Figure 8G). Similarly, ABCA12 knockdown OTCCs all showed an almost total absence of CTSD staining (Figure 8H), whereas in control co-cultures CTSD expression was observed only in the uppermost layer of the OTCC (Figure 8D). To follow-up this observation, ABCA12 co-localization studies were performed in keratinocytes and demonstrated evidence of ABCA12 co-localizing with KLK5 and CTSD but not with KLK7 or LEKT1 (Figure 9).

**Discussion**

Analysis of HI skin and the in vitro HI skin model has provided some insight into how loss of ABCA12 affects the biology of the epidermis. The most striking abnormalities associated with HI were the thickness of the epidermis, premature terminal differentiation, an abnormal lipid profile, and an absence of specific LG proteins. The ABCA12 knockdown OTCC replicated many of the ob-
servations seen in HI patient material, indicating that nonfunctional ABCA12 is the cause of the HI-associated epidermal abnormalities identified.

It has previously been documented that one of the clinical features of HI is an increased thickness of the SC and the epidermis as a whole. H&E of the HI skin biopsy and ABCA12 knockdown OTCCs confirmed this increased epidermal thickness. The thickness of the SC associated with HI is most likely caused by aberrant desquamation resulting in the retention of the thick scale. This may be in part because certain proteases that are expressed or activated as products of late differentiation are not transported to the SC in HI skin because of an ineffective LG system.

We have identified that the LG components KLK5 and CTSD are significantly reduced/absent from HI skin and the OTCC HI model. Both of these proteins are important for desquamation; KLK-5 is expressed in the stratum granulosum and transported to the SC, via the LG network, where it is thought to form a proteolytic cascade where KLK5 activates itself as well as KLK7. Once active it is thought that both these enzymes perform desquamation by digesting desmoglein 1, desmocollin-1, and corneodesmosin. CTSD also has a role in epidermal desquamation through corneodesmosome degradation. CTSD is activated by ceramides derived from acid sphingomyelinase. The fact that ceramides may be significantly reduced/absent from the SC of HI skin could additionally hinder the role of CTSD during desquamation. Therefore, without the transport of these proteins to the SC in HI, it is likely that normal desquamation cannot occur and the SC remains abnormally thick.

Abnormal synthesis or metabolism of LG-related lipids has previously been suspected as the main pathological cause of HI. The structure of ABCA12 and the fact that many of the proteins in the ABC superfamily have a role in energy-dependent active transport of substances across membranes led to the hypothesis that HI may in part be attributable to defective lipid transport. The Akiyama group demonstrated defective lipid transport by a congested pattern of glucosylceramide (GlcCer) staining in keratinocytes with defective ABCA12. The authors then show recovery after corrective gene transfer. Nile Red analysis supports the finding that ABCA12 is likely to transport GlcCer into the LG system in preparation for transport to the intercellular space. GlcCer is enzymatically hydrolyzed into different ceramides in the transition from the SG to the SC. It is likely that ceramides are absent from the HI skin biopsy (and the OTCCs to some degree) because no nonpolar lipids were detected. The nonpolar ceramides are a key part of
the skin barrier so their loss would have a profound effect on barrier function.

However, a key question raised is why are there diminished or absent LGs in HI skin as other ABC transporters are also expressed in the skin and have been shown to be involved in the transportation of lipids in the epidermis. Overexpression of ABCA7 in HeLa cells results in an increased expression of intracellular and cell-surface ceramide as well as intracellular phosphatidylserine. It is suggested by the authors that ABCA7 may play a functional role in LG lipid homeostasis in the epidermis. It has also been suggested that ABCB1 and ABCC1, ABCC3, and ABCC4 translocate amphiphilic anions, such as conjugates of lipophilic compounds with glutathione, glucuronate, and sulfate. It has also been suggested that ABCB1 and ABCG1 may be involved in the translocation of cholesterol. Therefore if these, and other, ABC transporters are expressed in the skin and are involved in lipid transport why is it not possible for them to compensate for the lack of ABCA12 found in HI? In this study, we provide evidence that ABCA12 may have other functions in the skin in addition to lipid loading.

ABC with the markers of late epidermal differentiation (involutin, K2e, TGase1, and filaggrin) identified that the expression pattern of these proteins is highly abnormal in HI compared with normal control skin. Using both HI skin and ABCA12 knockdown OTCCs, the localization of TGase 1, involucrin, and K2e was irregular with expression seen throughout the epidermis compared with their localization in the top layers of controls. Similarly, filaggrin expression was observed throughout the upper spinous layer in HI skin, but was absent from the SC suggesting filaggrin is not being processed effectively. It has previously been reported that there is a defect in the conversion of profilaggrin to filaggrin in patient skin. The highly abnormal expression pattern of late epidermal differentiation markers suggests that nonfunctional ABCA12 affects the tightly controlled program of early epidermal differentiation and terminal differentiation is initiated prematurely.

The proteins LEKT1, KLK7, KLK5, and CTSD are all markers of the LG system. Expression of these LG proteins was analyzed in the HI skin biopsy and ABCA12 knockdown OTCCs. CTSD and KLK5 expression was significantly reduced/absent in both HI skin and ABCA12 knockdown OTCCs but LEKT1 and KLK7 were expressed. These observations are in accordance with the immunocytochemical co-localization experiments. There was a degree of co-localization between ABCA12 and both KLK5 and CTSD but no evidence of co-localization between ABCA12 and either KLK7 or LEKT1. These results add to the hypothesis that CTSD and KLK5 are localized together with ABCA12 in the LG system. It suggests that LEKT1 and KLK7 are separately localized to ABCA12 and are still translocated within the LG system indicating that some areas of the LG system are still formed in HI. Some LGs are present in HI patients even though they are diminished and some appear abnormal. It is possible that ABCA12 is important for the formation process of some but not all parts of the LG system. The lack of KLK5 and CTSD transport to the SC via ABCA12-mediated LG may in part be responsible for the defect in desquamation detected in HI. In conclusion we provide evidence that the function of ABCA12 is not limited to just lipid loading of LG but also in the formation of at least some of the LG network and in the complex regulation of early epidermal differentiation.

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