Glucosylceramide metabolism is regulated during normal and hormonally stimulated epidermal barrier development in the rat

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Abstract Glucosylceramides, delivered to the stratum corneum interstices by exocytosis of lamellar body contents, are enzymatically hydrolyzed to ceramides, which are major components of the lipid lamellar bilayers that mediate epidermal barrier function. Because this conversion is critical for permeability barrier homeostasis in the adult animal, in this study we measured the changes in activities of the enzymes responsible for the synthesis of glucosylceramide and its conversion to ceramide, UDP-glucose:ceramide glucosyltransferase (GC synthase) and β-glucocerebrosidase (β-GlcCer′ase), respectively, during fetal barrier formation. In epidermis from rats of gestational age 17–21 days, GC synthase activity peaked on day 19, prior to barrier competence, whereas β-GlcCer′ase activity rose throughout barrier formation, exhibiting a 5-fold increase over this time period. β-GlcCer′ase protein rose in parallel with activity, as did mRNA levels. Enzyme activities in skin explants from 17-day fetal rats, incubated up to 4 days in hormone- and serum-free media, paralleled those measured at corresponding time points in utero. Incubation with hormones that accelerate barrier development had minimal effects on GC synthase activity, whereas β-GlcCer′ase activity was significantly increased after 1 or 2 days in culture. Finally, inhibition of β-GlcCer′ase with conduritol B epoxide prevented barrier development in vitro and was accompanied by abnormalities in the lamellar bilayer ultrastructure of the stratum corneum. These data indicate that both synthesis and hydrolysis of glucosylceramide are regulated during fetal development. Furthermore, the enzymatic hydrolysis of glucosylceramide to ceramide is essential for fetal barrier ontogenesis.—**Hanley, K., Y. Jiang, W. M. Holleran, P. M. Elias, M. L. Williams, and K. R. Feingold.** Glucosylceramide metabolism is regulated during normal and hormonally stimulated epidermal barrier development in the rat. *J. Lipid Res.* 1997. **38:** 576–584.

Supplementary key words ceramides • epidermal barrier ontogenesis • glucocerebrosidase • glucosylceramide synthase • stratum corneum

Both the composition and structural arrangement of the extracellular lipids in the stratum corneum (SC) are critical for normal mammalian epidermal permeability barrier function. Whereas the predominant lipid species in the SC interstices are ceramides, cholesterol, and free fatty acids, the lipids delivered to the SC, via lamellar body exocytosis, are primarily glucosylceramides, cholesterol, and phospholipids (reviewed in ref. 1). Therefore, both the formation of the precursor lipids and their subsequent metabolism into the species required for mature lamellar bilayer structure formation are important steps in the development and maintenance of a competent barrier.

Glucosylceramides, which are the principal lipid components of epidermal lamellar bodies, are synthesized from ceramide and UDP-glucose in a reaction catalyzed by UDP-glucose:ceramide D-glucosyltransferase (GC synthase; EC 2.4.1.80), the enzyme responsible for the initial step in the formation of all glycosphingolipids. GC synthase activity has been localized to the cytosolic surface of the Golgi (2–4), and the human cDNA sequence has been recently described (5). Although GC synthase activity increases with human keratinocyte differentiation in vitro (6), the role of this enzyme in epidermal barrier formation has not been established.

The ceramides of the SC are derived in large part from the subsequent deglycosylation of glucosylceramides after their secretion into the SC extracellular domains. The enzyme β-glucocerebrosidase (β-GlcCer′ase; EC 3.2.1.45), which is present in the SC (7), is responsible for this conversion. In extracutaneous tissues, β-GlcCer′ase is a lysosomal enzyme, and a deficiency of this enzyme results in the most common lipid storage disorder, Gauchers disease. Abundant evidence sug-

Abbreviations: β-GlcCer′ase, β-glucocerebrosidase; CBE, conduritol bromoepoxide; GC synthase, UDP-glucose:ceramide glucosyltransferase; SC, stratum corneum.

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gests a critical role for β-GlcCer′ase in barrier homeostasis and repair. For example, inhibition of this enzyme by topical applications of conduritol-type inhibitors results in structural defects in lamellar bilayers, abnormal barrier function, and retarded barrier repair (8, 9). A similar barrier defect and altered ultrastructure is evident in patients and transgenic mice with severe β-GlcCer′ase deficiency (Gauchers disease, type II) (10). These studies suggest that β-GlcCer′ase catalyzes the final step in the formation of mature lamellar unit structures in the SC interstices.

A competent barrier is an essential requirement for terrestrial life. In mammals, the permeability barrier forms during late gestation in order that a competent barrier is present at birth (reviewed in ref. 11). Using the fetal rat as an experimental model, our laboratory has shown previously that the formation of a competent barrier between d 19 and 21 of gestation (term = 22 d) correlates both with increased SC lipid content, particularly ceramides, and with the reorganization of lamellar body-derived membranes into mature lamellar bilayer unit structures in the extracellular spaces of the SC (12). Because of their putative importance for ceramide synthesis and glucosylceramide to ceramide processing, in the present study, we measured the changes in activities of these two key enzymes, GC synthase and β-GlcCer′ase, in fetal rat epidermis in utero during barrier ontogenesis. Moreover, we also measured the changes in activities of these two enzymes in vitro, using a fetal skin explant model that closely mimics in utero development (13), and then examined the effects of various hormones, which either accelerate or inhibit barrier formation in vitro (13, 14), on enzyme activity. Finally, we determined the effects of inhibition of β-GlcCer′ase activity on barrier development. Our studies show that both enzymes are regulated in relation to barrier development, but these changes do not occur in parallel.

MATERIALS AND METHODS

Materials

[^{14}C]UDP glucose (263 mCi/mmol) was obtained from NEN Research Products (Boston, MA). 4-Methylumbellifery-β-D-glucopyranoside (4-MUG), 4-methylumbelliferone (4-MU), sodium taurocholate, triiodothyronine (T3), dexamethasone (DEX), diethylstilbestrol (DES), and dihydrotestosterone (DHT) were from Sigma Chemical Co. (St. Louis, MO). Conduritol B epoxide (CBE) was from Toronto Research Chemicals, Inc. (Toronto, Canada). M199 media was from Gibco (Grand Isle., NY) and organ culture inserts (COL-wells) were from Costar (Cambridge, MA). Molecular grade chemicals were from Sigma or from Fisher Scientific (Fairlawn, NJ). Oligo(dT)-cellulose was from Pharmacia LKB Biotechnology (Uppsala, Sweden), and nitrocellulose was from Schleicher and Schuell (Keene, NH). The multiprime DNA Labeling System was from Amersham International (Amersham, UK) and (α-^{32}P)dCTP (3000Ci/mmol, 10mCi/ml) was from NEN. The murine β-GlcCer′ase cDNA probe was kindly provided by R. O'Neill, NIH.

Tissue and enzyme preparation

Flank skin was excised from fetal Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) of gestational age 17–21 days. Epidermis was separated from dermis after incubation in 10 mM EDTA in Ca^{2+}- and Mg^{2+}-free PBS, pH 7.4, at 37°C for 30–40 min. The tissues were then minced and homogenized (3 × 15 s with a Polytron homogenizer, followed by 2 × 10 s sonication at 35% power) on ice. The homogenization buffer used to prepare the enzyme source for GC synthase consisted of 0.25 M sucrose, 0.05 M MOPS, 1 mM EDTA, 1 mM EGTA, and 0.5 mM PMSF, pH 7.4. Crude homogenates were first centrifuged at 10,000 *g* for 10 min., then at 100,000 *g* for 60 min, both at 4°C, and the microsomal pellet was retained for GC synthase activity (3).

Tissues for β-GlcCer′ase determination were homogenized in PBS containing 0.1 mM PMSF and 0.1% Triton X-100, and centrifuged at 10,000 *g* for 15 min at 4°C. Enzyme activity was measured in the supernatant.

Protein content was measured by the method of Bradford (15).

Organ culture model and measurement of barrier function

Full-thickness flank skin was removed from day 17 rats and incubated dermis-side down on collagen membrane inserts (3 μm pore size), in hormone- and serum-free media, as described previously (13). Epidermal water loss was measured gravimetrically and reported as mg water lost/mm^2 per h, as previously described (13). Barrier function was measured in explants incubated in 10 nM DEX (in ethanol), 10 nM T3 (in dimethylsulfoxide (DMSO)), 100 nM DES (in ethanol), 100 nM DHT (in DMSO), or vehicle alone (final concentration 0.05%) after 1 or 2 d of incubation. CBE, in concentrations of 0.1 μM–50 μM, or ethanol alone (0.05%), was added to the medium for 48 h beginning on culture day 2.

UDP-glucose:ceramide glucosyltransferase (GC synthase) assay

The synthesis of glucosylceramide from exogenous ceramide was assayed as described (16). Briefly, the total assay volume of 110 μl contained 50 μM UDP [U-

[14C]glucose (70 mCi/mmol), 50 mM MOPS (pH 6.5), 5 mM $MnCl_2$, 2.5 mM $MgCl_2$, 1 mM NADH, 5 mM dimercaptopropanol, and 1% w/v CHAPS. The solid substrate was prepared by adsorbing 20 μg ceramide (Type IV; Sigma) onto 1 mg silica gel, and the reaction was initiated with the addition of 0.1–0.2 mg protein. Because microsomal preparations of epidermis gave slightly higher specific enzyme activities than did crude homogenates or membrane preparations obtained by centrifugation at 10,000 *g* for 30 min (data not shown), the former were used in all subsequent experiments. Reactions were carried out at 37°C for 30 min, and were terminated by the addition of ice-cold PBS. Pellets were then washed 4 times by centrifugation and resuspended in ice-cold PBS, transferred to scintillation vials, and radioactivity was measured.

β-Glucocerebrosidase assay

β-GlcCer′ase activity was assayed using the synthetic substrate 4-MUG as described (7). Assays were performed in 5 mM sodium taurocholate in citrate–phosphate buffer (pH 5.6) with 0.5 mM 4-MUG for 60 min at 37°C, with a final assay volume of 100 μl, and protein concentration of 1–2 mg/ml. The reaction was terminated with 2 ml carbonate–bicarbonate buffer (pH 10.5). The fluorescence was then measured at 360 λ (excitation) and 450 λ (emission) and compared with a standard (4MU) curve.

Immunocytochemistry

Full-thickness skin was excised from fetal rats of gestational age d 17 and d 21. Samples were snap-frozen in O.C.T. embedding medium (Miles Scientific, Naperville, IL) and stored at −80°C. Ten-μm sections were prepared and fixed in ethanol–acetone 1:1 at −20°C for 10 min, washed with PBS, and then incubated with 2% normal goat serum (Sigma) for 10 min at room temperature. Sections were then incubated with rabbit anti-human glucocerebrosidase antibody (polyclonal antibody against human placental β-glucocerebrosidase provided by B. Martin and E. Ginns, NIH) (17) diluted in 1 mg/ml BSA in PBS to a final concentration of 1:300 or 1:500. Sections on control slides were incubated with PBS/BSA alone. Sections were then washed with PBS, and incubated with fluorescein-conjugated goat affinity-purified F(AB′)2 fragment to rabbit IgG (Cappel, Durham, NC) at a final concentration of 10 μg/ml in PBS/BSA at room temperature for 1 h. Slides were viewed and photographed with a Leitz Ortholux II Microphot-FX light and fluorescent microscope (Garden City, NY).

Isolation of epidermal mRNA and Northern analysis

Fetal epidermis was pooled (0.1–0.2 g), snap-frozen, and stored at −70°C. Total RNA was isolated as described (18), using a modification of the guanidinium thiocyanate method, and poly A(+) RNA was isolated using oligo (dT) cellulose. Eight μg of poly A(+) RNA in sample buffer was run on agarose/formaldehyde (1%/6.1%) gels. RNA was then transferred to nitrocellulose and fixed by baking at 80°C for 2 h. Blots were prehybridized at 60°C for 1 h, and hybridized overnight at 60°C with a [32P]dCTP-radiolabeled murine cDNA probe for β-glucocerebrosidase. After washing, blots were exposed to X-ray film, and bands were quantified by scanning densitometry.

Electron microscopy

Samples were minced into 1-mm^3 pieces, fixed in modified Karnovsky's fixative, and processed for electron microscopy. Sections were stained with uranyl acetate and lead citrate, post-fixed in either reduced osmium or ruthenium tetroxide, and examined using a Zeiss 10A electron microscope, as described previously (12).

Statistical analysis

Statistical evaluation was performed using a Student's *t* test.

RESULTS

GC synthase and β-GlcCer′ase activities change during barrier development in utero

Epidermal differentiation begins in the fetal rat on d 17, culminating in the formation of a competent epidermal barrier to water loss by gestational d 21 (no measurable barrier is present on d 19; barrier function is variable on d 20) (12–14). To determine whether the specific activity of GC synthase or of β-GlcCer′ase changes during barrier ontogenesis, we measured the activities of these enzymes in whole skin and in epidermis from fetal rats of gestational ages d 17 through 21. The activity of GC synthase in both the epidermis and in whole skin reaches a maximum at d 19, and then either plateaus or declines slightly through d 21 (**Fig. 1A**). In contrast, the specific activity of β-GlcCer′ase in both epidermis and whole skin rises steadily from d 17 to 21 (Fig. 1B). Whereas β-GlcCer′ase activity increases approximately 2.5-fold in whole skin ($P < 0.01$, n = 5), in epidermis it increases nearly 5-fold ($P < 0.01$, n = 5) during this time period. These results show that β-GlcCer′ase activity increases concurrently with formation of the epidermal permeability barrier in utero, whereas GC synthase activity peaks just prior to formation of a competent barrier.

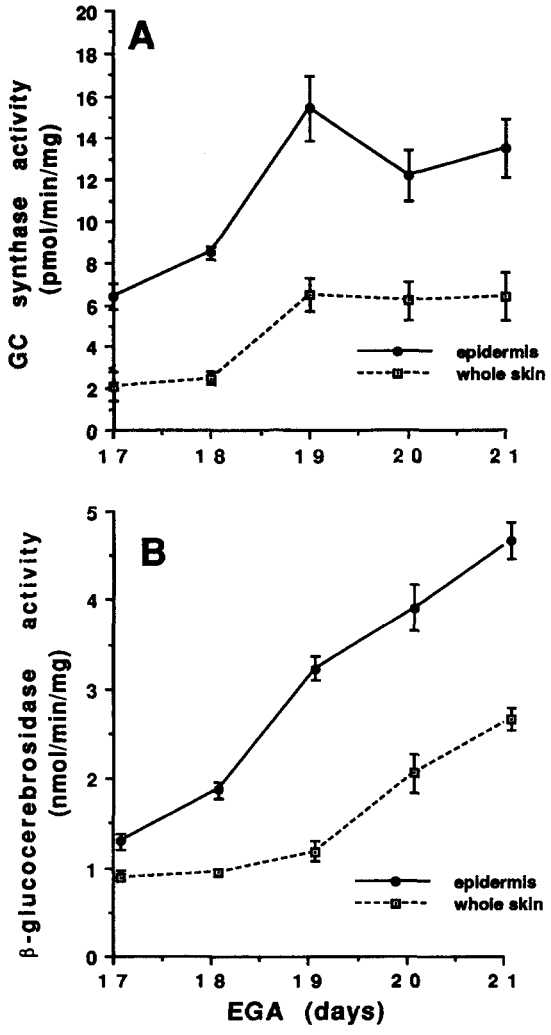

Fig. 1. The specific activities of GC synthase (A) and β-glucocerebrosidase (B) are separately regulated during barrier development. Enzyme activity in whole skin (open squares) and in epidermis (closed circles) taken from rats of gestational age d 17 through d 21 was measured as described in Methods. Data are mean ± SEM, n = 5.

Levels of β-GlcCer'ase increase during barrier development in utero

We next used immunohistochemistry to examine the quantity and location of β-GlcCer'ase protein in the epidermis of fetal rat skin excised from animals of gestational age d 17 (no measurable barrier) in comparison to d 21 animals (competent barriers). Immunostaining was cytoplasmic and generalized in the 2–3 cell layer epidermis of d 17 animals (**Fig. 2A**). Staining was increased in d 21 epidermis (Fig. 2B), with intense staining in the stratum granulosum and the stratum corneum (SC) (Fig. 2C), consistent with reports of 2-fold higher activity in the upper epidermal layers (stratum granulosum and corneum) than in the lower (basal and spinosum) layers (7). No staining was observed in control samples (no first antibody) (data not shown). These results indicate that the increased β-GlcCer'ase activity during barrier development is paralleled by increased protein levels.

β-GlcCer'ase mRNA levels increase during barrier development in utero

Increased mRNA levels correlate with increased β-GlcCer'ase activity in adult murine epidermis during barrier repair (9). We next determined whether the increase in β-GlcCer'ase activity during fetal development could be accounted for by a corresponding increase in epidermal mRNA levels. As shown in **Fig. 3,** β-GlcCer'ase mRNA levels increase approximately 5-fold between d 17 and 21, paralleling the changes in enzyme activity. These results indicate that the increase in β-GlcCer'ase activity during barrier development can be attributed to increased mRNA levels.

Epidermal β-GlcCer'ase activity in vitro is increased by DEX, T3, or estrogens

We have shown previously that full-thickness skin explants obtained from EGA d 17 fetal rats and incubated in hormone- and serum-free media for 4 d stratify to form a SC and barrier to water loss, in a manner that precisely mirrors that observed in utero (13, 14). Using this model, we next examined changes in epidermal activity of GC synthase and β-GlcCer'ase during in vitro ontogenesis. As seen in **Fig. 4,** the time course of activity for each enzyme paralleled that measured for in utero samples, (c.f., Fig. 1): whereas GC synthase peaked at d 19 (Fig. 4A), β-GlcCer'ase exhibited a 5-fold increase between d 17 and 21 ($P < 0.01$, n = 6) (Fig. 4B).

Previous studies have shown that barrier maturation in fetal rat skin in utero is accelerated by maternal glucocorticoid or estrogen treatment and inhibited by androgens (14, 19), and that barrier development in fetal rat skin explants is accelerated by glucocorticoids, thyroid hormone, or estrogens, and inhibited by androgens (13, 14). We next investigated whether these hormones that regulate the rate of barrier ontogenesis influence GC synthase or β-GlcCer'ase activities in vitro. As shown in **Fig. 5A,** the addition of 10 nM dexamethasone (DEX), 10 nM thyroid hormone (T3), or 100 nM diethylstilbestrol (DES) to the incubation medium resulted in modest but significant increases in GC synthase activity after 1 d of incubation (approximately 10–30% increase over controls). No effect on activity was observed in explants incubated for 2 d in the presence of DEX, T3, or DES. DHT had no effect on GC synthase activity after 1 or 2 d of incubation. In contrast, the addition of DEX, T3, or DES to the medium significantly

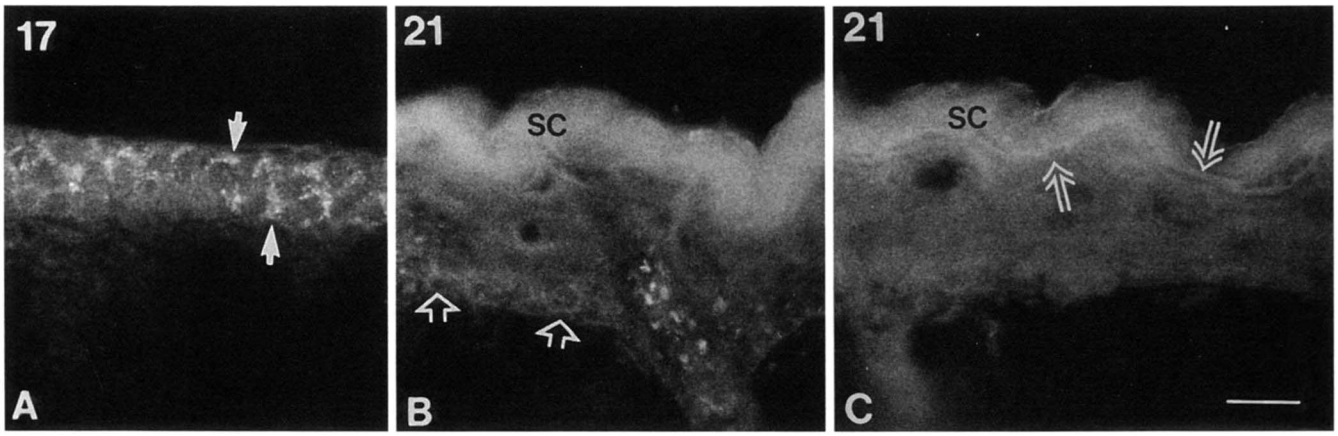

Fig. 2. β-Glucocerebrosidase protein levels are increased during barrier development in utero. Seventeen- and 21-day fetal rat skin was stained with anti-β-GlcCer′ase antibody. Seventeen-day epidermis is unstratified and reveals speckled cytosolic staining (A, solid arrows). Twenty-one-day epidermis displays a prominent stratum corneum (SC) and an overall increase in staining intensity (B and C). Labeling is seen in the basal layer (B, open arrows), and is very intense in the outer regions of the epidermis, which display a membrane pattern (C, double arrows). Control samples (no first antibody) revealed no staining at either time point (not shown). A, 1:300 dilution, B and C, 1:500 dilution; bar, 5 μm, all panels.

increased β-GlcCer′ase activity after 1 and 2 d in culture (approximately 75–100% increase over controls) (Fig. 5B). Indeed, these treatments increased the activity of β-GlcCer′ase to levels close to that observed in control explants after 4 d in culture, when a competent barrier is present. β-GlcCer′ase activity was unaffected by 1 d of DHT treatment; however, in explants incubated for 2 d in the presence of DHT, β-GlcCer′ase activity was significantly inhibited (75% of control, $P < 0.02$, n = 6). These results indicate that hormones that either accelerate or delay barrier formation induce corresponding changes in epidermal β-GlcCer′ase activity, but have less impact on the activity of GC synthase during this period of development.

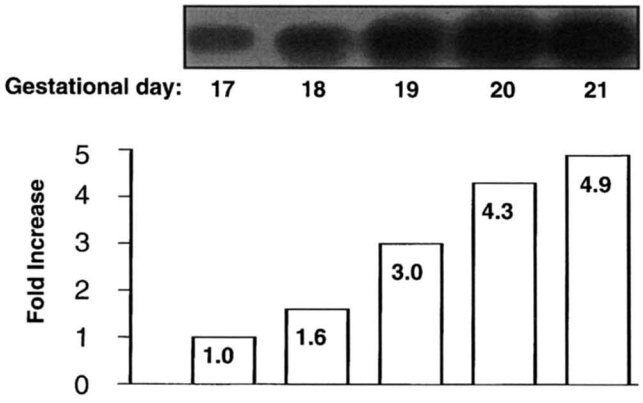

Fig. 3. β-Glucocerebrosidase mRNA levels increase during barrier ontogenesis. Isolation of epidermal mRNA from rats of gestational age d 17–21 and Northern blot analysis using a cDNA probe for β-glucocerebrosidase were performed as described in Methods. Eight μg poly (A)+ was loaded in each lane. RNA integrity and equal loading was verified by gel electrophoresis. Autoradiogram shows results of one representative experiment. The corresponding diagram shows the quantification of mRNA levels with the level at d 17 arbitrarily set to 1.0. Similar results were observed in two independent experiments.

CBE inhibits both β-GlcCer′ase activity and barrier formation in vitro

As β-GlcCer′ase activity is critical for barrier function in mature animals (7, 9), we examined the effects of inhibition of this enzyme on barrier development in vitro. Topical applications of CBE significantly inhibit β-GlcCer′ase activity in adult murine skin (7). To determine first whether CBE also inhibits β-GlcCer′ase activity in fetal skin explants, enzyme activity was measured in various concentrations of CBE (0–50 μM), added during day 2 to 4 of culture, with activity determined on day 4. As shown in **Fig. 6A,** addition of CBE to the culture medium resulted in a dose-dependent inhibition of β-GlcCer′ase activity, with 20 μM CBE producing maximal inhibition (13% of control).

We next determined whether inhibition of β-GlcCer′ase impairs formation of the barrier in vitro by measuring water loss gravimetrically in fetal skin explants incubated with CBE (Fig. 6B). As reported previously (13, 14), transepidermal water loss decreased in control explants between d 2 and 4 of culture, signifying development of a competent barrier (Fig. 6B, dashed lines). In contrast, addition of CBE to the medium prevented the decline in water loss in a dose-dependent manner (20 μM CBE produced the maximum inhibitory effect on barrier formation). The concordance of the dose-response curves for inhibition of β-GlcCer′ase activity and inhibition of barrier formation suggest that β-GlcCer′ase activity is required for barrier ontogenesis.

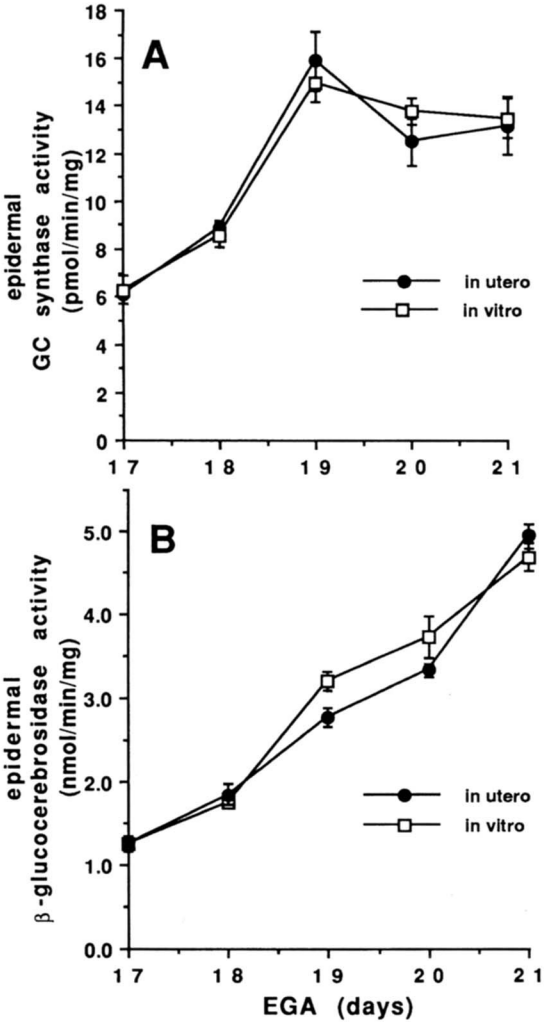

Fig. 4. Specific activities of GC synthase (A) and β-glucocerebrosidase (B) in vitro parallel in utero. Epidermal activity was measured in explants from gestational age d 17 fetal rats and incubated 0–4 days in serum- and hormone-free media (open squares) or in epidermis taken from pups of the corresponding gestational age (d 17–21) (closed circles) (see Methods). Day 18 corresponds to culture day 1, day 19 to culture day 2, etc. Data are presented as mean ± SEM, n = 6.

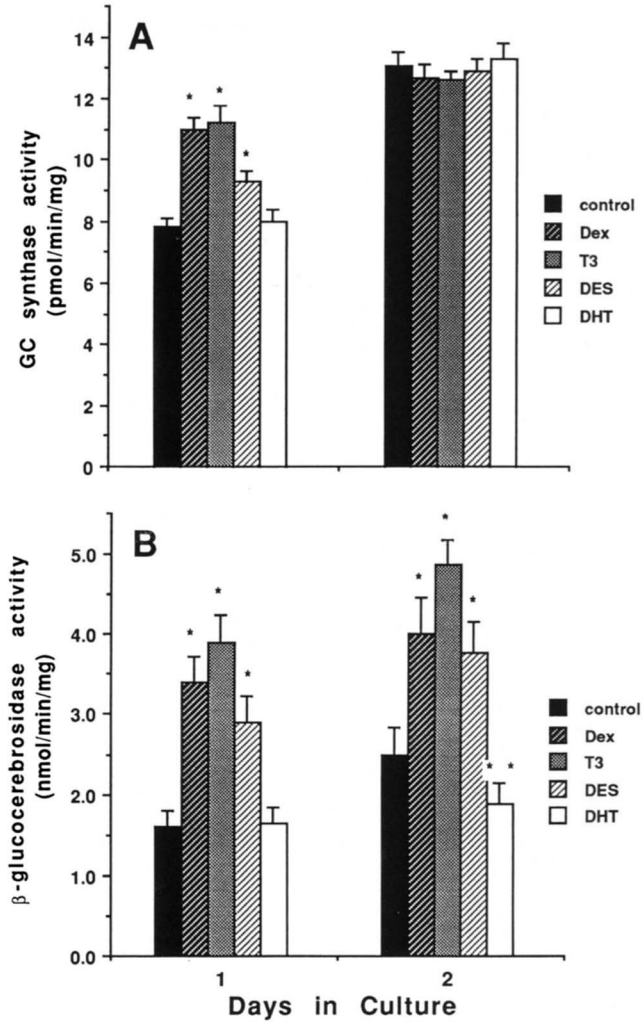

Fig. 5. GC synthase (A) and β-glucocerebrosidase (B) activities are regulated by hormones that affect barrier development. Fetal skin explants from gestational age d 17 rats were incubated with 10 nM dexamethasone (Dex), 10 nM thyroid hormone (T3), 100 nM diethylstilbestrol (DES), or 100 nM dihydrotestosterone (DHT) and enzyme activity was measured on d 1 or d 2 as described in Methods. $*P < 0.005$ (n = 6), $**P < 0.02$ (n = 6), compared with controls. Data are expressed as mean ± SEM.

CBE inhibition of β-GlcCer′ase interferes with the maturation of SC extracellular membrane structure

We next determined whether inhibition of β-GlcCer′ase activity during fetal development in vitro results in structural alterations in the lamellar body secretory system. Lamellar body substructure (**Fig. 7A**) and lamellar body-derived secreted material at the stratum granulosum–SC interface (Fig. 7B) in fetal skin incubated with 20 μM CBE appeared normal, with no evidence of cellular toxicity observed. However, the SC interstices in CBE-treated explants were filled with secreted, incompletely processed lamellar material, with a paucity of mature lamellar unit structures (Fig. 7C). In contrast, control explants displayed multiple arrays of mature lamellar unit structures throughout the extracellular spaces of the SC (Fig. 7D), corresponding to previous descriptions of d 21 SC (12–14). Thus, inhibition of β-GlcCer′ase activity in fetal skin explants interferes with the final processing of secreted lamellar body-derived membranes into mature lamellar unit structures.

DISCUSSION

During late gestation, the skin generates a competent cutaneous permeability barrier that is capable of pre-

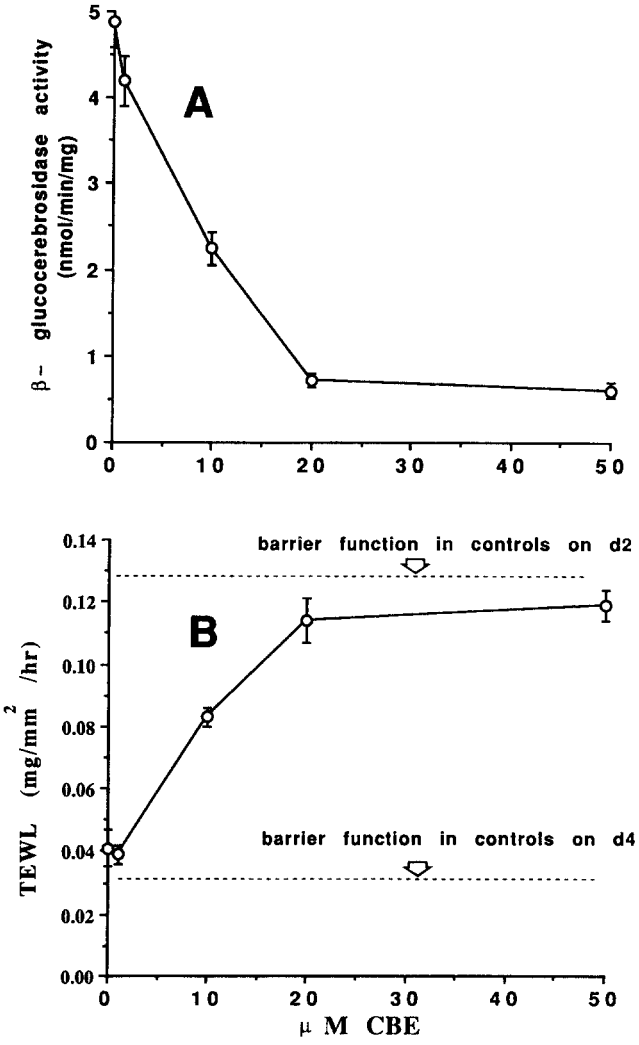

Fig. 6. CBE inhibition of β-glucocerebrosidase activity and barrier development is dose-dependent. (A): Epidermal enzyme activity was measured on d 4 (see Methods) in explants incubated from day 2 to 4 in the presence of various concentrations of CBE. (B): Water loss was measured gravimetrically in parallel samples. Data are mean ± SEM (n = 4, β-glucocerebrosidase activity), (n = 6, water loss).

venting excessive water loss in the neonate while protecting the infant from systemic infection (11, 20, 21). In view of the importance of ceramides for barrier competence in adult skin, in the present study we examined the regulation of two key enzymes involved in the generation of ceramide destined for the SC interstices: GC synthase and β-glucocerebrosidase. We utilized the well-characterized fetal rat model in which the barrier is formed between gestational d 19 and 21 (term is 22 d) (12, 19), and an in vitro model that displays comparable developmental milestones (13, 14).

GC synthase activity peaks on gestational d 19, as cornification begins but prior to the presence of a measurable barrier, and plateaus or declines during barrier development on d 20 and d 21. GC synthase activity has been previously measured in brain, kidney, liver, and the submaxillary gland (2–4, 16, 22), and recently in neonatal foreskin (6). A positive correlation between keratinocyte differentiation in vitro and increasing GC synthase activity has been reported (6). The rise in GC synthase activity between d 17 and 19, seen in our studies, correlates with the onset of epidermal stratification, and, most importantly, with the appearance of replete epidermal lamellar bodies. We previously reported that activities of other key lipid synthetic enzymes, such as hydroxymethylglutaryl Co A reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway, acetyl CoA carboxylase, one of two key regulatory enzymes of fatty acid biosynthesis, and serine palmitoyl transferase, which catalyzes the first committed step in ceramide biosynthesis, also peak prior to or on d 19 (23). Thus the enzymes that form the lipid constituents of lamellar bodies appear to be most active immediately prior to the development of a competent barrier, paralleling formation of the initial pool of lamellar bodies.

In contrast to those enzymes active in lipogenesis in the nucleated epidermal cell layers, the activity of β-GlcCer′ase, whose activity is greatest in the SC, rises throughout fetal barrier development, exhibiting an over 4-fold increase in activity between d 17 and 21. This increase in activity is paralleled by an increase in β-GlcCer′ase mRNA levels, suggesting that the increase in activity is due to increased gene transcription. Moreover, inhibition of β-GlcCer′ase activity with CBE prevents the formation of a competent barrier, resulting in the persistence of immature lamellar structures throughout the SC interstices, without any apparent effect on lamellar body substructure or secretion, suggesting a specific role for β-GlcCer′ase during barrier formation. A major role for β-GlcCer′ase in fetal barrier formation is also suggested by studies in a transgenic homozygous Gaucher mouse, produced by null mutations of the β-GlcCer′ase gene (24). These animals are born with incompetent barriers, explicable by abnormalities in lamellar bilayer structure and a failure of glucosylceramide to ceramide processing (10). The appearance of the SC membranes is similar in our CBE-treated explants and the Gaucher mouse. Our current studies provide further evidence that β-GlcCer′ase activity is required for the late stages of fetal barrier formation, and they indicate that the processing of glucosylceramide to ceramide is essential for the formation of mature lamellar unit structures.

We have shown previously that glucocorticoids, thyroid hormone, and estrogens accelerate, while testosterone inhibits, barrier ontogenesis in vitro (13, 14). In the present study, we found that these hormones did not greatly affect the activity of GC synthase but mark-

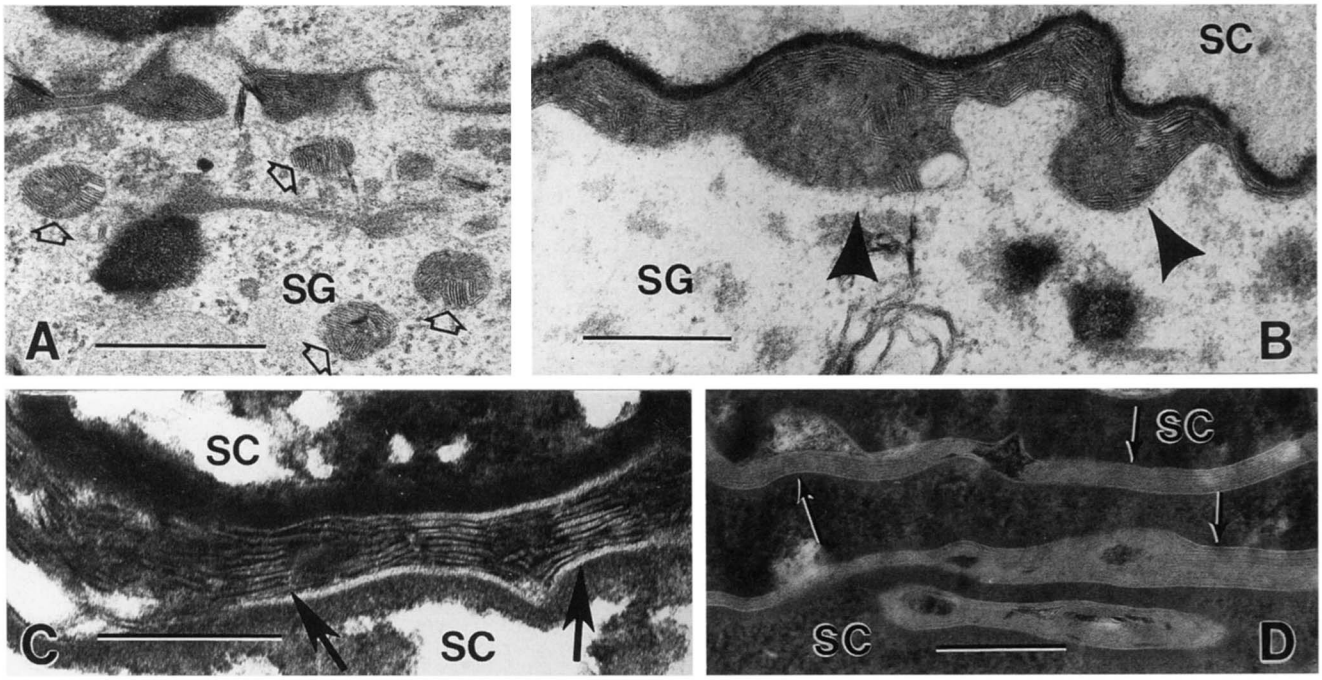

Fig. 7. CBE induces ultrastructural changes in SC membranes. Representative electron micrographs of upper epidermis of CBE-treated (A–C) versus vehicle-treated (D) explants. CBE treatment does not alter lamellar body substructure (A, arrows) or the appearance of secreted lamellar body contents at the stratum granulosum–stratum corneum (SC) interface (B, arrows). CBE-treated samples reveal immature, foreshortened, extracellular lamellae (C, arrows), interspersed with areas of phase separation. Vehicle-treated controls reveal extensive, mature lamellar unit structures throughout the SC interstices (D, arrows). A, B, reduced osmium postfixation. C, D, ruthenium tetroxide postfixation; bar, 0.05 μm.

edly increased β-GlcCer′ase activity between d 17–21. This provides further evidence linking β-GlcCer′ase activity and the formation of the epidermal permeability barrier. Consistent with these findings, hormonal regulation of these enzymes in extracutaneous tissues has been reported. For example, 17β-estradiol increased GC synthase activity in mouse kidney, while testosterone elevated GC synthase and decreased β-GlcCer′ase activity (25). Whether these hormones directly affect β-GlcCer′ase gene transcription or indirectly affect enzyme activity by activating other genes involved in epidermal terminal differentiation remains to be determined. As the promoter region of β-GlcCer′ase has not yet been characterized (5, 26), whether hormone response elements are present remains unknown.

The series of events that lead to the formation of a competent barrier to water loss are tightly regulated, as evidenced in the intrauterine growth-retarded fetal rat. Despite epidermal growth retardation including a thinner SC and delayed accumulation of keratins and profilaggrin, the lipid content in relation to SC dry weight is relatively unaffected and the time course of barrier development is not disrupted (27). Because ceramides play such an important role in mammalian barrier function, it is not surprising that the activities of the key enzymes in this pathway are also developmentally regulated. In this study we have shown that the syntheses of glucosylceramides and ceramides are separately regulated during fetal barrier development, and that the final processing of glucosylceramides to ceramides in the extracellular spaces of the SC is critical for the prenatal formation of a competent epidermal barrier.

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