Endogenous β-glucocerebrosidase activity in Abca12−/− epidermis elevates ceramide levels after topical lipid application but does not restore barrier function

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Abstract ABCA12 mutations disrupt the skin barrier and cause harlequin ichthyosis. We previously showed Abca12−/− skin has increased glucosylceramide (GlcCer) and correspondingly lower amounts of ceramide (Cer). To examine why loss of ABCA12 leads to accumulation of GlcCer, de novo sphingolipid synthesis was assayed using [14C]serine labeling in ex vivo skin cultures. A defect was found in β-glucocerebrosidase (GCase) processing of newly synthesized GlcCer species. This was not due to a decline in GCase function. Abca12−/− epidermis had 5-fold more GCase protein (n = 4, P < 0.01), and a 5-fold increase in GCase activity (n = 3, P < 0.05). As with Abca12−/+ epidermis, immunostaining in null skin showed a typical interstitial distribution of the GCase protein in the Abca12−/− stratum corneum. Hence, we tested whether the block in GlcCer conversion could be circumvented by topically providing GlcCer. This approach restored up to 15% of the lost Cer products of GCase activity in the Abca12−/− epidermis. However, this level of barrier ceramide replacement did not significantly reduce trans-epidermal water loss function. Our results indicate loss of ABCA12 function results in a failure of precursor GlcCer substrate to productively interact with an intact GCase enzyme, and they support a model of ABCA12 function that is critical for transporting GlcCer into lamellar bodies.—Haller, J. F., P. Cavallaro, N. J. Hernandez, L. Dolat, S. J. Soscia, R. Welti, G. A. Grabowski, M. L. Fitzgerald, and M. W. Freeman. Endogenous β-glucocerebrosidase activity in Abca12−/− epidermis elevates ceramide levels after topical lipid application but does not restore barrier function. J. Lipid Res. 2014, 55: 493–503.

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Harlequin ichthyosis (HI) is a severe autosomal recessive disease caused by mutations in the ABCA12 lipid transporter (1, 2). HI patients present with a drastically hyperkeratotic epidermis and have a complete loss of the skin permeability barrier function. Poor temperature regulation, enhanced water loss, and bacterial super-infections develop as a consequence of defects in the barrier function of HI skin, making neonatal survival difficult without intensive treatment (3–6).

The epidermis is responsible for the formation and maintenance of the skin barrier function (7). A critical component of this barrier is the extracellular lipid domains that surround the corneocytes of the stratum corneum (SC). These interstitial lipid domains are organized in lamellar structures and consist primarily of cholesterol, fatty acid, and ceramides (8). Ceramides comprise about half of the total lipids in the SC and are essential for the lamellar structure of this extracellular lipid domain (8–10). They are synthesized exclusively from glucosylceramide (GlcCer) and sphingomyelin (SM) precursors, which are generated in nucleated keratinocytes and stored in lamellar bodies (LB). As the keratinocyte matures, the contents of the LB are extruded into the interstices of the SC where the GlcCer and SM are enzymatically hydrolyzed to Cer by β-glucocerebrosidase (GCase) and sphingomyelinase.

Abbreviations: CBE, conduritol β-epoxide (small-molecule inhibitor of GCase); Cer, ceramide; E18.5, day 18.5 mouse embryo; GCase, β-glucocerebrosidase; GlcCer, glucosylceramide; HI, harlequin ichthyosis; LB, lamellar body; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (small-molecule inhibitor of GlcCer synthase); SC, stratum corneum; SG, stratum granulosum; TEWL, trans-epidermal water loss.

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To model HI and define how a loss of ABCA12 function disrupts the epidermal barrier, we and others have developed mice with inactivating mutations in the Abca12 locus (11–13). These animals recapitulate key features of the HI syndrome in that Abca12−/− pups do not survive ex utero and display a marked hyperkeratosis of the epidermis. Phenotyping of the Abca12−/− pups shows they lack a proper permeability barrier, which is associated with a profound reduction in skin linoleic esters of ω-hydroxy- ceramides (Cer-EOS) and a corresponding increase in their glucosylceramide precursors (GlcCer-EOS) (13). Consistent with these lipids playing a critical role in forming the SC interstitial lamellae, ultrastructural analysis of the Abca12−/− epidermis showed no SC lamellae, and the stratum granulosum (SG) lacked intact lamellar bodies (12, 13). Furthermore, ABCA12 has been shown to localize to the lamellar bodies (14). These observations are consistent with a model of ABCA12 function that posits the transporter resides on the limiting membrane of the lamellar body where it acts to specifically move GlcCer-EOS across the bilayer for eventual secretion at the SC/SG interface. Though it is well established that ABCA12 is essential for forming the epidermal lipid permeability barrier, little biochemical data exists as to how ABCA12 affects the metabolism of skin ceramides, including the enzymes involved in their synthesis.

Using a new N-terminal anti-ABCA12 antibody that shows no cross-reactivity to other ABCA proteins, we show that ABCA12 is highly expressed in the skin, with the stomach expressing detectable, but markedly less, ABCA12 protein. Given the restricted pattern of ABCA12 expression, we focused on developing a skin organ culture system to explore the metabolic function of ABCA12 in this tissue. With this system, we show novel biochemical evidence that indicates that loss of ABCA12 blocks the hydrolysis of endogenous GlcCer species, despite the presence of high levels of active GCase enzyme. Moreover, we show that topical application of GlcCer can partially circumvent this blockage, enabling Abca12−/− skin to enhance synthesis of ceramides critical for epidermal barrier function. However, exogenous delivery of GlcCer was only able to restore approximately 15% of the ceramides found in wild-type skin, and this change was insufficient to improve the water permeability barrier of the Abca12−/− epidermis.

MATERIAL AND METHODS

Reagents

Reagents were purchased from the indicated suppliers: [14C] serine (NEN-PerkinElmer), rabbit anti-GCase (Abcam), cell culture media (Lonza), and lipid standards (Avanti Polar Lipids). ABCA1, ABCA2, ABCA3, and ABCA7 antibodies were generated in our laboratory and previously described (14–16). Protein was measured by Bradford assay and BCA (bicinchoninic acid) protein assay kit (Pierce). All other reagents, unless otherwise specified, were purchased from Sigma-Aldrich.

Abca12−/− mice

Mice that were heterozygous for a null allele at the Abca12 locus have been previously described (13). Procedures were approved by the Massachusetts General Hospital Committee on Research Animal Care and conducted in accordance with the USDA Animal Welfare Act and the PHS Policy for the Humane Care and Use of Laboratory Animals.

Skin, epidermis, and lipid isolation

For all the described studies, day 18.5 mouse embryos (E18.5) were obtained using timed pregnancies and Caesarean section. After euthanasia, limbs and tails were removed, a dorsal cut was made at the neck down to the hypodermis and was extended to the tail stump, and then the skin was peeled off in one piece using dissecting forceps (17). This skin sample, which contains dermis and epidermis, is referred throughout this article as an “embryo whole-skin peel.” The histological presentation of these late gestational skin samples is shown in Fig. 2D. To isolate epidermis, the embryonic whole-skin peels were floated dermis-side down in 0.5% trypsin (GIBCO) in Dulbecco’s phosphate-buffered saline (PBS) at 4°C overnight, and then epidermis was separated from the dermis using tweezers. Total lipid was extracted from the samples by the method of Bligh and Dyer (18). Briefly, the whole skin or epidermis derived from one embryo was finely minced with scissors in 3.75 ml of a single-phase solution of methanol/chloroform/PBS (2:1:0.75). After 30 min sonication at 40°C using a Branson water bath sonicator, 1 ml of chloroform and 1 ml of PBS were added to achieve phase separation. The organic phase separated upon centrifugation was collected and dried under a gentle nitrogen gas stream.

Immunoblotting procedures and generation of an ABCA12 antibody

An anti-ABCA12 serum was raised against amino acids 47–315 by methods previously described (14). Specificity was confirmed with lysates from HEK293 cells transfected with cDNA encoding ABCA1, ABCA2, ABCA3, ABCA7, or ABCA12. Immunoblots of protein lysates were carried out as described previously (13). Membranes were probed with anti-ABCA12 serum (1:1000 dilution), at room temperature for 2 h, and detected using

![Diagram](image-url)
Circumventing the Abca12 metabolic defect

Skin organ culture and labeling procedures

E18.5 embryo whole-skin peels were cultured dermis-side down for 16 h in 2 ml of EMEM with 10% FBS, 0.6 mM CaCl2, 100 U/ml of penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO2 containing 1.24 µCi of [14C]serine in the presence of 100 µM conduritol B-epoxide (CBE, Sigma Catalog # C5424), or 30 µM (±)-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, Sigma Catalog # P7340), or no inhibitor. Media was aspirated, and skins were floated in 0.5% trypsin overnight at 4°C to isolate the epidermis.

Histology and immunohistochemistry

E18.5 embryo whole-skins peels were fixed in Bouin’s solution and paraffin embedded. For immunohistochemistry, slides were deparaffinized in xylene and ethanol and rehydrated in water. Slides were blocked with 10% normal horse serum and 1% BSA/PBS, and then incubated with primary antibody (1:75 dilution) in 1% BSA/PBS overnight at 4°C. The slides were then washed, and a secondary biotinylated goat-anti-mouse IgG antibody was applied (1:250 dilution). The avidin-biotin peroxidase complex and DAB chromophore-staining method was used (ABC kit, DAB kit; Vector Laboratories), and the slides counterstained with hematoxylin.

Fig. 2. ABCA12 protein is largely restricted to the epidermis. A: Tissue expression of ABCA12 mRNA in the adult mouse as determined by reverse transcription and quantitative PCR (RT-QPCR) (± SD, n = 3). B: A newly produced rabbit anti-ABCA12 polyclonal antibody specifically recognizes ABCA12 in the epidermis of neonatal mice and does not cross-react with other ABCA transporters transiently expressed in HEK 293 cells, including ABCA1, ABCA2, ABCA3, and ABCA7. Panels show signals derived from one immune-blot sequentially probed for the indicated transporters, involucrin and β-actin. C, ABCA12 expression in E18.5 embryos is largely restricted to the epidermis, though expression in the stomach was detected at markedly lower levels (15). D: Histological skin section stained with H and E, depicting the epidermal structures of E18.5 embryos used in our experiments. E: Histological section of the stomach stained with H and E identifying the hyperkeratosis of the gastric mucosa in the forestomach region in Abca12−/− animals (arrowheads point to the keratosis in the Abca12−/− tissues. L, lumen; SB, stratum basale; SQ, stratified squamous epithelium; SS, stratum spinosum).

TLC procedures

Lipids extracted from the epidermis of E18.5 mouse embryos were separated by TLC using a three-solvent system described previously (19), radiolabeled lipids were visualized and quantitated by phosphor imaging (Amersham Biosciences), and total lipids were subsequently quantified by densitometry of the plates charred with cupric sulfate in aqueous phosphoric acid (19). For preparative extraction, bands were located with iodine vapor, then scraped and extracted in methanol/chloroform 1:2 (v/v).

Mass spectrometry lipid profiling procedures

Lipid structure was determined using an ESI-MS/MS approach as reported previously (13). Scans were performed for precursors of m/z 264 in the positive mode (collision energy, 50 V) for GlcCers and Cers. Internal standards for quantification were d18:1/14:0Cer and d18:1/12:0GalCer. The amounts of the analyte lipids are indicated in units of normalized mass spectral signal with one unit representing the amount of lipid producing the same amount of signal as 1 nmol of the internal standard.

Skin organ culture and labeling procedures

E18.5 embryo whole-skin peels were cultured dermis-side down for 16 h in 2 ml of EMEM with 10% FBS, 0.6 mM CaCl2, 100 U/ml of penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO2 containing 1.24 µCi of [14C]serine in the presence of 100 µM conduritol β-epoxide (CBE, Sigma Catalog # C5424), or 30 µM (±)-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, Sigma Catalog # P7340), or no inhibitor. Media was aspirated, and skins were floated in 0.5% trypsin overnight at 4°C to isolate the epidermis.
β-Glucocerebrosidase assays

SC samples were scraped from E18.5 embryo skins, and then tissue lysates and in vitro measurements were performed as described previously (20, 21). For in situ activity, embryo whole-skin peels were preincubated with 0.54% sodium taurocholate in McIlvaine citrate-phosphate buffer pH 5.6 (taurocholate buffer) (21) in the presence or absence of 10 µM CBE for 30 min. Then 100 µl of a solution of 0.5 mM 4-methylumbelliferyl-β-D-glucopyranoside (Sigma Catalog # M3633) in taurocholate buffer was added onto the epidermis, and samples were incubated in the dark. The reaction was stopped with 1 ml of 200 mM carbonate-bicarbonate buffer pH 10.5. Samples were vortexed and centrifuged at 14,000 g at 4°C, and fluorescence (Ex/Em = 355/460 nm) was measured in the supernatant. In situ GCase activity represents the difference in activity obtained with or without CBE.

Topical [14C]GlcCer conversion

For generation of labeled [14C]GlcCer precursor, embryo whole skins were cultured as described above with 20 µg/ skin of [14C]serine for 24 h and purified as described in the TLC procedures. A solution of 40,000 dpm of [14C]GlcCer/ml in taurocholate buffer was prepared by water bath sonication. One-hundred microliters of this solution was added to the epidermal surface of an E18.5 embryo whole-skin peel. After 1 h at room temperature and an overnight incubation at 4°C, lipids were extracted from the whole-skin peel as described in the lipid isolation section above. Generated [14C]Cer products were separated from the input GlcCer substrate by silica gel column chromatography (22) with the following modification: Ceramides were eluted with 7 ml chloroform/methanol 98:2 (v/v) followed by 1.5 ml chloroform/methanol 92:8 (v/v)/. Then unhydrolyzed GlcCer was subsequently eluted with an additional 6.5 ml chloroform/methanol 92:8 (v/v). An aliquot of these fractions was quantified with liquid scintillation counting and the remainder analyzed by TLC as described above. To control for nonenzymatic GlcCer hydrolysis that may occur during the extraction process, radiolabeled GlcCer was added to a control skin sample immediately before lipid extraction during each experiment. These controls showed that observed ceramide formation was not due to the extraction process in either the wild-type or Abca12−/− samples.

Acute GlcCer treatment

to isomilligram GlcCer quantities that were needed to conduct the exogenous treatment assays, E18.5 Abca12−/− and Abca12+/+ embryo total epidermal lipid, dissolved in chloroform, was applied to a column of hexane-equilibrated silica gel 60 (6 mg of lipid/g of silica). After washing the column with 18 ml of chloroform/acetic acid 100:1 (v/v) per gram of silica, and 18 ml of chloroform/methanol 191:9 (v/v), GlcCers were eluted with 14 ml of chloroform/methanol 92:8 (v/v). This method yielded 0.125 mg of GlcCer/embryo epidermis of a purity that ranged 91–95% as assayed by TLC. The isolated fraction contained both GlcCer and GlcCer-EOS species. A suspension of 1 mg/ml these GlcCers in taurocholate buffer was prepared by water sonication. Freshly isolated E18.5 embryo whole-skin samples of 0.5 × 0.5 cm were placed dermis-side down onto a 1 cm diameter Millicell-PCF 0.4 µm membrane inserts (Millipore). A dose of 25 µl of the 1 mg/ml GlcCer suspension or 25 µl of vehicle (taurocholate buffer) was added onto the epidermal side of the skin samples. Inserts were incubated at 37°C in 60 mm dishes containing 3.6 ml of G1T-02-3DP1 Epidermal Keratinocyte 3D Prime medium (CellnTec) for 6, 12, 24, and 48 h. At the specified treatment times, epidermal barrier function was assayed as previously described using the gravimetric method (11, 13). Experiments run in parallel conditions using this GlcCer preparation spiked with a radioactive GlcCer tracer, and estimated 3.2 ± 0.5 µg and 3.7 ± 0.6 µg of Cer were formed during 24 h and 48 h incubation, respectively. This is equivalent to a replenishment of 13% and 15% of wild-type Cer levels.

Statistical analysis

All statistical analyses were performed using a Student t-test. P < 0.05 was considered statistically significant.

RESULTS

ABCA12 tissue expression

We first examined the tissue distribution of ABCA12. When assessed in the adult mouse, the expression of ABCA12 mRNA was detectable in a number of other tissues besides skin, including the heart, intestine, stomach, and kidney (Fig. 2A). To corroborate the mRNA findings, protein expression was assayed using a novel antibody that we had generated against the first putative N-terminal loop of ABCA12. This antibody detected ABCA12 in wild-type but not Abca12−/− skin and showed no cross-reactivity against multiple ABCA transporters, including ABCA1, ABCA2, ABCA3, and ABCA7 expressed in HER 293 cells (Fig. 2B). Given this lack of cross-reactivity, we probed a tissue blot derived from E18.5 mouse embryos with this antibody. Compared with ABCA12 expression in the epidermis, there was much reduced but detectable expression of ABCA12 in the stomach, whereas no signal was detected in the intestine or any of the other tissues analyzed, including the lung (Fig. 2C). When the blots were stripped and reprobed for ABCA1, ABCA2, ABCA3, and ABCA7, no expression of any of those transporters in the epidermis was evident, providing evidence that a compensatory upregulation of alternative ABCA transporters does not occur in the absence of ABCA12 (Fig. 2B, first two lanes). As reported previously (11–13), the loss of ABCA12 function results in a marked hyperkeratosis of the SC in E18.5 mouse embryos (Fig. 2D). Given our detection of ABCA12 protein in the stomach, we tested whether there were histological abnormalities in this organ of the Abca12−/− mouse. Histological analysis of day 18.5 mouse embryo stomach revealed a hyperkeratosis in the gastric mucosa, which was confined to the forestomach region of Abca12−/− animals (Fig. 2E). No other anatomical or histological differences were identified in the stomach. In composite, these results demonstrate a very restricted distribution pattern of ABCA12 protein, with epidermal expression predominating.

De novo ceramide synthesis

Having established that ABCA12 expression is largely confined to the epidermis, we sought to clarify the function it plays in the skin. To this end, skin samples from E18.5 embryos were cultured in media containing [14C] serine. Because serine palmitoyltransferase initiates de novo sphingolipid synthesis by condensing serine and palmitoyl-CoA (Fig. 1), use of radiolabeled serine allows for the monitoring of de novo synthesized glucosylceramide
and its conversion into ceramide. The charred TLC plate in Fig. 3A depicts total lipid profiles of the epidermis isolated from these skin cultures. Lipids from Abca12−/− epidermis showed a pattern that clearly differed from the lipids detected in Abca12+/+ epidermis. The most dramatic changes in these samples were in the species marked “a” to “d” (Fig. 3A). Species denoted “a” and “b” comigrated in the region of ceramide standards and lipids denoted “c” and “d” in the region of glucosylceramide standards. To define the molecular nature of these lipids, we ran preparative TLC plates to isolate microgram quantities of these lipids from an additional set of Abca12+/+ and Abca12−/− epidermal samples (n = 5). The isolated lipids were subjected to quantitative lipid profiling using an ESI-triple quadrupole tandem mass spectrometer. Lipid “a” was principally composed of very long chain ceramide esters. Scans of Abca12−/− samples showed there was a significant reduction (81%) in these lipids compared with amounts isolated from the Abca12+/+ samples, which we have previously shown, using collision induced product ion analysis, to be linoleic esters of ω-hydroxy very long-chain ceramides (Cer-EOS, Fig. 3B) (13). Profiling lipids extracted from the region of the plates where lipid “b” migrated indicated that “b” was principally composed of 16 to 22 carbon acyl chain ceramides (40%, Cer-d18:1(16:0), 30% Cer-d18:1(18:0), Fig. 3C). These two shorter acyl chain ceramides were significantly increased in the absence of ABCA12 (by 6.5- and 18.6-fold, respectively). These lipids have been previously reported to be generated by sphingomyelinase (10). Scans of lipid “c” showed

![Fig. 3. Alteration of endogenous sphingolipid levels in the Abca12−/− epidermis. A: Shown are endogenous lipids levels detected in extracts of Abca12+/+ and Abca12−/− day 18.5 embryonic skin cultures analyzed by copper charring of TLC plates. Lipids labeled “a” to “d” mark the most altered sphingolipid species between the genotypes. These were isolated by preparative TLC from additional Abca12+/+ and Abca12−/− day 18.5 embryonic epidermis and subjected to quantitative mass spectrometry. B–D: Graphed are the three most predominate species identified for each of the analyzed samples (n = 5, ± SD, *P < 0.05). The nomenclature, e.g., d18:1(50:2 ester), indicates a sphingosine base (d18:1) in an amide linkage to a very long chain fatty acid, which is ester-linked to another fatty acid; these two acyl chains contain a total of 50 carbons and two bonds. Previous data indicate that 50:2 is a 32-carbon saturated fatty amide linked to linoleic acid (18:2) (13).]
Furthermore, the modest effect of the inhibitors on the amount of labeled Cer in the null epidermis (Fig. 4) suggests that the ceramides that persist in the null skin are mainly processed through SM precursors and not from GlcCer. This is further supported by the behavior of serine labeled lipid “b.” Though present in significantly greater amounts in the \( \text{Abca12}^{+/+} \) epidermis, its levels were not significantly altered by either CBE or PDMP treatment in either ABCA12 wild-type or null skin.

GCase expression and activity

One possible explanation for our results is that a loss of ABCA12 inhibits GCase expression or enzymatic function, leading to the observed GlcCer buildup. This possibility was tested by measuring GCase activity and protein in the \( \text{Abca12}^{-/-} \) epidermis and shown not to be the case. In fact, 5-fold higher levels of GCase were detected in the \( \text{Abca12}^{-/-} \) epidermis of E18.5 mouse embryos by immunoblot analysis of tissue lysates (Fig. 5A, \( P < 0.01 \)). Furthermore, immunostaining of \( \text{Abca12}^{+/+} \) and \( \text{Abca12}^{-/-} \) whole-skin sections for GCase levels was used to assess the distribution of the enzyme. In \( \text{Abca12}^{+/+} \) epidermis, staining was evident in the nucleated cellular layers in a cytoplasmic distribution, whereas in the stratum corneum, the staining localized to the interstitial spaces. Immunostaining of the \( \text{Abca12}^{-/-} \) embryo whole skins confirmed that levels of GCase were increased. As with the \( \text{Abca12}^{+/+} \) tissue, however, this abundant GCase staining in the hyperkeratotic \( \text{Abca12}^{-/-} \) stratum corneum maintained a largely interstitial distribution (Fig. 5C).

![Fig. 4. Loss of ABCA12 activity causes a defect in the GCase-mediated conversion of de novo synthesized GlcCer to Cer. \( \text{Abca12}^{+/+} \) and \( \text{Abca12}^{-/-} \) day 18.5 embryonic skin cultured in the presence of \([\text{14C}]\)serine were untreated or treated with either an inhibitor of GCase (CBE, 100 µM) or glucosylceramide synthase (PDMP, 30 µM). Lipids from the isolated epidermis were separated on TLC plates. A: TLC plates of \([\text{14C}]\)serine-labeled lipids are representative samples. B: Graphed is the amount of labeled lipid quantitated by phosphor imaging and normalized to the amount of labeled phosphatidylethanolamine (n = 3, ± SD, *\( P < 0.05 \) control versus CBE- or PDMP-treated, **\( P < 0.05 \) control \( \text{Abca12}^{+/+} \) versus control \( \text{Abca12}^{-/-} \)).]
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mice, even after normalization of the activity to total SC protein levels (Fig. 5E). Thus our data indicate that loss of ABCA12 function is associated with increased GCase protein and activity in the SC. Finally, the enhanced levels of GCase protein in Abca12+/−/− epidermis appear to result from a posttranscriptional process, as loss of ABCA12 caused no significant change in the amount of Gba1 mRNA, which encodes GCase (Fig. 5B).

Topical application of GlcCer precursors

The above data indicated that the enzyme activity required for the conversion of ceramides from glucosylceramide precursors was not impaired in the Abca12−/− skin. This suggests that in the absence of ABCA12, endogenously synthesized GlcCer fails to localize to the intercellular lamellar region of the stratum corneum, which is rich in GCase activity. If this hypothesis were correct, we
reasoned that topical application of GlcCer should be able to bypass this transport defect and would restore ceramide production in the *Abca12*–/– stratum corneum. To test this hypothesis, we isolated [14C]GlcCer from [14C] serine-radiolabeled mouse skin cultures by preparative TLC and prepared a solution of this radiolabeled precursor. The solution was applied topically to the stratum corneum of unlabeled E18.5 embryo whole-skin samples. After incubation for 1 h at room temperature and overnight incubation at 4°C, lipids were extracted from the whole-skin sample, and ceramides were separated from their nonhydrolyzed precursors using a Silica Gel column (22). Significantly, the *Abca12*–/– skins converted 21–25% of the applied GlcCer into ceramides, while the *Abca12*+/+ skin converted 11–14% (Fig. 6A). Additional controls performed in parallel during these experiments showed GlcCer hydrolysis did not occur when the skin samples were extracted immediately after the addition of radiolabeled substrate. Thus, these controls show the ceramides produced during this incubation period were catalyzed by endogenous GCase. Remarkably, considering the near complete block in GCase processing of endogenously synthesized GlcCers, the *Abca12*–/– skin synthesized all major ceramides from the topically applied precursors. Importantly, this included the generation of the missing ceramide “a” or Cer-EOS (Fig. 6B). Thus, circumvention of the block in endogenous GlcCer processing and restoration of ceramide production by topical administration of GlcCer precursors onto skin lacking ABCA12 activity are possible.

Given endogenous GCase in *Abca12*–/– stratum corneum could generate lost barrier ceramides when provided an exogenous source of GlcCer substrate, we tested whether this improved performance of the barrier. As above, a suspension of GlcCer in buffer or buffer alone was applied to the epidermal side of freshly excised E18.5

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**Fig. 6.** Topical application of GlcCer precursors to the SC restores ceramide production. [14C]GlcCer was applied onto the SC of *Abca12*+/+ and *Abca12*–/– whole-skin samples obtained from day 18.5 mouse embryos. After 1 h incubation at room temperature followed by an overnight incubation at 4°C, total lipids were extracted. Ceramides were separated from their unhydrolyzed precursors using a silica gel column. An aliquot was quantitated by liquid scintillation counting (A). Values at the top of the bars represent the average percentage of substrate converted into ceramides (n = 3, ± SD, P < 0.05). B: Shown are the converted ceramides and unhydrolyzed precursors resolved on a TLC plate and visualized by phosphor imaging. The “Ctrl” lane shows a wild-type skin which was processed immediately after addition of the radioactive substrate to control for hydrolysis of the substrate during the lipid extraction. C: TEWL measurements of E18.5 *Abca12*–/– skins grown on an air/liquid interface and treated with an acute dose of GlcCer for the indicated periods did not show a significant improvement of the skin permeability barrier function (n = 6 for times 12 and 24 h, n = 2 for times 6 and 48 h).


Abca12\(^{-/-}\) embryo whole-skin samples. These were grown dermis-side down in a millipore-PCF membrane insert at an air/liquid interface in CnT-02-3DP1 Epidermal Keratinocyte 3D Prime medium (CellLine) for 6, 12, 24, and 48 h at 37°C in a humidified tissue culture incubator. At the specified times, the ability of the skin to retain water was measured using a trans-epidermal water loss (TEWL) assay. Application of GlcCer onto Abca12\(^{-/-}\) whole-skin samples under the assayed conditions did not show any statistical significant improvement in permeability as compared with the vehicle treatment at any of the measured times (Fig. 6C). To confirm that significant amounts of ceramides are generated and persist during the longer time frame of the 37°C functional assays, we conducted parallel biochemical assays using a radioactive GlcCer tracer spiked into the GlcCer preparation. These control experiments confirmed this approach was replenishing approximately 13% and 15% of the wild-type epidermal Cer levels in the Abca12\(^{-/-}\) epidermis at the 24 h and 48 h incubation points, respectively.

**DISCUSSION**

In these studies, skin organ cultures from Abca12\(^{-/-}\) late-term embryos were used to interrogate ABCA12 function within the epidermis, in which we show the transporter is most abundant. Ex vivo culture of embryonic whole skin, combined with metabolic labeling, small-molecule inhibition, and mass spectrometry lipid profiling, demonstrates that the absence of ABCA12 induces a block in the hydrolysis of de novo synthesized GlcCer-EOS, as well as other nonester glucosylceramides species. Importantly, the GlcCer processing block is not explained by a decline in either the amount or activity of GCase enzyme. Indeed, Abca12\(^{-/-}\) skin contains significantly enhanced levels of GCase protein and activity, particularly in the stratum corneum. Moreover, we demonstrate that Abca12\(^{-/-}\) stratum corneum, when presented with exogenous GlcCer precursors, is able to overcome the GCase-mediated GlcCer conversion defect and regenerate some of the lost ceramides that are known to be critical for formation of the skin permeability barrier.

These results provide novel biochemical data into how glucosylceramides accumulate in the Abca12\(^{-/-}\) skin. By exploring the enzymatic processing of de novo-synthesized GlcCer, we show that flux of this lipid processed by GCase was significantly diminished. These observations now identify a key processing step in the conversion of GlcCer to Cer that, in the absence of ABCA12, accounts for our and other observations of the enhanced steady-state levels of glucosylceramides in the Abca12\(^{-/-}\) epidermis (11–13). Gaucher’s disease, caused by mutations in the GCase gene, is also characterized by accumulation of GlcCer, and a subset of Gaucher’s patients present with an ichthyotic phenotype (26). Likewise, mice lacking GCase display ichthyotic skin and accumulate excess GlcCer (27). Moreover, in the absence of GCase, these animals maintain ceramide levels by inducing sphingomyelin hydrolysis (28), which results in the generation of shorter acyl chain ceramides of the class we have identified to be contained in the band “b” ceramides that are elevated in the Abca12\(^{-/-}\) epidermis. Thus, as with genetic loss of GCase activity, we now demonstrate specific alterations in sphingolipid metabolism in the Abca12\(^{-/-}\) epidermis that are occurring despite intact GCase activity.

A substantial amount of literature demonstrates that inhibiting cholesterol, fatty acid, ceramide, or glucosylceramide synthesis leads to abnormal lamellar bodies and impaired barrier function (7, 29–32). Thus, stratum granulosum cells must generate all these lipids and then transport them into the LB where they can be assembled into a lamellar structure required for barrier function (7). In normal skin, ABCA12 localizes to LB (33), and keratinocytes demonstrate a widely distributed pattern of cellular GlcCer expression (1, 12). In contrast, in HI skin, GlcCer fails to localize to the periphery of the keratinocyte cytoplasm (34), and there is a loss of lamellae structure in the stratum corneum and LB (1, 11–13). These data, together with our observations of the enzymatic processing of de novo-synthesized ceramides in the Abca12\(^{-/-}\) skin and the partial circumvention of the GlcCer processing defect via topical application of exogenous precursors, are consistent with a critical role for ABCA12 in transporting GlcCer to the extracellular space via its action at the lamellar body membrane (1, 7, 11–13, 35, 36). With loss of ABCA12, an accumulation of GlcCer species is observed that is due not to a block in GCase enzyme activity, as with Gaucher’s patients, but from a defect in the trafficking of GlcCer into the LB and hence to the extracellular domains of the stratum corneum. Without proper supply of this precursor to the interstices of the stratum corneum, there is a loss in the production of ceramides via the GCase pathway. Since some ceramide species can only be generated via the activity of GCase (28, 37), the result is loss of specific epidermal ceramides that are critical for maintaining the permeability barrier function of the skin (7).

ABCA12 tissue distribution in the neonate is largely epidermal, and in the absence of ABCA12 function, no compensatory upregulation of other ABCA-class transporters, including ABCA1, ABCA2, ABCA3, and ABCA7, was detected in the Abca12\(^{-/-}\) epidermis. Expression of ABCA12 was also observed in the stomach; as in skin, the Abca12\(^{-/-}\) gastric epithelia presented evidence of a marked hyperkeratosis. This finding suggests that ABCA12 has a role at the affected region of the stomach that results in a similar thickening of the keratinized layers as is found in the skin. Keratinized epithelia in certain regions of the stomach is characteristic of rodent gut but not of human stomach. Therefore, these findings cannot be translated to explain the gastrointestinal problems described in HI patients (38).

Lastly, supplying GlcCer to the epidermis under the conditions assayed was not sufficient to restore the permeability barrier. As our experiments were limited to a short treatment period and resulted in only up to 15% restoration of the wild-type ceramide levels, it is possible that
different treatment paradigms, such as using higher levels of topical exogenous lipid or applying GlcCer as part of a three-component mixture that includes cholesterol and fatty acids, could result in higher ceramide levels and perhaps greater restoration of function. Alternatively, exogenous GlcCer may need first to be taken up into granular cells, and then secreted via the lamellar body secretory system to impact barrier function (39). Moreover, the loss of ABCA12, which is associated with a number of other pathological skin features, including hyperkeratotic stratum corneum and abnormal protein distribution in the epidermis involving involucrin, transglutaminase 1, and filaggrin (11, 13, 34, 40), may cause disruptions that no amount of topical lipid replacement can correct. These deficits could arise as a consequence of cellular GlcCer accumulation in keratinocytes that may cause lipotrophic effects (41). Therefore, supplementation of ceramide production by itself may not suffice to correct skin barrier function, even if it were to more completely restore levels of extracellular Cer. In a recent report on congenital hemidysplasia with ichthyosiform erythroderma and limb defect (CHILD) syndrome, an X-linked dominant disorder of distal cholesterol metabolism, treatments targeted to reduce the accumulation of precursors were required to restore skin function in patients that was not restored by the provision of topical cholesterol alone (42). Thus, as in the CHILD syndrome, dual therapies may be required to correct the cutaneous phenotype of ABCA12-null skin.

In summary, we present novel evidence that strengthens the view that ABCA12 is required to transport de novo-synthesized GlcCer to the interstices of the stratum corneum. The results provide biochemical data that advance our understanding of the defect in epidermal sphingolipid metabolism due to loss of ABCA12 activity. These findings suggest new strategies that may be required to ameliorate defects in skin function in individuals with harlequin ichthyosis.

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REFERENCES

1. Akiyama, M., Y. Sugiyama-Nakagiri, K. Sakai, J. R. McMillan, M. Goto, K. Arita, Y. Tsuji-Abe, N. Tabata, K. Matsuoka, R. Sasaki, et al. 2005. Mutations in lipid transporter ABCA12 in harlequin ichthyosis and functional recovery by corrective gene transfer. J. Clin. Invest. 115: 1777–1784.

2. Kelsell, D. P., E. E. Norgert, H. Unsworth, M. T. Teh, T. Cullup, C. A. Mein, P. J. Dopping-Hepenstal, B. A. Dale, G. Tadini, P. Fleckman, et al. 2005. Mutations in ABCA12 underlie the severe congenital skin disease harlequin ichthyosis. Am. J. Hum. Genet. 76: 794–805.

3. Elias, P. M., M. Fartasch, D. Crumrine, M. Behne, Y. Uchida, and W. M. Holleran. 2000. Origin of the corneocyte lipid envelope (CLE): observations in harlequin ichthyosis and cultured human keratinocytes. J. Invest. Dermatol. 115: 765–769.

4. Hsu, W. Y., J. Y. Chen, W. L. Lin, and C. H. Tsay. 1989. Harlequin fetus–a case report [article in Chinese]. Zhonghua Yi Xue Za Zhi (Taipei) 43: 63–66.

5. Moreau, S., E. Salame, M. Goulet de Rugy, and P. Delmas. 1999. Harlequin fetus: a case report. Surg. Radiol. Anat. 21: 215–216.

6. Sarkar, R. C., R. Sharma, S. Sethi, S. Basu, R. Das, V. Mendiattara, K. Sardana, and N. Kakar. 2000. Three unusual siblings with Harlequin ichthyosis in an Indian family. J. Dermatol. 27: 609–611.

7. Feingold, K. R. 2007. Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis. J. Lipid Res. 48: 2551–2561.

8. Gray, G. M., and H. J. Yardley. 1975. Lipid compositions of cells isolated from pig, human, and rat epidermis. J. Lipid Res. 16: 434–440.

9. Schoephoester, R. T., P. W. Wertz, K. C. Madison, and D. T. Downing. 1985. A survey of polar and non-polar lipids of mouse organs. Comp. Biochem. Physiol. B 82: 229–232.

10. Uchida, Y., and W. M. Holleran. 2008. Omega-3-acylceramide, a lipid essential for mammalian survival, J. Dermatol. Sci. 51: 77–87.

11. Smyth, I., D. F. Hacking, A. A. Hilton, N. Mukhamedova, P. J. Meikle, S. Ellis, K. Satterley, J. E. Collinge, C. A. de Graaf, M. Bahlo, et al. 2008. A mouse model of harlequin ichthyosis delineates a key role for Abca12 in lipid homeostasis. PLoS Genet. 4: e1000192.

12. Yang, T., M. Akikiw, H. Nishihara, K. Sakai, W. Nishie, S. Tanaka, and H. Shimizu. 2008. Harlequin ichthyosis model mouse reveals alveolar collapse and severe fetal skin barrier defects. Hum. Mol. Genet. 17: 3075–3083.

13. Zuo, Y. D., Z. Zhuang, R. Han, G. Isaac, J. J. Tobin, M. McKee, R. Welti, J. L. Brisette, M. L. Fitzgerald, and M. W. Freeman. 2008. ABCA12 maintains the epidermal lipid permeability barrier by facilitating for-mation of ceramide insoluble esters. J. Biol. Chem. 283: 66521–66526.

14. Fitzgerald, M. L., A. J. Mendez, K. J. Moore, L. P. Andersson, H. A. Panjeton, and M. W. Freeman. 2001. ATP-binding cassette transporter 1A contains an NH2-terminal signal anchor sequence that translocates the protein’s first hydrophobic domain to the exoplasmic space. J. Biol. Chem. 276: 15137–15145.

15. Fitzgerald, M. L., R. Xavier, K. J. Hale, R. Welti, J. L. Goss, C. E. Brown, D. Z. Zhuang, S. A. Bell, N. Lu, M. McKee, et al. 2007. ABCA3 inactivation in mice causes respiratory failure, loss of pulmonary surfactant, and depletion of lung phosphatidylglycerol. J. Lipid Res. 48: 621–632.

16. Kim, W. S., M. I. Fitzgerald, K. Kang, K. Okuhira, S. A. Bell, J. J. Manning, S. L. Koehn, N. Lu, K. J. Moore, and M. W. Freeman. 2007. Aca7 null mice retain normal macrophase phosphorylcholine and cholesterol efflux activity despite alterations in adipose mass and serum cholesterol levels. J. Biol. Chem. 280: 3989–3995.

17. Pirrone, A., B. Hager, and P. Fleckman. 2005. Primary mouse keratinocyte culture. Methods Mol. Biol. 289: 3–14.

18. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911–917.

19. Pappinen, S. M., H. Hermansson, J. Kuntsche, P. Somerharju, P. Wertz, A. Urtti, and M. Suuronen. 2008. Comparison of rat epidermal keratinocyte organotypic culture (ROC) with intact human skin: lipid composition and thermal phase behavior of the stratum corneum. Biochim. Biophys. Acta 1778: 824–836.

20. Raviv, A. P. J., J. A. Bouwstra, G. S. Gooris, A. Weerheim, H. E. Boede, and M. Ponec. 1995. Reduced skin barrier function parallels abnormal stratum corneum lipid organization in patients with lamellar ichthyosis. J. Invest. Dermatol. 105: 619–624.

21. Takagi, Y., E. Kriehuber, G. Imokawa, P. M. Elias, and W. M. Holleran. 1999. Beta-glucocerebrosidase activity in mammalian stratum corneum. J. Lipid Res. 40: 861–869.

22. Erickson, J. S., and N. S. Radin. 1973. N-hexyl-O-glucosyl sphingosine, an inhibitor of glucosyl ceramide-glucosidase. J. Lipid Res. 14: 133–137.

23. Premkumar, L., A. R. Sawkar, S. Boldin-Adamsky, L. Toker, I. Silman, W. J. Kelly, A. H. Futerman, and J. L. Sussman. 2005. X-ray structure of human acid-beta-glucosidase covalently bound to con-durabenz-Le-biose. Implications for Gaucher disease. J. Biol. Chem. 280: 23815–23819.

24. Abe, A., J. Inokuchi, M. Jimbo, H. Shimen, A. Nagamatsu, J. Ya Shayman, G. S. Shukla, and N. S. Radin. 1992. Improved inhibitors of glucocerebrosidase synthase. J. Biochem. 111: 191–196.

25. Lee, L., A. Abe, and J. A. Shayman. 1999. Improved inhibitors of glucocerebrosidase synthase. J. Biochem. 239: 911–917.

26. Fujimoto, A., N. Tavebi, and E. Sidransky. 1995. Congenital ichthyosis preceding neurologic symptoms in two siblings with type 2 Gaucher disease. Am. J. Med. Genet. 59: 356–358.

27. Holleran, W. M., E. I. Ginnis, G. K. Menon, J. U. Grundmann, M. Fartasch, C. E. McKinney, P. M. Elias, and E. Sidransky. 1994. Consequences of beta-glucocerebrosidase deficiency in epidermis. Ultrastructure and permeability barrier alterations in Gaucher disease. J. Clin. Invest. 93: 1756–1764.
28. Uchida, Y., M. Hara, H. Nishio, E. Sidransky, S. Inoue, F. Otsuka, A. Suzuki, P. M. Elias, W. M. Holleran, and S. Hamanaka. 2000. Epidermal sphingomyelins are precursors for selected stratum corneum ceramides. *J. Lipid Res.* **41**:2071–2082.
29. Feingold, K. R., M. Q. Man, G. K. Menon, S. S. Cho, B. E. Brown, and P. M. Elias. 1990. Cholesterol synthesis is required for cutaneous barrier function in mice. *J. Clin. Invest.* **86**:1738–1745.
30. Mao-Qiang, M., P. M. Elias, and K. R. Feingold. 1993. Fatty acids are required for epidermal permeability barrier function. *J. Clin. Invest.* **92**:791–798.
31. Jennemann, R., R. Sandhoff, L. Langbein, S. Kaden, U. Rothermel, H. Gallala, K. Sandhoff, H. Wiegandt, and H. J. Grone. 2007. Integrity and barrier function of the epidermis critically depend on glucosylceramide synthesis. *J. Biol. Chem.* **282**:3083–3094.
32. Chujor, C. S., K. R. Feingold, P. M. Elias, and W. M. Holleran. 1998. Glucosylceramide synthase activity in murine epidermis: quantitation, localization, regulation, and requirement for barrier homeostasis. *J. Lipid Res.* **39**:277–285.
33. Sakai, K., M. Akiyama, Y. Sugiyama-Nakagiri, J. R. McMillan, D. Sawamura, and H. Shimizu. 2007. Localization of ABCA12 from Golgi apparatus to lamellar granules in human upper epidermal keratinocytes. *Exp. Dermatol.* **16**:920–926.
34. Yanagi, T., M. Akiyama, H. Nishihara, J. Ishikawa, K. Sakai, Y. Miyamura, A. Naoe, T. Kitahara, S. Tanaka, and H. Shimizu. 2010. Self-improvement of keratinocyte differentiation defects during skin maturation in ABCA12-deficient harlequin ichthyosis model mice. *Am. J. Pathol.* **177**:106–118.
35. Scott, C. A., S. Rajpopat, and W. L. Di. 2013. Harlequin ichthyosis: ABCA12 mutations underlie defective lipid transport, reduced protease regulation and skin-barrier dysfunction. *Cell Tissue Res.* **351**:281–288.
36. Mitsutake, S., C. Suzuki, M. Akiyama, K. Tsuji, T. Yanagi, H. Shimizu, and Y. Igarashi. 2010. ABCA12 dysfunction causes a disorder in glucosylceramide accumulation during keratinocyte differentiation. *J. Dermatol. Sci.* **60**:128–129.
37. Hamanaka, S., M. Hara, H. Nishio, F. Otsuka, A. Suzuki, and Y. Uchida. 2002. Human epidermal glucosylceramides are major precursors of stratum corneum ceramides. *J. Invest. Dermatol.* **119**:416–423.
38. Rajpopat, S., C. Moss, J. Mellerio, A. Vahlquist, A. Ganemo, M. Hellstrom-Pigg, A. Ilyashyn, N. Burrows, G. Lestringant, A. Taylor, et al. 2011. Harlequin ichthyosis: a review of clinical and molecular findings in 45 cases. *Arch. Dermatol.* **147**:681–686.
39. Man, M. Q., K. R. Feingold, and P. M. Elias. 1993. Exogenous lipids influence permeability barrier recovery in acetone-treated murine skin. *Arch. Dermatol.* **129**:728–738.
40. Thomas, A. C., D. Tattersall, E. E. Norgett, E. A. O’Toole, and D. P. Kebell. 2009. Premature terminal differentiation and a reduction in specific proteases associated with loss of ABCA12 in Harlequin ichthyosis. *Am. J. Pathol.* **174**:970–978.
41. Akiyama, M. 2011. The roles of ABCA12 in keratinocyte differentiation and lipid barrier formation in the epidermis. *Dermatologica* **3**:107–112.
42. Paller, A. S., M. A. van Steensel, M. Rodriguez-Martin, J. Sorrell, C. Heath, D. Crumrine, M. van Geel, A. N. Cabrera, and P. M. Elias. 2011. Pathogenesis-based therapy reverses cutaneous abnormalities in an inherited disorder of distal cholesterol metabolism. *J. Invest. Dermatol.* **131**:2242–2248.