Permeability barrier requirements regulate epidermal β-glucocerebrosidase

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Abstract The intercellular spaces of the outermost layers of the epidermis (stratum corneum, SC) of terrestrial mammals contain a mixture of lipids, enriched in ceramides that are critical for the epidermal permeability barrier. Whereas glucosylceramides (GlcCer) are synthesized in abundance in the epidermis, they disappear coincident with an increase of ceramides (Cer) in the SC. Hence, hydrolysis of GlcCer to Cer by β-glucocerebrosidase (GlcCer'ase), may be required for permeability barrier homeostasis. We determined first whether modulations in epidermal GlcCer'ase activity and mRNA levels occur in response to barrier disruption; and second, how GlcCer'ase inhibitors influence barrier function and SC membrane ultrastructure. Barrier disruption significantly increased epidermal GlcCer'ase mRNA levels, with a 2.8-fold increase over untreated control levels at 8 h ($P < 0.01$). GlcCer'ase activity was increased in whole epidermis (34%; $P < 0.02$) 24 h after barrier disruption. Localization of GlcCer'ase activity showed an increase (33%; $P < 0.05$) in the outer epidermis (SC and stratum granulosum), without a change in lower epidermal activity (stratum spinosum and stratum basale). Furthermore, a single topical application of the GlcCer'ase inhibitor, bromoconduritol-B-epoxide (BrCBE), inhibited enzyme activity (98%) and significantly delayed permeability barrier recovery after acetone treatment. In addition, BrCBE treatment disrupted SC intercellular lamellar bilayers, without evidence of cellular toxicity. These results indicate that epidermal processing of GlcCer to Cer by GlcCer'ase is required for barrier homeostasis, and that this important enzymatic step is regulated by barrier requirements.—**Holleran, W. M., Y. Takagi, G. K. Menon, S. M. Jackson, J. M. Lee, K. R. Feingold, and P. M. Elias.** Permeability barrier requirements regulate epidermal β-glucocerebrosidase. *J. Lipid Res.* 1994. **35:** 905-912.

Supplementary key words epidermis • glucosylceramides • ceramides • glucocerebrosidase • stratum corneum • sphingolipids • ultrastructure

The permeability barrier of mammalian epidermis is mediated by a system of highly organized lamellar bilayers localized to the intercellular spaces of the stratum corneum (reviewed in ref. 1). These bilayer structures are enriched in relatively hydrophobic species; i.e., ceramides, cholesterol, and free fatty acids, and depleted in the phospholipids associated with bilayer formation in other cellular membranes (2–4). These hydrophobic species derive, in part, from a mixture of precursors, including phospholipids and glucosylceramides, contained in the epidermal lamellar body (5, 6), a distinctive secretory organelle that delivers these lipids, along with co-packaged hydrolytic enzymes (7, 8), to the intercellular spaces at the base of the stratum corneum. After secretion, lamellar body contents undergo a series of morphologic transformations leading to the formation of intercellular lamellar unit structures characteristic of the stratum corneum of terrestrial mammals (1, 9). These structural alterations are accompanied by depletion of glucosylceramides and phospholipids, with an increase in ceramides and free fatty acids within the stratum corneum interstices (2–4, 10). In both mucosal epithelia (11) and marine cetacean epidermis (12), which display less stringent barrier requirement, glycosylceramides predominate over ceramides, perhaps due to a paucity of epithelial glycosidases (13), and basic lamellar unit structures do not form.

These observations suggest that transformation of glucosylceramides to ceramides may be an important prerequisite of permeability barrier competence. We recently demonstrated large quantities of β-glucocerebrosidase (GlcCer'ase) in murine epidermis, with the highest levels of activity in stratum corneum (14). Moreover, when bromoconduritol-B-epoxide (BrCBE), a covalent inhibitor of GlcCer'ase (15), was applied topically to intact skin, a progressive defect in permeability barrier function occurred, which was accompanied by both the accumulation

Abbreviations: GlcCer'ase, β-glucocerebrosidase; SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; BrCBE, bromoconduritol-B-epoxide; TEWL, transepidermal water loss; 4MU, 4-methylumbelliferone; HPTLC, high performance thin-layer chromatography; PBS, phosphate-buffered saline.

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of glucosylceramides and the persistence of incompletely processed lamellar body-derived bilayers (16).

In the present study, we asked whether GlcCer'ase activity is regulated by permeability barrier requirements, in which layer(s) of the epidermis such an increase in enzyme activity is (are) localized, and whether inhibition of GlcCer'ase would delay permeability barrier repair, demonstrating its requirement for barrier homeostasis. In an acute model of permeability barrier disruption, the acetone-treated mouse (17), we provide evidence first, that barrier requirements regulate GlcCer'ase mRNA levels and enzyme activity; and second, that GlcCer'ase activity is required for normal permeability barrier homeostasis.

MATERIALS AND METHODS

Materials

4-Methylumbelliferone (4MU), 4MU-β-D-glucoside, α-hydroxy and non-α-hydroxy galactosylceramides (I and II, respectively) (as standards for HPTLC), α-hydroxy and non-α-hydroxyceramides (IV and III, respectively), and sodium taurocholate were obtained from Sigma Chemical Co. (St. Louis, MO). Bromoconduritol B-epoxide (BrCBE) was kindly provided by Prof. Gunter Legler (University of Köln, Germany) and synthesized as described previously (18). Bio-Rad Protein Assay Kit and BSA were obtained from Bio-Rad (Richmond, CA). All solvents were of reagent or HPLC grade.

Animals

Hairless male mice (hr/hr) were purchased from Simonsen Laboratories (Gilroy, CA), and fed Purina mouse diet and water ad libitum. All animals were 8–12 weeks old at the time of study. Transepidermal water loss (TEWL) was measured at various time points with an electrolytic moisture analyzer (Meeco, Warrington, PA), and data are expressed as g/m²/h (17, 19).

Experimental groups

Cohorts of five or six mice each were gently treated with acetone-soaked cotton balls until TEWL rates exceeded 3.0 g/m²/h (pretreatment levels $\leq$0.15 g/m²/h). Immediately after acetone treatment, animals were treated on one flank with either BrCBE (325 nmol in 25 μl propylene glycol–ethanol 70:30, v/v) or BrCBE plus an equimolar concentration of ceramides (III and IV). The contralateral side was either left untreated or received vehicle alone. TEWL was measured in both inhibitor and vehicle-treated animals at 2, 4, 6, 12, 14, 18, and 24 h after treatment. At various, parallel time points skin samples were taken for enzyme assay and electron microscopy (see below).

Preparation of epidermal samples

Epidermal sheets were obtained from hairless mice by exposure of full-thickness skin samples first to dry heat (60°C, 60 sec) followed by removal of the epidermis by gentle scraping with a scalpel blade (#15). Prior studies showed no loss of GlcCer'ase activity after these procedures (14). All subsequent steps were performed at 4°C, unless otherwise noted. Epidermal sheets were homogenized in 10 vol PBS (with 0.1 mM PMSF) using a Polytron tissue homogenizer (15 sec × 3 bursts), followed by sonication (35% power, 10 sec × 2 bursts). Protein concentrations were measured using the Bradford procedure (20), using bovine serum albumin (BSA) as the standard.

To obtain viable upper versus lower epidermal cell layer preparations, the staphylococcal epidermolytic toxin separation was used, as described previously (3). Briefly, groups of adult hairless mice (n = 3–11) were injected intradermally with 50 μg staphylococcal epidermolytic toxin, dissolved in 100 μl PBS. After 2 h the mice were killed, the upper epidermis, comprising stratum corneum (SC) and stratum granulosum (SG) with some residual stratum spinosum (SS), was removed in one piece. The subjacent piece of skin, comprising lower epidermis, i.e., SB with some SS plus the dermis, was excised and immediately placed epidermis-side downward onto a plastic Petri dish on crushed ice. The subcutaneous fat was scraped from the undersurface with a scalpel blade, and the pieces were immersed in 10 mM EDTA in Dulbecco's PBS, calcium (Ca^{2+})- and magnesium (Mg^{2+})-free (pH 7.4) at 37°C. After incubation for 30 min, the lower epidermis was peeled off the dermis by gentle scraping with a scalpel blade and blotted dry. Upper and lower epidermal sheets then were weighed, minced into small pieces (<1 mm³), and stored in small plastic tubes overnight at −70°C. Neither epidermolytic toxin treatment nor EDTA incubations affect residual enzyme activity (14).

Isolation and analysis of epidermal mRNA

Total RNA was isolated by a variation of the guanidinium thiocyanate method (21), as we have described recently (14). Briefly, epidermis (0.2–0.4 g from whole area of two mice) was homogenized in 4 ml Solution A (4 M guanidinium thiocyanate, 24 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) on ice. After the addition of acid phenol and chloroform–isoamylalcohol 24:1, RNA was reprecipitated three times from the aqueous phase with isopropanol. Oligo(dT)-cellulose (10 mg) was then added to the total RNA extract, and poly A(+) RNA was eluted from the oligo(dT)-cellulose with three successive washes in RNA-H_2O. mRNA was then precipitated from solution by adding 3 M sodium acetate (60 μl) and 1.2 ml 100% ethanol and incubating at −80°C for 1 h. mRNA was resuspended in 50 μl of RNA-H_2O and

the absorbance was determined at both 260 and 280 nm. The entire epidermis of two mice yields between 10 and 30 μg of epidermal poly A(+) RNA.

Northern analysis

Aliquots of poly A(+) RNA (4–8 μg) were run on agarose/formaldehyde (1%/6.1%) gels for three h at 75 V as previously described (14). RNA was transferred to nitrocellulose (0.45 μm pore size), and probed with a murine cDNA probe for β-glucocerebrosidase (kindly provided by Dr. R. O'Neill; NIH, Bethesda, MD). [^{32}P]dCTP-radiolabeled cDNA probes were prepared by random priming (Amersham Multiprime DNA Labeling System). Blots were prehybridized at 42°C for 30 min, and hybridization with radiolabeled probes was performed overnight at 42°C followed by a 30-min room temperature wash and a 60-min 65°C wash in wash buffer (0.2 × SSC, 0.1% SDS, pH 7.0). Air-dried blots were exposed to X-ray film, the film was developed, and bands were quantified by scanning densitometry.

Epidermal β-glucocerebrosidase assay

Our assay method for β-glucosidase is modified from that of Mier and van den Hurk (22), as described recently (14, 16). Briefly, assays were performed at 37°C in citrate-phosphate buffer (pH 5.6), containing 5 mM (0.54%) sodium taurocholate, with 0.5 mM 4MU-β-D-glucoside as substrate, and a 60-min reaction time. Enzyme solutions in assay buffer (50 μl) were preheated in siliconized glass culture tubes, and reactions were initiated by the addition of 50 μl substrate solution in assay buffer. Reactions were terminated by adding 1.25 ml 200 mM carbonate-bicarbonate buffer (pH 10.5). The fluorescence (ex = 360 nm, em = 450 nm) was measured with a fluorescence spectrophotometer (Perkin-Elmer, Model 204). A standard 4MU solution (0–300 nM) in carbonate-bicarbonate buffer was used for calibration of each assay.

Electron microscopy

At various time points after treatment with the protocols described above, skin biopsies were minced to <0.5 mm^3, and fixed in modified Karnovsky's fixative overnight. Samples then were post-fixed in both 0.2% ruthenium tetroxide (23), using a recently described protocol (24), and 2% aqueous osmium tetroxide both containing 1.5% potassium ferrocyanide. After fixation, all samples were dehydrated in graded ethanol solutions and embedded in an Epon-epoxy mixture (25). Thin sections were examined after either lead citrate or double-staining with uranyl acetate and lead citrate in a Zeiss 10A electron microscope, operated at 60 kV.

Statistical analysis

Statistical evaluation of data was performed using either a two-tailed Student's *t*-test or a paired *t*-test.

RESULTS

β-Glucocerebrosidase mRNA levels in relation to barrier repair

To determine whether epidermal GlcCer'ase activity changes in relation to permeability barrier function, we first determined the effect of acute barrier disruption with acetone on both enzyme mRNA levels and activity at various timepoints during barrier repair. Northern analyses of poly-A+ mRNA extracts from whole epidermal samples (6 μg per lane), exposed to a cDNA probe specific for the murine β-GlcCer'ase sequence, revealed a single band for GlcCer'ase mRNA (approximately 1.2 kb) (data not shown). As seen in **Fig. 1,** the mRNA levels for GlcCer'ase were significantly increased at 8 and 24 h after acetone treatment in comparison to controls ($P <$ 0.01 and 0.01, respectively). The maximal difference between acetone-treated and control mRNA levels occurred at 8 h after acetone treatment, with quantities returning toward control levels at 24 h. These results show that permeability barrier disruption with acetone results in increased GlcCer'ase mRNA levels.

Total enzyme activity and localization in relation to barrier repair

We next determined whether the increase in mRNA levels during barrier repair results in changes in GlcCer'ase activity, and the localization of these changes within the epidermis. In these studies, we first evaluated changes in

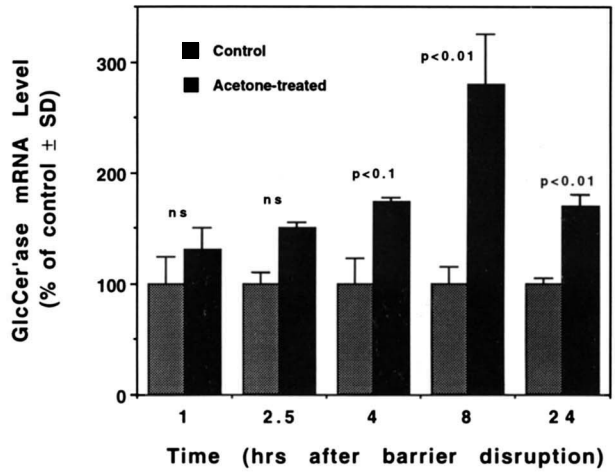

Fig. 1. Quantitation of β-glucocerebrosidase mRNA levels at various time points after permeability barrier disruption by acetone treatment. Total RNA was isolated from whole epidermis by a variation of the guanidinium thiocyanate method (21), with poly A(+) mRNA further isolated using oligo (dT)-cellulose. Northern analysis (6 μg/lane) was performed using a cDNA probe for murine GlcCer'ase (14). Results are presented as the mean absorbance (by scanning densitometry ± SD); n ≥ 4 for each sample. Control values at each timepoint represent parallel untreated epidermal samples probed on the same blot with treated epidermal samples. GlcCer'ase mRNA levels increased after barrier disruption, with a peak at 8.0 h ($P <$ 0.01), returning toward control levels at 24 h.

total enzyme activity in acetone-treated versus control animals. Total epidermal GlcCer'ase activity was significantly increased ($P < 0.02$) in the treated versus untreated epidermis only at 24 h after permeability barrier disruption with topical acetone (**Fig. 2**). Although a trend toward increasing GlcCer'ase activity was observed at earlier timepoints, these changes did not achieve statistical significance (data not shown). The increase in GlcCer'ase activity at 24 h occurred in the outer epidermis (stratum corneum and stratum granulosum) after barrier disruption, with no change in the lower epidermis (stratum spinosum and stratum basale) (Fig. 2). Again, the increased activity in the outer epidermis was only evident at 24 h, and not at earlier timepoints (data not shown). These data suggest that the barrier-induced increase in GlcCer'ase mRNA leads to higher epidermal enzyme activity, with the increase localized primarily to the outer epidermis.

Effect of BrCBE on epidermal permeability barrier recovery

In order to ascertain further whether GlcCer'ase activity is required for barrier recovery, we next measured the rate of recovery of barrier function after a single topical application of BrCBE to acetone-treated skin. In vehicle-treated control animals, barrier function recovered by 45, 65, and 85% within the first 3, 7, and 24 h, respectively, after acetone treatment (**Fig. 3**). In contrast, a single topical application of BrCBE (325 nmol/5 cm²) significantly delayed barrier recovery after acetone treatment at all timepoints (Fig. 3), returning to normal after

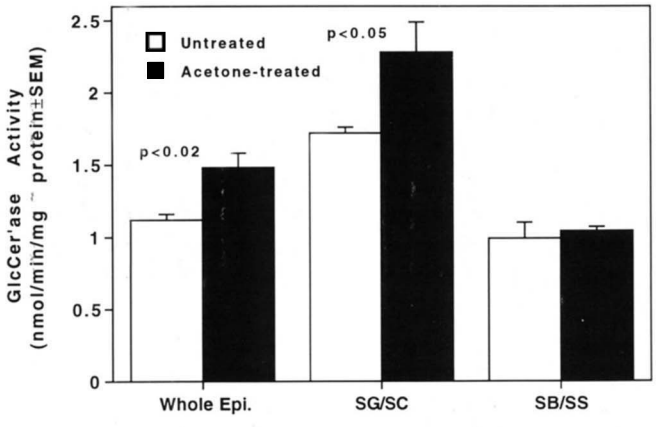

Fig. 2. β-Glucocerebrosidase activity after barrier disruption with acetone. After acetone treatment, the specific activity of GlcCer'ase was determined in whole epidermal homogenates, and in homogenates of upper (stratum corneum and stratum granulosum) and lower (stratum spinosum and stratum basale) epidermis. GlcCer'ase activity was increased in whole epidermis (34%; $P < 0.02$) at 24 h after barrier disruption. Elevated GlcCer'ase activity was localized to the upper epidermis (33%; $P < 0.05$), with no change occurring in the lower epidermis. Values represent the mean (± SEM) activities (n = 6 animals).

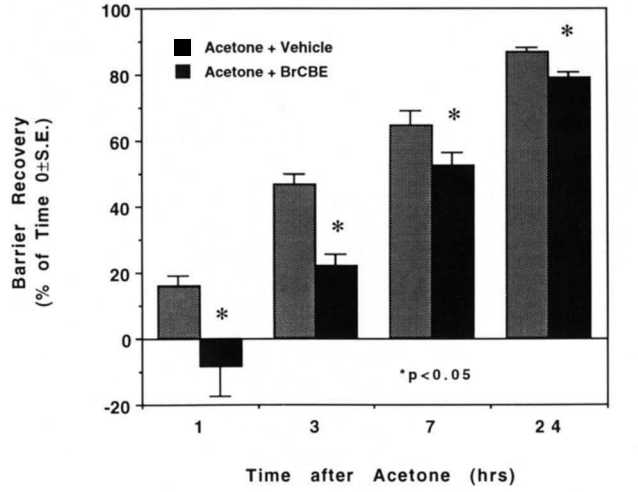

Fig. 3. Effect of topical BrCBE effect on permeability barrier recovery in acetone- versus vehicle-treated animals. TEWL measurements were obtained at various timepoints after barrier disruption and during the subsequent repair process (see Methods). Topical BrCBE treatment resulted in significant slowing of permeability barrier recovery at all timepoints ($P < 0.05$).

24 h. Moreover, topical coapplications of ceramides with BrCBE did not correct the BrCBE-induced delay in barrier repair (data not shown). A single topical dose of BrCBE inhibited epidermal GlcCer'ase activity by 98% (1.77 ± 0.3 vs. 0.03 ± 0.007; $P < 0.001$) 1 h after application. These data show that inhibition of epidermal GlcCer'ase leads to a delay in the repair of the permeability barrier.

Structural alterations induced by BrCBE treatment

In order to determine the structural basis for the BrCBE-induced delay in barrier recovery after acetone treatment, we next examined the ultrastructural abnormalities that result from the inhibition of epidermal GlcCer'ase (**Figs. 4A** and **4B**). The normal lamellar bilayer unit structure was largely restored by 24 h post-treatment in stratum corneum from animals treated with acetone plus vehicle alone (Fig. 4A). In contrast, in samples obtained at 24 h after BrCBE application to acetone-treated skin, disruption of the lamellar bilayer structures was evident, with focal extension of these abnormalities into the outer stratum corneum. These changes included persistence of immature (unprocessed) lamellar body-derived sheets, as well as separation (loose packing) of individual membrane leaflets through expansion of hydrophilic domains (Fig. 4B). These structural abnormalities were not corrected when ceramides were co-applied with BrCBE (data not shown). Yet, the intercellular domains at the stratum granulosum–stratum corneum interface, and within the first two layers of the stratum corneum, appeared comparable to controls, containing a full complement of normal-appearing, extruded

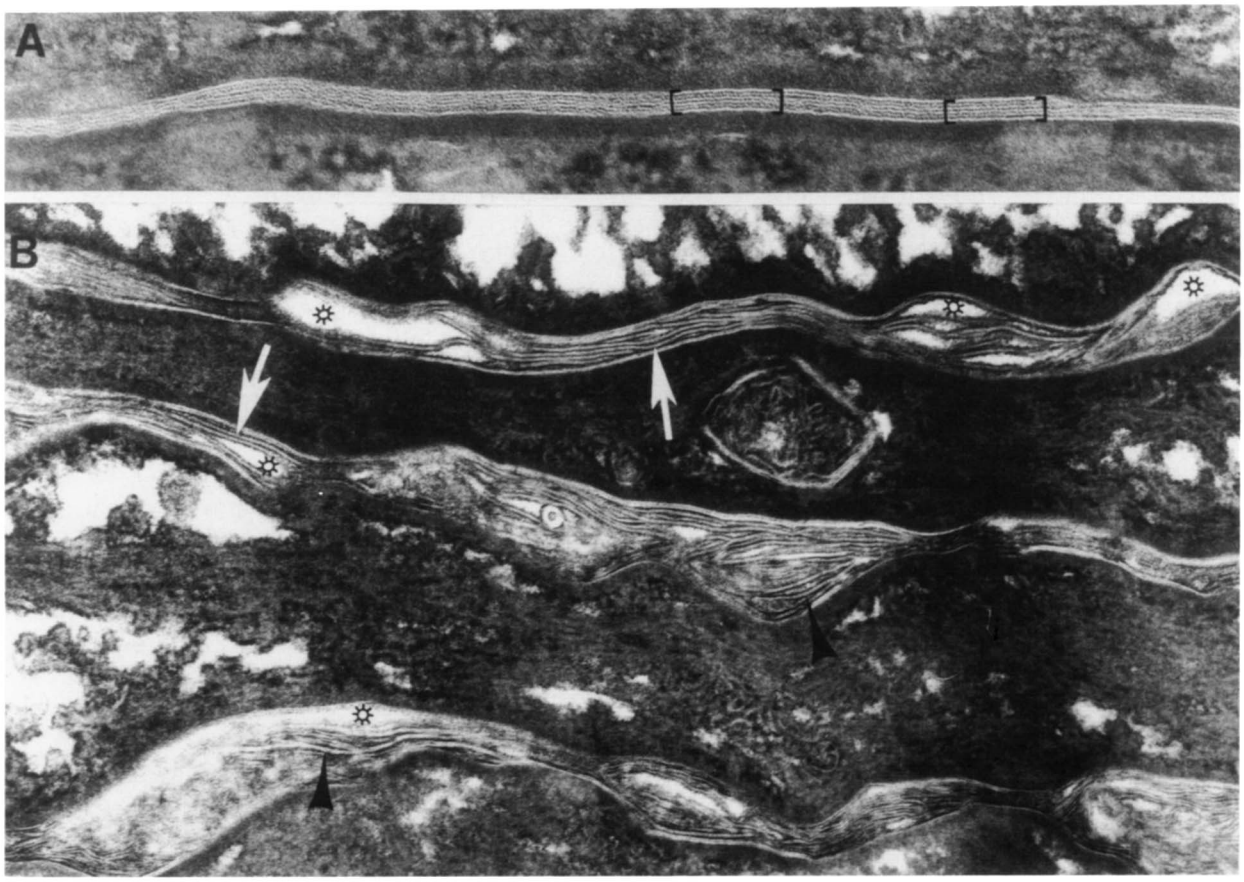

Fig. 4. Effect of a single topical application of BrCBE versus vehicle on stratum corneum intercellular membrane structures in acetone-treated stratum corneum at 24 h. Whereas the intercellular spaces contain abundant lamellar material (4B, white arrows), they show no sign of the structural transformation into the lamellar basic unit structures seen in controls (4A, brackets) (compare 4B-arrows with 4A-brackets). Asterisks show areas of membrane separation. Ruthenium tetroxide post-fixation; A ×63,000; B ×31,500.

lamellar body-derived contents (Fig. 4B). Finally, no evidence of cellular toxicity was observed. These results indicate that inhibition of GlcCer'ase activity interferes with the transformation of secreted lamellar body contents into mature lamellar bilayer unit structures.

DISCUSSION

Striking alterations in both lipid composition and distribution occur during the terminal differentiation of mammalian epidermis (1, 26), changes that are presumed to be important for permeability barrier function (1, 9, 27, 28). In the stratum corneum of terrestrial mammals, a lipid mixture enriched in ceramides, cholesterol, and free fatty acids, localized to the intercellular spaces, replaces the phospholipid, neutral lipid, and glycosphingolipid mixture of the subjacent nucleated cell layers of the epidermis (26, 29). As epidermal glucosylceramides disappear, ceramide content simultaneously increases to 40–50% of stratum corneum lipid by weight (2–4, 10). To investigate the relationship between epidermal ceramides and the other key lipids for barrier function, we have used a metabolic approach, correlating changes in lipid synthesis with barrier recovery after acute and chronic perturbations. These studies have shown that permeability barrier repair is paralleled by increased ceramide synthesis (19), as well as enhanced synthesis of cholesterol and fatty acids (17, 30). Furthermore, the increase in sphingolipid generation has been attributed to increased serine-palmitoyl transferase (SPT) activity (19, 28), while the increased cholesterol synthesis is paralleled by elevations in hydroxymethylglutaryl-CoA (HMG-CoA) reductase (31, 32). As both SPT and HMG-CoA reductase are rate-limiting for their respective biosynthetic pathways (33, 34), these studies clearly show that the key early biosynthetic steps for ceramide, cholesterol, and fatty acid formation are regulated by barrier requirements.

In the present study we have begun to address whether more distal, processing steps, late in the formation of the key barrier lipids, also are regulated by barrier requirements. One such distal step, the conversion of glucosylceramides to ceramides in the epidermis, which is mediated by GlcCer'ase, may be critical for permeability barrier formation. GlcCer'ase activity accounts for over 80% of epidermal β-glucosidase activity, and increases in the outer layers of murine epidermis (14), in parallel with the disappearance of glucosylceramide and the increase in ceramides (26, 29). Moreover, repeated topical applications of BrCBE to intact murine skin cause a progressive abnormality in permeability barrier function, in association with GlcCer'ase inhibition (16). Therefore, in the present study we first assessed GlcCer'ase activity and mRNA levels during permeability barrier repair. Northern analyses showed a striking increase in GlcCer'ase mRNA levels after acute disruption of the barrier by acetone treatment. This increase in message levels was reflected by a commensurate increase in total epidermal GlcCer'ase activity, an increase that was localized to the outer epidermal layers (SC and SG). The increase in mRNA levels that precedes the increase in GlcCer'ase activity at 24 h may reflect a high level of baseline epidermal GlcCer'ase activity (14, 16). Although increased de novo synthesis is likely occurring, the change in total epidermal GlcCer'ase activity is not evident until 24 h after barrier abrogation. These results demonstrate that GlcCer'ase activity is regulated by epidermal barrier requirements, and imply that localized changes, presumably in SC extracellular domains, may be a critical element in the recovery of a functional barrier.

A variety of genes appear to be activated during the epidermal response to permeability barrier disruption, including those of HMG-CoA reductase, LDL-receptor, and apoE (32), specific cytokines (35), and now GlcCer'ase. The increase in epidermal GlcCer'ase mRNA and the resultant increase in enzyme activity are components of the complex physiologic response to barrier perturbation. These results are reminiscent of other physiologic processes in which groups of genes are activated. For example, during the acute phase response, a number of distinct genes are activated within liver cells, leading to the appropriate cellular response (36, 37). Thus, understanding the coordinate regulation of the array of genes involved in epidermal permeability barrier homeostasis will not only give insight into the regulation of normal epidermal physiology, but also improve our understanding of pathologic conditions in which barrier dysfunction plays a role.

The importance of GlcCer'ase in permeability barrier homeostasis is further emphasized by the topical inhibitor studies. A single application of BrCBE, which inhibited GlcCer'ase activity by >98% (16), led to a delay in barrier recovery after acetone treatment. The delay in barrier recovery was accompanied by the formation of abnormal intercellular lamellar leaflets during barrier repair, comparable to those observed following repeated application of inhibitor to intact skin (16). We recently found similar functional and membrane structural abnormalities (38) in homozygous offspring of transgenic mice with deletion of the GlcCer'ase gene (39, 40). These findings also are consistent with the observations of decreased glucosidase levels (13), and an increased glycosylceramide:ceramide ratio (11) in mucous membranes, and recent studies that suggest that keratinizing oral epithelium retains untransformed lamellar body contents into the outer SC (41). Furthermore, immature membrane structures and elevated glucosylceramide:ceramide ratios are present in marine cetacean epidermis (12). Both of these types of keratinizing epithelia require a lesser degree of barrier competence than the epidermis of terrestrial mammals. Together, these studies demonstrate the importance of GlcCer'ase activity in the maintenance of permeability barrier integrity.

In summary, these studies demonstrate that the activity of epidermal GlcCer'ase is regulated by permeability barrier requirements, and inhibition of GlcCer'ase results in a delayed recovery of the permeability barrier after an acute challenge. Moreover, failure to hydrolyze glucosylceramides results in a disruption of the normal stratum corneum intercellular architecture and altered permeability barrier homeostasis.

This work was supported by NIH grants AR 39448, AR 19098, AR 39639, and the Medical Research Service of the Veterans Administration. Mr. Raymond Pelayo provided capable editorial assistance. Dr. Holleran is a recipient of the Thomas B. Fitzpatrick Research Award for 1993–94, sponsored by Kao Corporation of Japan.

Manuscript received 1 September 1993 and in revised form 27 December 1993.

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