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Research Article

Hydrolysis of glucosylceramides (GlcCer) by beta-glucocerebrosidase generates ceramides, critical components of the epidermal permeability barrier. Ceramides also are involved in the regulation of cellular proliferation and differentiation in a variety of cell types. Whereas most studies have focused on ceramides and their sphingoid base metabolites as growth inhibitors, GlcCer apparently acts oppositely (i.e., as a mitogen). To determine whether enhancement of GlcCer content stimulates epidermal mitogenesis, we examined the response of hairless mouse epidermis to alterations in endogenous and/or exogenous GlcCer. Topical applications of conduritol B epoxide, a specific irreversible inhibitor of beta-glucocerebrosidase, increased epidermal GlcCer levels twofold, an alteration localized largely to the basal, proliferative cell layer (fourfold increase); and stimulated epidermal proliferation (2.3-fold elevation in [3H]thymidine incorporation; $P <$ or $= 0.001$), localized autoradiographically again to the basal layer, and resulting in epidermal hyperplasia. Intracutaneous administration of GlcCer (2.0 mg) also stimulated epidermal DNA synthesis, while simultaneous treatment with conduritol B epoxide plus GlcCer resulted in an additive increase in DNA synthesis. These increases in epidermal proliferation could not be attributed either to altered epidermal permeability barrier function, or to nonspecific irritant effects, as determined by four separate criteria. These results strongly suggest that GlcCer directly stimulates epidermal mitogenesis.

Glucosylceramides Stimulate Murine Epidermal Hyperproliferation

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Abstract

Hydrolysis of glucosylceramides (GlcCer) by β-glucocerebrosidase generates ceramides, critical components of the epidermal permeability barrier. Ceramides also are involved in the regulation of cellular proliferation and differentiation in a variety of cell types. Whereas most studies have focused on ceramides and their sphingoid base metabolites as growth inhibitors, GlcCer apparently acts oppositely (i.e., as a mitogen). To determine whether enhancement of GlcCer content stimulates epidermal mitogenesis, we examined the response of hairless mouse epidermis to alterations in endogenous and/or exogenous GlcCer. Topical applications of conduritol B epoxide, a specific irreversible inhibitor of β-glucocerebrosidase, increased epidermal GlcCer levels twofold, an alteration localized largely to the basal, proliferative cell layer (fourfold increase); and stimulated epidermal proliferation (2.3-fold elevation in [^{3}H]thymidine incorporation; $P \leq 0.001$), localized autoradiographically again to the basal layer, and resulting in epidermal hyperplasia. Intracutaneous administration of GlcCer (2.0 mg) also stimulated epidermal DNA synthesis, while simultaneous treatment with conduritol B epoxide plus GlcCer resulted in an additive increase in DNA synthesis. These increases in epidermal proliferation could not be attributed either to altered epidermal permeability barrier function, or to nonspecific irritant effects, as determined by four separate criteria. These results strongly suggest that GlcCer directly stimulates epidermal mitogenesis. (*J. Clin. Invest.* 1995. 95:2903–2909.) Key words: epidermis • ceramides • glucosylceramides • Gaucher disease • epidermal DNA synthesis

Introduction

Mammalian epidermis generates large quantities of ceramides (Cer)[1] as critical extracellular constituents of the epidermal permeability barrier (1, 2). Whereas the nucleated cell layers generate glucosylceramides (GlcCer), the proportions of GlcCer to Cer decrease late in epidermal differentiation, with ceramide (Cer) content peaking in the stratum corneum (1, 3). Thus, inhibition or deficiency of epidermal β-glucocerebrosidase (GlcCer'ase; E.C. 3.2.1.45), which catalyzes the conversion of GlcCer to Cer, results in altered barrier function (4, 5). Furthermore, complete deficiency of GlcCer'ase which occurs in severe neuronopathic Type II Gaucher disease, is characterized not only by the accumulation of GlcCer in the cells of the reticuloendothelial system (6–8), but also by a collodion baby phenotype (9–12). Comparable skin abnormalities occur in a GlcCer'ase-deficient null allele mouse model of Gaucher disease (9), which also displays profound barrier abnormalities in association with an altered GlcCer to Cer ratio (5).

However, the alterations in sphingolipid composition which occur late in epidermal differentiation may be required not only for the formation of a competent barrier, but also for modulating epidermal proliferation and differentiation. In addition to their structural properties, sphingolipids, including both Cer and GlcCer, appear to regulate cellular proliferation and differentiation. Exogenously supplied ceramides both inhibit cellular proliferation, and induce differentiation in a variety of cell types (13, 14). Moreover, the hydrolysis of sphingomyelin to Cer (via neutral sphingomyelinase) has been linked to cellular differentiation through a novel cellular signaling pathway (15, 16). In contrast, evidence is accumulating that increased cellular GlcCer levels induce cellular proliferation. For example, the organomegaly in Gaucher disease has been attributed not only to the accumulation of tissue GlcCer, but also to possible growth-stimulating properties of GlcCer (17). Likewise, administration of the specific GlcCer'ase inhibitor, conduritol B epoxide, to mice causes elevations in cellular GlcCer content, in association with tissue hyperplasia (18). Moreover, intraperitoneal injection of emulsified GlcCer into rats induces tissue hypertrophy, including an 18–24% increase in liver mass (17). In addition, blockade of GlcCer generation from Cer by application of the glucosyl-transferase inhibitor, PDMP, blocks the development of renal hypertrophy in streptozotocin-treated rats (19). Finally, modulations in GlcCer content, either by exogenous lipid applications or administration of conduritol compounds, result in proliferation of MDCK and other cell types in cell culture (20). To determine whether changes in endogenous and/or exogenous GlcCer content influence epidermal homeostasis, we assessed here the effects of altered GlcCer levels on epidermal cell kinetics. Our results suggest that GlcCer is a potent modulator of murine epidermal mitogenesis.

Methods

Materials. Glucocerebrosides were obtained from Matreya Inc. (Philadelphia, PA); conduritol B (CB) and conduritol B epoxide (CBE) were from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada); and Myrj 52 was from ICI America, Inc. (Wilmington, DE). High performance thin-layer chromatography (HPTLC) plates (Silica Gel 60) were purchased from Merck (Darmstadt, FRG). Calf thymus DNA, deoxynojirimycin (dNJM), phorbol 12,13-dibutyrate (PdiBu), lacto-

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1. *Abbreviations used in this paper:* BrCBE, bromoconduritol B epoxide; CB, conduritol B; CBE, conduritol B epoxide; Cer, ceramide; GlcCer, β-D-glucosylceramide; GM3, monosialylganglioside-3; LacCer, lactosylceramide; PdiBu, phorbol 12,13-dibutyrate; β-GlcCer'ase, β-glucocerebrosidase; TEWL, transepidermal water loss.

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sylceramides (LacCer), monosialylganglioside-GM3, and standards for HPTLC (including sphingomyelin, ceramides III and IV and cerebrosides I and II) were obtained from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol was obtained from Boehringer Mannheim (Indianapolis, IN) and [methyl-^{3}H]thymidine was from Amersham Life Sciences (Arlington Heights, IL). All solvents were of reagent or HPLC grade.

Animals and experimental protocols. Hairless male mice (h/h) were purchased from Simonsen Laboratories (Gilroy, CA), and fed Purina mouse diet and water ad libitum. All animals were 8–12 wk old at the time of study. Barrier function was assessed as transepidermal water loss (TEWL) measured at various time points with an electrolytic water analyzer (Meeco, Warrington, PA), as described in detail previously (2, 21). Water loss measurements were obtained over a small area of skin (0.5 cm^2), recorded in parts per million/0.5 cm^2 per hour over background, and the data were converted to mg/cm^2 per hour. At each time point, 3–5 readings were performed on each flank (both treated and untreated), with 6–8 animals in each test group.

Two doses of each conduritol compound were applied topically to mouse epidermis (40 μl of a 250 nmol/μl solution) in vehicle (propylene glycol:ethanol; 70:30, vol/vol) 4–5 h apart. After assessing the ability of the various conduritol compounds to stimulate mitogenesis in relation to their ability to inhibit β-GlcCer'ase, CBE was used in most subsequent experiments both because of its efficacy and its commercial availability. To assess directly the effects of occlusion, some CBE-treated mice were immediately covered with a snugly fitting, water-impermeable membrane (one finger of a Latex glove) (2). The wrap was removed just prior to excision of the whole skin samples for the biochemical studies described below. Some mice were treated with topical betamethasone dipropionate (Diprolene® AF; Schering) 0.05% cream following application of CBE and/or GlcCer. Other groups of mice were injected subcutaneously twice daily with glucocerebrosides (0.5 to 2 mg) emulsified with 0.38 to 1.5 mg Myrj 52 in saline solution, as described previously by Datta and Radin (17). 24 h after application of the initial dose of CBE and/or GlcCer, the animals were sacrificed, the epidermis was separated from the dermis, and the epidermis was homogenized, as described below.

[^{3}H]Thymidine incorporation/DNA synthesis measurements. Each animal received an intraperitoneal injection of [^{3}H]thymidine (1 μCi/g body weight) 1 h before sacrifice. Skin samples (5 cm^2) were removed from both the treated and control sides, scraped free of excess subcutaneous fat, and the epidermis was separated from the dermis by incubation in a 10 mM EDTA solution at 37°C for 40 min. Epidermal sheets were weighed, minced, and homogenized in 0.8 ml phosphate-buffered saline (calcium-magnesium free, containing 10 mM EDTA) with a Sonic Dismembranator (Fisher Scientific Co., Pittsburgh, PA). 100 μl was removed from each sample for DNA assay (see below). One ml of cold 20% TCA was added to the remaining samples, and they were centrifuged at 2,000 *g* (4°C; 5 min). The supernatant was discarded and the resultant precipitant was washed twice with 500 μl of 5% cold TCA followed by centrifugation. The precipitated material then was suspended in 1.0 ml of 1N NaOH and centrifuged (see above). Duplicate aliquots of the supernatant containing the resuspended DNA were counted by liquid scintillation spectrometry.

Total cellular DNA was assayed by the method of Labarca and Paigan (22). Samples for DNA assay were brought to 1 ml with Hoechst buffer (pH 7.4). One ml of bisbenzimidazole (Sigma Chemical Co.; diluted 1:1,000 with distilled H_2O) was added. Standards were prepared using calf thymus DNA (134 μg/ml) diluted 1:10 with Hoechst buffer. All samples and standards were kept in the dark for two hours, and fluorescence measured using a spectrophotometer.

β-Glucocerebrosidase assay. Epidermal samples were homogenized in 300 μl of extraction buffer (60 mM potassium phosphate, containing 0.1% Triton X-100 by volume, pH 5.96) with a Potter-Elvehjem grinder. The extracts were sonicated (80%; 2 × 10 s), and cell debris was pelleted in a microfuge at 10,000 *g* for 5 min. Aliquots of epidermal supernatants were assayed for 1 h at 37°C in citrate-phosphate buffer (pH 5.6), using 4-methylumbelliferyl-β-D-glucopyranoside (MUG) as the substrate, as described previously (23). Protein determinations were performed with either the BCA Protein Assay Kit (Bio-Rad, Richmond, CA) or the Bradford procedure (24), using BSA as the standard. The specific activity vs. protein concentration was determined for each sample, with 4-methylumbelliferone used to generate a standard curve.

Endotoxin assay. To determine whether any of the topical or injected solutions were contaminated with potentially mitogenic bacterial products, we assayed endotoxin activity using of 0.1 ml of amebocyte lysate and injected samples (e.g., 0.1 ml of sphingolipid) at 37°C, as described previously (25). All components to be assayed were vortexed briefly and samples were monitored initially at 5–10 min intervals for 45 min, and then less frequently up to 8 h. The development of a marked increase in viscosity or gelation indicates the presence of endotoxin in the test sample. The rate of gelation of amebocyte lysate was further quantitated by measurement of light scattering at room temperature with a photofluorometer. None of the injected solutions used in these studies demonstrated significant endotoxin activity (i.e., < 50 pg/injection).

Assessment of inflammation. The murine ear model was used to determine whether the doses of topical CBE used in this study were able to induce acute inflammatory response (26). CBE (5 μmol in 20 μl of vehicle, as above) or phorbol ester (PdiBu; 20 nmol in 20 μl acetone) were applied separately to the external aspect of mouse ears (3–6 animals in each group). The contralateral ears served as controls and were treated with either vehicle alone or PdiBu plus a potent corticosteroid (0.05% Diprolene® AF cream). Ear thickness was measured with a microcaliper prior to treatment, and at 18 h after topical applications.

Histology and autoradiography. Fresh, full-thickness skin samples were obtained before treatment, and at 24 and/or 72 h after initial treatment with compound or vehicle. Biopsies were taken for both light microscopy (hematoxylin and eosin or Feulgen staining) and autoradiography two hrs after subcutaneous administration of [^{3}H]thymidine (diluted 1:10 with 0.15 M NaCl; 10 μCi/site). Samples were fixed in buffered formalin, paraffin-embedded, and sectioned (5 μm). For autoradiography, sections were stained with hematoxylin/eosin and coated with Ilford K5-D emulsion for autoradiography. All sections were examined and photographed with a Leitz Ortholux II microscope.

Epidermal lipid quantitation. Skin was excised (5 cm^2) and both subcutaneous fascia and fat were removed by scraping with a surgical blade. Skin samples were placed dermis-side-downward either onto 10 mM EDTA (as above for whole epidermis) or onto 10 mM dithiothreitol (DTT) in phosphate-buffered saline (calcium-magnesium free; pH 7.4) at room temperature and incubated for 40 min at 37°C. The upper epidermis, comprising stratum corneum, stratum granulosum, and stratum spinosum, was removed by peeling with forceps, and the lower epidermis (stratum basale) was removed by scraping with a surgical blade (27). Both upper and lower epidermal samples were minced and placed into 7.6 ml of Bligh/Dyer (28) extraction medium (chloroform:methanol:water, 4:2:1.6 vols) overnight at 4°C, extracted to obtain total lipids by a modification of the Bligh-Dyer method (29), dried, weighed, and stored in chloroform at −70°C until use. Separation and quantitation of individual sphingolipid species were performed, as described previously (30, 31). After the final solvent fractionation, the dried plates were dipped in charring solution, (1.5% cupric sulfate in acetic acid:sulfuric acid:orthophosphoric acid:distilled water; 50:10:10:95, vols) as described previously (4), dried (40°C, 10 min), and then charred at 180°C for 15 min. The plates were scanned with a variable wavelength scanning densitometer (Camag; Muttenz, SWI), and the lipid fractions were quantitated by comparison to known standards run in parallel with the experimental samples. Quantitation of individual species was performed using CATS II software (Camag).

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

Results

Selected glucocerebrosidase inhibitors alter epidermal glucocerebrosidase activity. We first determined the effects of inhibitors on epidermal β-glucocerebrosidase (GlcCer'ase) activity. Single topical applications of CBE, CB, and dNJM to intact

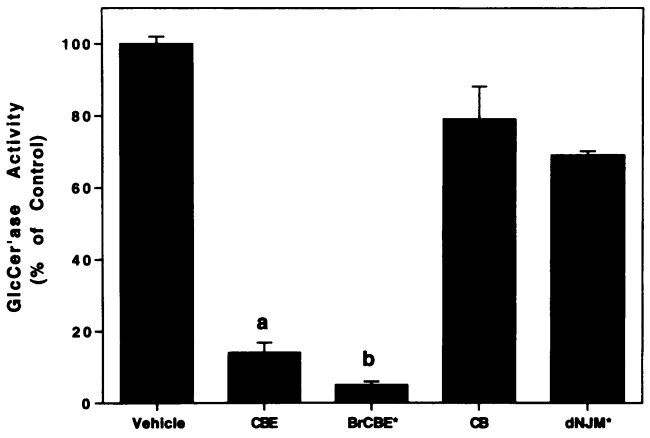

Figure 1. Inhibition of epidermal β-glucocerebrosidase activity. Glucocerebrosidase activity was assessed in murine epidermal homogenates 1 h after treatment with either vehicle, CBE, CB, or dNJM. Activity of BrCBE-treated epidermis was measured 1 h after a single topical dose (375 nmol/5 cm^2), as described previously (4). Results are presented as percentage of control epidermal enzyme activity (mean±SEM; $n \geq 3$). ^{a,b} $P \leq 0.001$ vs. vehicle control.

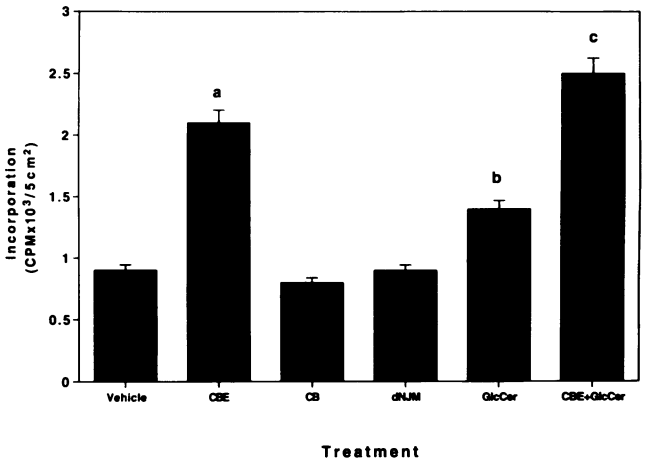

Figure 2. Stimulation of [^{3}H]thymidine incorporation by GlcCer and β-GlcCer'ase inhibitors. 23 h after initial treatment with CBE, CB, dNJM, GlcCer, or CBE plus GlcCer (see Methods), [^{3}H]thymidine was injected intraperitoneally. 1 h later, epidermis was isolated and [^{3}H] incorporated into DNA was quantitated (see Methods). Increased incorporation was observed in animals treated with CBE and/or GlcCer ($n \geq 7$; ^{a} CBE: $P \leq 0.001$ vs. vehicle control; ^{b} GlcCer: $P \leq 0.001$; ^{c} CBE + GlcCer: $P \leq 0.001$).

hairless mouse skin resulted in 86, 21, and 30% inhibition, respectively, of epidermal GlcCer'ase activity one hr following treatment (Fig. 1). Thus, only CBE (10 μmol/5 cm^2) provided a comparable degree of inhibition (86±0.5%) to the levels described previously with topical BrCBE (i.e., 95±3%; 4). Vehicle treatment of epidermis did not alter GlcCer'ase activity. Based upon these results, the commercially available CBE was used in all subsequent studies.

Glucocerebrosidase inhibitors and exogenous glucosylceramides induce epidermal proliferation. With the demonstration that topical conduritol compounds inhibit epidermal GlcCer'ase activity, we next determined whether topical CBE induces epidermal hyperplasia. As seen in Fig. 2, topical CBE increased epidermal [^{3}H]thymidine incorporation by twofold over vehicle-treated controls ($P \leq 0.001$) 24 h after application. In contrast, equal doses of chemically related, but less-potent inhibitors of GlcCer'ase, CB and dNJM, did not significantly alter [^{3}H]thymidine incorporation. These results show first, that the conduritol inhibitors stimulate epidermal DNA synthesis, an effect which requires significant inhibition of GlcCer'ase activity; and second, that the changes in epidermal proliferation are not likely to be due to non-specific irritant effects of these agents.

We next examined whether intracutaneous administration of GlcCer stimulates epidermal mitogenesis. Two subcutaneous injections (time 0 and 6 h) of emulsified GlcCer (2.0 mg each) resulted in a 1.5-fold increase in [^{3}H]thymidine incorporation at 24 h (Fig. 2; $P \leq 0.001$). In contrast, subcutaneous administration of equivalent concentrations of emulsified galactocerebrosides, ceramides, or lower doses of GlcCer did not significantly increase [^{3}H]thymidine incorporation (data not shown). Furthermore, neither LacCer or GM3 ganglioside increased [^{3}H]thymidine incorporation (i.e., < 10% increase, P = NS). Finally, coadministration of topical CBE with intracutaneous GlcCer resulted in an additive increase (~ 2.8-fold) in [^{3}H]-thymidine incorporation at 24 h (Fig. 2; $P \leq 0.001$). These results show that either topical applications of a β-GlcCer'ase inhibitor and/or intracutaneous administration of GlcCer induce epidermal proliferation.

Localization of mitotic response and resultant epidermal alterations. To determine further whether the CBE- and GlcCer-induced increase in epidermal thymidine incorporation could be attributed to stimulation of epidermal proliferation, we next localized epidermal mitotic activity using both [^{3}H]thymidine incorporation and a DNA-specific stain (Feulgen). Feulgen-stained nuclei were markedly increased (Fig. 3) in CBE plus GlcCer-treated (*B*) vs. control (*A*) epidermis, and the increase again was localized primarily to the basal layer of the epidermis (Fig. 3 *B, arrows*; data for CBE and GlcCer alone not shown). Similarly, autoradiographic studies independently demonstrated increased thymidine incorporation in CBE plus GlcCer-treated (*D*) vs untreated control (*C*) epidermis, with labeling localized to the basal layer (Fig. 3 *D; arrows;* data for CBE and GlcCer alone not shown). Whereas 27% of basal cells were labeled in treated samples, only 6% of basal cells were labeled in control samples. Finally, histologic sections taken 72 h after twice daily CBE applications showed marked epidermal hyperplasia in the CBE-treated vs. the control samples (Fig. 4, *A* vs. *B*). These results confirm by two independent techniques that (*a*) both CBE and GlcCer increase epidermal proliferation; (*b*) the increase is localized to the basal layer; and (*c*) the increase in DNA synthesis results in epidermal hyperplasia.

Conduritol B epoxide increases epidermal glucosylceramide content, localized to the basal layer. To determine whether the stimulation in epidermal DNA synthesis induced by inhibition of GlcCer'ase could be attributed to changes in endogenous levels of epidermal sphingolipids, we next determined whether topical CBE applications alter epidermal sphingolipid content. Twice daily applications of CBE for three days resulted in significantly elevated epidermal GlcCer levels (i.e., twofold increased), in comparison to vehicle-treated controls (Fig. 5; $P \leq 0.005$). In contrast, epidermal ceramide and sphingomyelin content were not altered significantly at this time point (Fig. 5; sphingomyelin data not shown).

In order to determine whether the increased GlcCer was localized to the epidermal basal layer (i.e., the putative target of the mitotic stimuli), we next quantitated GlcCer and Cer

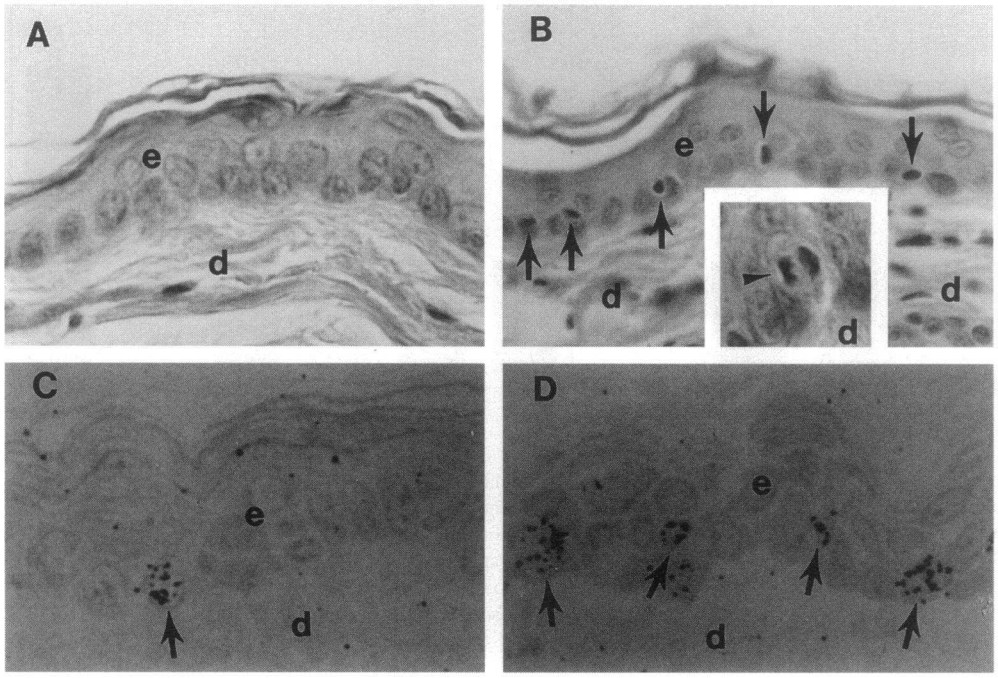

Figure 3. Feulgen stain and autoradiography of GlcCer and CBE-treated epidermis. (*A* and *B*) Feulgen-stained, vehicle-treated (*A*) and GlcCer + CBE-treated (*B*) murine epidermis at 24 h. Increased mitotic figures are present throughout the basal layer of treated mice (*B, arrows*), in comparison to vehicle-treated epidermis (*A*). Inset shows anaphase nucleus (*B, arrowhead*). (*C* and *D*) Autoradiography of epidermis showing increased labeling of basal cells after treatment with GlcCer plus CBE (*D*) vs. normal, vehicle-treated controls (*C*) (*e,* epidermis; *d,* dermis).

content in both the upper (stratum corneum, stratum granulosum, and stratum spinosum) vs. lower (stratum basale) epidermis 24 h after CBE treatment. As seen in Fig. 6, GlcCer levels were significantly increased in the lower layer of murine epidermis ($P \leq 0.001$), while GlcCer levels in the upper epidermis were not significantly altered. Whereas ceramide levels did not change significantly in the lower epidermis in response to CBE, they increased comparably in the outer layers (vs. the lower layer) of both treated and untreated epidermis (Fig. 6), a change consistent with epidermal differentiation (1, 3). Furthermore, although CBE induced marked increases in GlcCer content in the lower epidermis (i.e., 5–10-fold, $P \leq 0.001$ for two separate experiments), no significant change was observed for either lactosylceramide or GM3 ganglioside (i.e., ratio of treated to untreated = 0.8±0.2 and 1.4±0.4, respectively). These results demonstrate that CBE treatment induces an overall increase in epidermal GlcCer content, which is localized to the lower, mitotically active layer of the epidermis.

Epidermal mitotic response can not be attributed to barrier disruption. Since prior studies have demonstrated first, that daily topical applications of BrCBE (low dose) or CBE (higher doses) induce disruption of the epidermal permeability barrier (4); and second, that permeability barrier abrogation regulates epidermal DNA synthesis (32), we next asked whether either the CBE- and/or GlcCer-induced increase in epidermal proliferation could be attributed to an abnormality in permeability barrier function rather than to an independent, mitogenic effect of altered GlcCer content. Whereas transepidermal water loss (TEWL) levels increase significantly after three consecutive days of CBE treatment, only minor changes in barrier function (TEWL < 0.25 vs. 0.10 to 0.18 mg/cm^2 per h in normal controls) were observed at 24 h after treatment with CBE (Fig. 7), a time when mitogenesis is stimulated significantly.

We next determined whether application of a water-impermeable, Latex wrap, which artificially restores barrier function, as well as decreasing the DNA synthesis that occurs in response to barrier abrogation (32), would inhibit the proliferative response in either CBE- or GlcCer-treated mice. Occlusion did not diminish the proliferative response to either CBE, GlcCer, or CBE plus GlcCer (data not shown). These findings together demonstrate that the hyperprolifera-

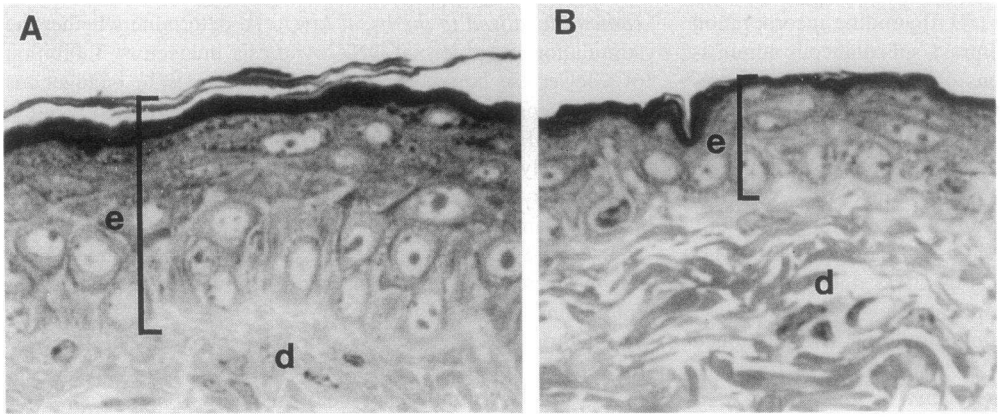

Figure 4. Histology of GlcCer + CBE-treated epidermis. Hematoxylin-and eosin-stain of GlcCer + CBE-treated (*A*) and vehicle-treated (*B*) murine skin. Skin was treated topically for 3 d with topical CBE and subcutaneous GlcCer (see Methods). Hyperplasia is evident in treated epidermis (*A*), while vehicle-treated controls appear normal (*B*) (*e,* epidermis; *d,* dermis). ×150.

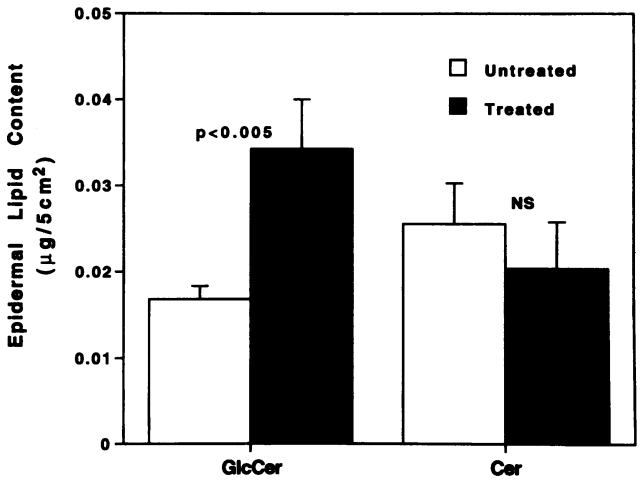

Figure 5. Effect of CBE on epidermal GlcCer and Cer content. Animals were treated daily with topical CBE (10 μmol bid) for 3 d, the epidermis was isolated, and lipids were extracted and quantitated by HPTLC and densitometry (see Methods). The untreated left flank of each animal served as a control. Results are presented as mean lipid weight (μg) per 5 cm^2 of epidermal surface area (±SEM; $n \geq 4$).

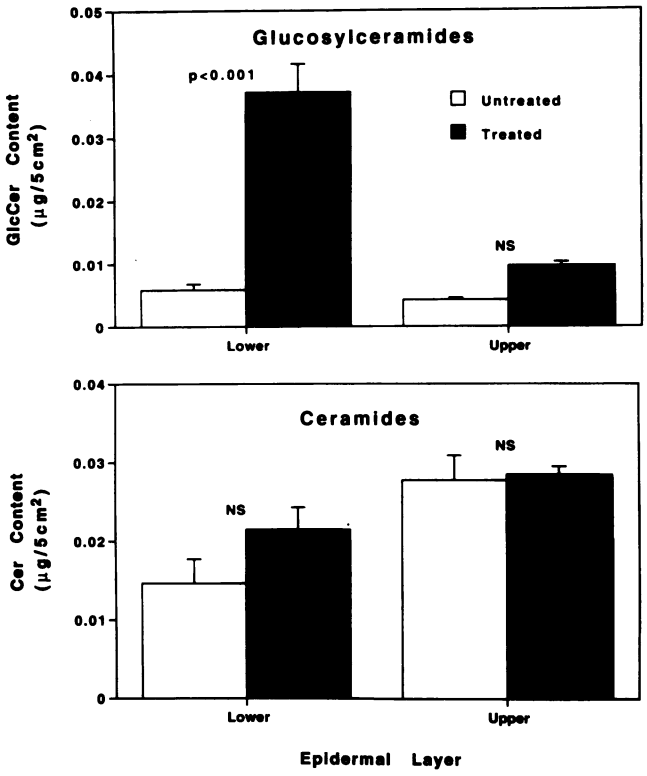

Figure 6. Localization of CBE-induced changes in GlcCer and Cer content in upper and lower murine epidermis. Animals were treated topically with two doses of topical CBE (10 μmol). Epidermis was isolated 24 h later and total lipids were extracted and quantitated by HPTLC and densitometry (see Methods). Epidermis from vehicle-treated animals served as controls. Results are presented as mean lipid weight per 5 cm^2 of epidermal surface area (±SEM; $n \geq 8$ for each value).

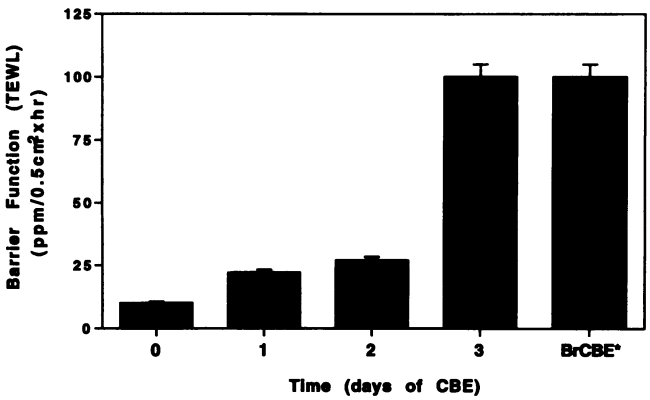

Figure 7. Effect of repeated topical CBE applications on epidermal permeability barrier function. Animals were treated with CBE (10 μmol bid) for up to 3 d. TEWL measurements were made daily immediately before the first daily application. Each value represents the mean (±SEM) of at least three TEWL readings on each of 6–8 animals. * Topical BrCBE for 5d results in equivalent barrier abrogation (4).

tive response to increases in either endogenous and/or exogenous GlcCer can not be attributed to alterations in epidermal permeability barrier function.

Glucocerebrosidase inhibitors do not induce inflammation. The studies described above show that neither chemically related, but ineffective conduritol compounds nor other monohexyl-ceramides induce hyperproliferation. To determine whether the hyperproliferative response to β-GlcCer'ase inhibition results from a non-specific irritant effect, we next applied CBE to mouse ears, utilizing PdiBu as a positive control (26). As seen in Fig. 8, PdiBu produced an expected, approximately twofold increase in ear thickness, an effect that was inhibited by potent topical corticosteroids. In contrast, topical CBE, at doses that induce hyperplasia, produced no increase in ear thickness over the vehicle-treated controls. These results show that the β-GlcCer'ase inhibitor, CBE, does not produce inflammation at a time when epidermal proliferation is stimulated.

Finally, to determine further whether the increase in epidermal [^{3}H]thymidine incorporation could be attributed to a non-

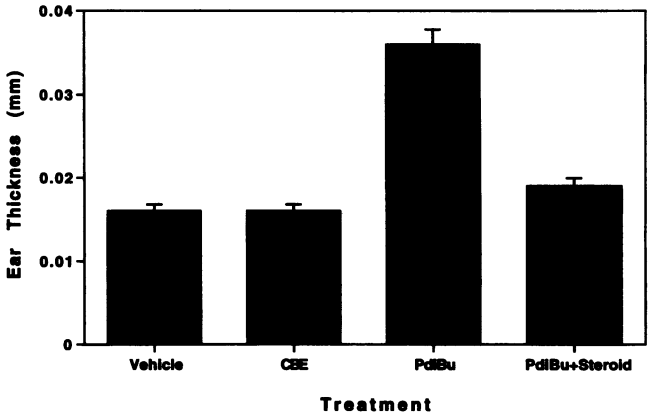

Figure 8. Topical CBE does not induce cutaneous inflammation. The external aspects of animal ears were treated either with vehicle, CBE (5 μmol/ear ×2), phorbol ester (20 nmol/ear), or PdiBu + steroid (0.05% Diprolene). Ear thickness was measured 18 h after treatment using a microcaliper. Results are presented as mean ear thickness±SEM ($n \geq 4$ for each value).

specific irritant effect, topical betamethasone dipropionate (a Class I "super-potent" topical steroid preparation) was applied to sites treated with either CBE and/or subcutaneous GlcCer. The potent steroid did not diminish either the GlcCer, CBE, or the GlcCer plus CBE-induced increase in [^{3}H]thymidine incorporation (data not shown). These results show further that the epidermal response to increased GlcCer can not be attributed to either non-specific irritant or pro-inflammatory effects of these compounds.

Discussion

Generation of Cer from GlcCer is required for the formation of a competent epidermal permeability barrier. In the absence of β-GlcCer'ase, GlcCer accumulates and Cer levels decrease in the stratum corneum intercellular membrane domains, leading to abnormal barrier function (4, 5). In addition to their critical role in the barrier, sphingolipids also are putative regulators of cell growth and division. Preliminary in vitro studies have shown that changes in both exogenous and endogenous GlcCer stimulate human keratinocyte proliferation, and have further shown that GlcCer opposes the antiproliferative effects of exogenous Cer (33). In the present study, we investigated whether alterations in either endogenous or exogenous levels of epidermal GlcCer result in epidermal hyperproliferation. Topical CBE, which inhibited epidermal β-GlcCer'ase by over 85% (Fig. 1), increased epidermal DNA synthesis, as assessed by three independent parameters. Moreover, equivalent doses of chemically-related conduritol compounds that are less potent inhibitors of epidermal GlcCer'ase did not stimulate epidermal DNA synthesis, suggesting strongly that the hyperplastic response is linked to inhibition of enzyme activity rather than to nonspecific effects of the conduritol compound. Likewise, only subcutaneous administration of GlcCer, not the closely-related cerebroside GalCer, nor the GlcCer metabolite Cer, stimulated epidermal growth, an effect which was enhanced by co-applications of CBE. Furthermore, coadministration of a potent, topical antiphlogistic steroid, betamethasone dipropionate, did not diminish the proliferative response to CBE, further arguing against a non-specific irritant mechanism. Together, these studies strongly suggest that GlcCer is a potent mitogen for keratinocytes in vivo.

It is well established that topical applications of a variety of compounds to the skin commonly provoke an inflammatory response (34). To eliminate inflammation as a potential cause of GlcCer-induced hyperproliferation, we used two different approaches. First, topical CBE did not produce evidence of inflammation using the murine ear inflammation model. Second, coapplication of a potent corticosteroid with CBE did not alter the mitogenic effect of the CBE treatment. Furthermore, chronic daily applications of either CBE (this study) or BrCBE (4) did not produce histologic or gross morphologic evidence of dermal inflammation. Thus, the epidermal mitogenic effects described here are not likely to result from a pro-inflammatory effect of these molecules.

Although the above-described studies provide a strong link between altered GlcCer content and epidermal DNA synthesis, they do not exclude the possibility that alterations in permeability barrier integrity could contribute to the proliferative response in vivo. Both acute abrogations of the barrier resulting from removal of extracellular stratum corneum lipids by either organic solvents or tape stripping and chronic barrier disruption (e.g., in essential fatty acid deficiency) lead to an increase in DNA synthesis that is normalized in these models by occlusion with a water-impermeable membrane (32). Whereas, hyperplasia was an incidental observation in our earlier studies in which repeated applications of BrCBE lead to increased GlcCer and barrier disruption (4), in the present study, the effects of either exogenous GlcCer or topical conduritol components on epidermal proliferation preceded any evidence of significant barrier alteration, suggesting that these two events are independent. Moreover, the hyperproliferative response to these compounds was not inhibited by occlusion. Thus, it appears highly unlikely that the increase in DNA synthesis observed with increased epidermal GlcCer content results from permeability barrier disruption.

The present results demonstrate that increased GlcCer levels within the mitogenic layers of the epidermis produce epidermal proliferation. These results are consistent with previous reports suggesting that the simple monohexanoyl-glycosphingolipid, GlcCer, is a promoter of cell growth (17, 20, 35). However, our studies do not exclude the possibility that the observed mitogenic response may be due to a GlcCer metabolite (i.e., either a more complex glycosphingolipid or a catabolic metabolite). In fact, exogenous lactosylceramide (36), gangliosides (37–39), sphingosine base (40) or sphingosine-1-PO_4 (41), each have been shown to stimulate cellular proliferation in vitro. In the present study, CBE induced significant increases in GlcCer content without alteration of lactosylceramide or GM3 ganglioside content 24 h after treatment. Moreover, previous lectin and antibody binding studies have demonstrated that a variety of glycoconjugates are expressed on the surface of basal and suprabasal keratinocytes (42, 43, 44). It is possible that one or more of these glycoconjugates could be involved in the regulation of epidermal mitogenesis. In contrast, other sphingoid base metabolites and Cer, the *N*-acetylated derivatives of sphingosine, appear to inhibit proliferation and induce cellular differentiation. Although stimulation of proliferation also has been noted in Swiss 3T3 fibroblasts (45), a preponderance of evidence suggests that exogenous Cer and Cer generated by sphingomyelin hydrolysis both inhibit proliferation and induce differentiation (13–16). Thus, the opposing effects of GlcCer and Cer suggest that the content of each of these compounds may be tightly regulated to produce optimal growth rates in epidermis, and presumably in extracutaneous tissues, as well.

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