Induction of Ceramide Glucosyltransferase Activity in Cultured Human Keratinocytes

CORRELATION WITH CULTURE DIFFERENTIATION*

Gloria N. Sando‡, Elizabeth J. Howard, and Kathi C. Madison

From the Marshall Dermatology Research Laboratories, Department of Dermatology, University of Iowa College of Medicine, Iowa City, Iowa 52242

Ceramides are the major component of the extracellular lipids that comprise the epidermal permeability barrier. They are derived from glucosylceramides (GlcCer) upon their extrusion from lamellar granules into the extracellular space in the upper layers of the epidermis. To better understand the regulation of the unique pathway for ceramide production in epidermis, we have studied the activity of the enzyme responsible for GlcCer synthesis, ceramide glucosyltransferase (CerGlc transferase), during keratinocyte culture differentiation. Human keratinocyte cultures were expanded in low calcium keratinocyte growth medium (KGM) and then switched to either normal calcium KGM (nKGM) or "complete" Dulbecco's modified Eagle's medium/Ham's F-12 (3:1) supplemented with 10% fetal bovine serum (cDMEM). At 7 and 10 days after the medium switch, electron microscopy revealed that cDMEM cultures were more fully differentiated morphologically and contained numerous lamellar granules. The GlcCer/DNA content of cDMEM cultures increased to 6 times that of day 0 cultures and was nearly 4 times greater than that of nKGM cultures, whereas the total lipid/DNA content of cDMEM cultures increased to 1.8 times that of day 0 cultures and was ~1.2 times that of nKGM cultures. CerGlc transferase activity/DNA increased 6 times in cDMEM cultures but ~1.5 times in nKGM cultures. By contrast, β-glucocerebrosidase activity, which is responsible for the conversion of GlcCer to ceramide, increased to a similar extent in both differentiating culture systems. Treatment of cultures with the reversible CerGlc transferase inhibitor, α-threo-1-phenyl-2-(palmitylamino)-3-morpholino-1-propanol, prevented the increase of GlcCer in cDMEM cultures, and blocked conversion of exogenously added ceramide to GlcCer. A low level of CerGlc transferase activity, relative to that in differentiated keratinocytes, was detected in cultures of other human cell types. These results indicate that CerGlc transferase activity is induced during epidermal differentiation and that regulation of this enzyme may be an important determinant of the specialized production and compartmentalization of epidermal sphingolipids.

The differentiation of epidermal epithelial cells (keratinocytes) is characterized by a programmed series of profound biochemical and morphological transformations that ultimately produce the protective barrier necessary for terrestrial life. The most thoroughly studied events are the expression of keratins and other proteins integral to the structure of the outermost nonviable layer of the epidermis, the stratum corneum (reviewed in Refs. 1 and 2). The regulation of the complex changes in lipid synthesis and organization that accompany these structural protein alterations and ultimately result in the water impermeability of skin is less understood. As keratinocytes progress from the basal proliferative layer outward toward the stratum corneum, they become enriched in specific lipids and form lamellar granules, specialized organelles that contain stacks of membranous disk-like structures (3). Lamellar granules are particularly enriched in glucosylceramides (GlcCer),1 and also contain acid hydrolases capable of processing their lipid contents (4–6). Upon extrusion of the lamellar granule contents into the intercellular spaces at the junction of the viable and nonviable cell layers, GlcCer are converted to ceramides. The extracellular lipids, which are devoid of phospholipids and contain ceramides as a major fraction, organize into lamellar sheets (2). These sheets surround the nonviable cornocytes in the stratum corneum and are responsible for the barrier function of the epidermis (7).

Despite the critical roles that sphingolipid metabolism and trafficking play in epidermal physiology, studies of these processes in keratinocytes have been limited. The increase in ceramide content during differentiation and the de novo synthesis of ceramides and GlcCer have been demonstrated in epidermis (8) and in organotypic keratinocyte cultures (9–11). Cultured keratinocytes were reported to have a high level of serine palmitoyltransferase activity, which catalyzes the synthesis of the long-chain base precursor of sphingolipids (12). A crucial role for the acid glucosidase, glucosylceramidase (β-glucocerebrosidase), in converting GlcCer to extracellular ceramides after extrusion of lamellar granule contents has recently been delineated (13). The enzyme responsible for the formation of GlcCer has not been studied previously with regard to its role in epidermal sphingolipid metabolism. CerGlc transferase (UDPglucose-N-acylsphingosine d-glucosyltransferase, EC 2.4.1.80), also

1 The abbreviations used are: GlcCer, glucosylceramide(s); cDMEM, complete Dulbecco's modified Eagle's medium (Dulbecco's modified Eagle's medium/Ham's F-12 (3:1) with 10% fetal bovine serum, 10 μg/ml insulin, 0.4 μg/ml hydrocortisone, 5 units/ml penicillin, and 5 μg/ml streptomycin); KGM, keratinocyte growth medium; nKGM, KGM supplemented with 1.4 mM Ca2⁺; POMP, α-threo-1-phenyl-2-(palmitoylamino)-3-morpholino-1-propanol; NBD-ceramide, 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)caproylsphingosine; NBD-GlcCer, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)decanoylceramide; MOPS, 3-(N-morpholino)propanesulfonic acid; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; CerGlc transferase, ceramide glucosyltransferase.

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† To whom correspondence should be addressed: 270 ML, University of Iowa, Iowa City, IA 52242. Tel.: 319-335-8088; Fax: 319-351-1644.
tered glucosylceramide synthase, catalyzes the reaction of UDPGlc and ceramide to form UDP and GlcCer, which, in most cell types other than keratinocytes, serves mainly as a precursor for complex glycolipids or as a plasma membrane component. The enzyme has been partially purified from Golgi fractions from porcine submaxillary gland and rat brain, and its dependence on phospholipids, detergents, metals, and other effectors has been studied (14–16). Just recently, a procedure for the purification of the enzyme to a high specific activity from rat liver Golgi membranes was published (17), but molecular properties of the transferase protein have not yet been described. CerGlc transferase resides on the cytoplasmic face of membranes in the cis-Golgi, and possibly, in a pre-Golgi compartment (18–20). Studies with O-2-threo-1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol and structural analogs (reviewed in Ref. 21), demonstrated that these specific, reversible inhibitors of CerGlc transferase activity reduced glycosphingolipid concentrations in a variety of cultured cells. Effects of these inhibitors on specific cellular processes underscored the importance of the transferase in ganglioside metabolism, cell surface recognition and adhesion properties, and regulation of cell growth (21). A mouse melanoma cell line with deficient GlcCer transferase activity and no detectable glycolipids was reported to show altered morphology and a reduced growth rate as compared to the parental line (22).

Little is known about in vivo regulation of CerGlc transferase. The demand for GlcCer as a precursor of structural components of plasma membrane lipids and glycoconjugates probably varies in different cell types and in different cell cycle phases. The demand might be increased in cells that are actively synthesizing membranes, such as developing erythrocytes, but otherwise, the cellular need for GlcCer as a lipid component of plasma membranes is less evident. The low activity of the enzyme in normal, untransformed cells might be due to a low rate of membrane turnover or to the presence of inhibitors. The enzyme activity in the skin of 24-h-old rats was 60-70% of that of adult rat skin (18), which suggests that the rate of skin membrane turnover is lower than in other tissues. The transferase might be involved in the de novo synthesis of ceramides in regenerating skin (19). GlcCer is a known intermediate in the synthesis of gangliosides (20) and is a precursor for the synthesis of sulfated glycosphingolipids (21).

EXPERIMENTAL PROCEDURES

Materials—[3-3H]UDPGlc, 11.8 GBq/mmol, was purchased from Du Pont NEN, Boston, MA. (6→2→3)-D-glucoside (NBD-GlcCer) was from Sigma. PPM (p-aminoethylamine)-3-palmitoyl-1-propanol (PPMP). Finally, the activity of CerGlc transferase in keratinocytes was compared to that in a variety of other types of cultured cells, to help assess their relative potential for GlcCer production. A portion of these results were reported earlier in a preliminary form (23).

Cell Culture—Keratinocytes were obtained from neonatal foreskins by overnight trypsinization in 0.2% trypsin, 0.1% sucrose in phosphate-buffered saline at 4°C. Cells were plated in 60 mm plastic tissue culture dishes using 0.07 mM calcium keratinocyte growth medium (KGM) (Clonetics, San Diego, CA) containing 2% fetal bovine serum (Sigma) and grown at 37°C in a humidified incubator under a 5% CO₂, 95% air atmosphere. The medium was changed 24 h later to 0.07 mM calcium KGM without serum, and the cultures were then fed every other day. Cultures were harvested after 3–4 days with 0.07% trypsin/0.02% EDTA solution, washed twice with 20 mM HEPES, and stored at −80°C. After a 30-min incubation with 10 μg/ml streptomycin, 100 U/ml penicillin, and 5 μg/ml hydrocortisone, 5 units/ml penicillin, and 5 μg/ml streptomycin solution. The standard reaction mixture contained, at a final concentration, in a volume of 110 μl: 1 mg of ceramide-silica gel, 50 μM UDPGlc (7.4 KBq), 50 μM nKGM, pH 6.5, 5
confirmed not to interfere with assay results.

conducted with amounts of sample that were within the range of standard amounts of individual samples. The latter was routinely verified by assaying increase of nKGM cultures increased 3 times before reaching a maximum at ~5 days, whereas that of cDMEM cultures plateaued at ~7 days at a level that was 2–3 times higher than in nKGM cultures. Cultures maintained in low calcium KGM had the same DNA content as those switched to nKGM for the first week and declined to half those values during the second week (Fig. 1 legend). The protein content of nKGM cultures increased ~10-fold to a plateau after 1 week, whereas that of cDMEM cultures continued to increase to a level ~3–4 times that of nKGM cultures after 2 weeks. Results of most of our biochemical analyses are normalized with respect to DNA, rather than protein content, because of the huge increase in structural proteins that characterizes keratinocyte differentiation.

Morphological Properties of Differentiated Keratinocyte Cultures—A comparison of nKGM with cDMEM cultures by electron microscopy after 7–10 days (Fig. 2) showed that cDMEM cultures had more and thicker cell layers (Fig. 2, a and b) and also demonstrated keratohyalin granules, a marker of terminal epidermal differentiation. There were numerous lamellar granules in cDMEM cultures (Fig. 2c), but only rare lamellar granules in nKGM cultures. By day 10, a prominent cornified envelope and extrusion of lamellar granule contents into the intercellular spaces was noted only in cDMEM cultures (Fig. 2d). By morphologic criteria, cDMEM cultures clearly demonstrate a much higher level of differentiation than do nKGM cultures even though a well-developed stratum corneum does not form.

Keratinocyte Lipids—Results from experiments in which the lipid composition of cDMEM cultures was analyzed over a 10-day period are presented in Table I. A nearly 3-fold increase in total lipids over the period from day 3–10 was accompanied by a marked increase in the neutral sphingolipid fractions, primarily at the expense of diacylglycerolceramide lipids fractions, with little change in sphenoglycerolipid. The ceramide fraction increased 6– to 9-fold over the time course to a level that was nearly 7% of total lipids by day 10 in one experiment. GlcCer, which is precursors of the extracellular ceramides in the stratum corneum, were increased ~3–8 times and represented 2.8–4.4% of total lipids by day 10. Of particular interest was the time-dependent appearance of acyl-GlcCer and its metabolite, acylceramide, lipids unique to epithelium and markers of
terminal epidermal differentiation (32, 33).

Time-dependent increases in the neutral sphingolipid fractions were much less pronounced to absent in nKGM cultures, as is illustrated for the GlcCer content in Fig. 3. By day 7 after the medium switch, the total lipid content, normalized to cell DNA, of nKGM and cDMEM cultures, was increased approximately 1.5 times and 2 times, respectively, over cultures that were maintained in low calcium KGM. In contrast to the relatively small change in total lipid/DNA, the GlcCer/DNA content of cDMEM cultures increased 6-fold, whereas that of nKGM cultures showed only a slight increase over the low calcium KGM. The increased GlcCer content in the more highly differentiated, cDMEM cultures was evident during periods when activity is changing the most and at extremes from the mean.

Properties of Keratinocyte CerGlC Transferase Activity—The preparation of homogeneous samples from the more differentiated keratinocyte cultures posed a difficulty in view of the instability of CerGlC transferase activity with prolonged homogenization. A homogenization protocol was maximized for prevention of CerGlC transferase activity, normalized to DNA content, in nKGM cultures was not significantly elevated over the day 0 value, although in some individual experiments up to 2 times increases in activity were observed during the second week. By contrast, after a lag of ~3 days, transferase activity in cDMEM cultures began to increase and reached a maximum level at 8 days that was ~6 times the activity at day 0 in the combined data and ~10 times in some individual experiments. Variability in the data are evident during periods when activity is changing the most and at pH values from ~6.3–7, with a shoulder at ~60% maximum extending to pH values up to 7.7.

Dependence of GlcCer Transferase Activity on Culture Differentiation—Values for CerGlC transferase activity over time in culture are shown in Fig. 4. Data from several experiments with overlapping sampling times were combined. Over a 2-week period following the medium switch, the mean transferase activity, normalized to DNA content, in nKGM cultures was not significantly elevated over the day 0 value, although in some individual experiments up to 2 times increases in activity were observed during the second week. By contrast, after a lag of ~3 days, transferase activity in cDMEM cultures began to increase and reached a maximum level at 8 days that was ~6 times the activity at day 0 in the combined data and ~10 times in some individual experiments. Variability in the data are evident during periods when activity is changing the most and

Table I

| Lipid composition of human keratinocyte cultures over time after switching to cDMEM. Keratinocyte cultures grown to near-confluence in low calcium KGM were switched to cDMEM and harvested for lipid analysis after 3, 7, and 10 days. Results shown are the average values of data from two separate experiments; four to seven 100-mm cultures were combined for each time point. Numbers in parentheses indicate the deviation of the extremes from the mean. |
| Lipid species | Lipid content | 3 days | 7 days | 10 days |
|---------------|---------------|--------|--------|--------|
| SM | 13.7 (0.2) | 13.6 (0.5) | 14.0 (0.5) |
| PC | 41.9 (2.2) | 37.5 (3.5) | 34.8 (0.4) |
| PE | 27.5 (2.5) | 22.8 (2.8) | 18.8 (3.2) |
| GlcCer | 0.97 (0.62) | 3.7 (0.6) | 3.6 (0.8) |
| AGlcCer | ND | 0.36 (0.12) | 0.56 (0.03) |
| Other Cer | 0.73 (0.06) | 2.8 (0.4) | 5.3 (0.6) |
| ACer | ND | 0.12 (0.03) | 0.52 (0.27) |
| CHOL | 12.6 (1.6) | 15.1 (1.0) | 17.9 (0.5) |
| FFA | 1.00 (0.31) | 0.57 (0.09) | 0.85 (0.15) |
| TG | 1.35 (0.05) | 2.7 (0.9) | 2.4 (0.3) |
| CME | 0.33 (0.02) | 0.88 (0.13) | 1.5 (0.2) |
| Total lipid, µg/100-mm culture | 802 (33) | 1728 (11) | 2169 (140) |

a. SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GlcCer, glucosylceramide; AGlcCer, acylglucosylceramide; Cer, ceramide; ACer, acylceramide; CHOL, cholesterol; FFA, free fatty acids; TG, triglycerides; CME, cholesterol monooesters. b. ND, not detected.

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Fig. 2. Electron micrographs of keratinocyte cultures. Cultures were grown as described under “Experimental Procedures” and the legend to Fig. 1 and switched to nKGM for 7 days (a) or cDMEM for 7 days (b and c) or 10 days (d). A segment (1 cm²) of each 100-mm culture was excised and processed for electron microscopy as was described under “Experimental Procedures.” Final magnification was 15,000 (a), 15,000 (b), 90,000 (c), and 105,000 (d). a, arrows, keratohyalin granules; arrowheads, lamellar granules. b, arrows, extruded lamellar granules; arrowheads, lamellar granules showing internal membrane structure. c, arrowheads, lamellar granule contents; arrows, cornified envelope.

Fig. 3. Total lipid and GlcCer content of keratinocyte cultures at day 0 or day 7 after switching to nKGM or cDMEM. Cells from 4–7 cultures (100 mm) from each experimental group were pooled. Procedures for lipid extraction and analysis by thin layer chromatography are described under “Experimental Procedures.” Cell lipid content was normalized with respect to DNA, as determined in cultures grown under identical conditions. Results are expressed as a percentage of the day 0 value for total lipid or GlcCer. Data from 2 separate experiments were averaged to obtain the results shown.
Behavior of glucocerebrosidase activity during differentiation—Cell isolation, cell harvesting, and homogenate preparation were as for the results shown in Fig. 1 and as described under “Experimental Procedures.” Data from 5 experiments with overlapping sampling times were combined. Results shown are the means ± S.E. for data from 2–4 determinations per sample from 3–6 separate cultures harvested at the indicated time points. Glucosyltransferase activity is expressed as (pmol of product)(min)(mg of DNA) 

Km values were 8.0 and 7.2 μM, with respect to UDPGlc, and 5.9 and 5.2 μM, with ceramide, for activity from cultures harvested at 0 and 7 days after the switch to cDMEM, respectively. The apparent Vmax values were 24 and 85, with respect to UDPGlc, and 14 and 71, with ceramide, for the transferase in the undifferentiated versus differentiated cultures, respectively. The near identity of Km values with each substrate for enzyme in the two culture systems and the similar change in Vmax values with each substrate is compatible with the notion that the amount of transferase is increased during differentiation. A less likely possibility is that the change in Vmax values reflects a differentiation-induced modification of the transferase structure that equally affects the catalytic efficiency with respect to both substrates, without affecting the Km values. To further explore the mechanism of the increased transferase activity during differentiation, cultures were treated with cycloheximide 4 days after the switch to cDMEM. Following two 4-h applications of cycloheximide over the range of 3–100 μg/ml, CerGlc transferase activity was reduced 60–80% from the control activity. A fit of data points obtained at 4, 8, 12, and 24 h after addition of 30 μg/ml cycloheximide to the culture medium, to the equation for first order decay, revealed a half-life for the transferase activity of approximately 9 h. These results suggest that during culture differentiation, the transferase activity undergoes a relatively rapid turnover, and that the fractional labeling of sphingomyelin and intracellular ceramide was examined (Fig. 6). On day 7 after the switch to cDMEM, cultures were pretreated for 2 h with PPMP, pulsed with NBD-ceramide, and then chased for 30 and 90 min in the presence of PPMP. Cultures treated with the inhibitor had 5 and 9%, respectively, of the label in GlcCer, compared to 46 and 66% in the untreated controls. PPMP stimulated an increase in the fractional labeling of sphingomyelin and intracellular ceramide in this short-term pulse-chase experiment. These results are consistent with the premise that addition of PPMP to keratinocyte cultures inhibited transferase catalysis, thus blocking the formation of GlcCer.

Comparison of Keratinocyte CerGlCer Transferase Activity with That in Other Cell Types and in Foreskin Tissue—Analysis of CerGlc transferase activity in extracts of selected human cell culture and keratinocytes revealed low levels of activity, in the general range of those found for undifferentiated keratinocyte cultures (Table III). The samples selected included foreskin fibroblasts, monocyte-macrophage cell lines before and after differentiation with phorbol ester, and the Caco-2 intes-

![Fig. 4. Ceramide glucosyltransferase activity in lysates of keratinocyte cultures with time after switching to nKGM or cDMEM. Culture conditions and data acquisition were as described for Fig. 4.](image1)

![Fig. 5. β-Glucocerebrosidase activity in lysates of keratinocyte cultures with time after switching to nKGM or cDMEM. Culture conditions and data acquisition were as described for Fig. 4.](image2)
TABLE II
Effect of PPMP treatment of keratinocyte cultures on lipid composition
Human keratinocyte cultures were switched to cDMEM after reaching near-confluence in low calcium KGM. Cultures were treated with a final concentration of 10 μM PPMP in ethanol or 0.1% solvent control on days 4, 5, and 6, and harvested on day 7. Results shown are from a single experiment in which four 100-mm cultures from each group were combined for lipid analysis. Abbreviations are as in Table I.

| Lipid species | Control | PPMP | % total |
|--------------|---------|------|---------|
| SM           | 13.7    | 13.2 |         |
| PC           | 32.3    | 33.8 |         |
| PE           | 28.7    | 32.5 |         |
| GlcCer       | 5.1     | 0.78 |         |
| AGlCer       | 0.58    | ND*  |         |
| Other Cer    | 1.21    | 0.85 |         |
| ACer         | ND      | ND   |         |
| CHOL         | 14.0    | 13.4 |         |
| FFA          | 0.45    | 0.54 |         |
| TG           | 3.2     | 4.8  |         |
| CME          | 0.73    | 0.15 |         |
| Total lipid, μg/100 mm culture | 2479 | 2335 | |

* ND, not detected.

Fig. 6. Effect of PPMP on NBD-sphingolipid synthesis from NBD-ceramide. Cultures were switched from low calcium KGM to cDMEM on day 0. On day 7, cultures were preincubated for 2 h with either 10 μM PPMP or 0.1% ethanol control, then labeled with NBD-ceramide for 1 h at 4 °C, rinsed, and placed at 37 °C for 30 or 90 min, with PPMP in the chase medium. Cultures were harvested and the lipids were extracted and analyzed. The results from determinations on duplicate cultures were averaged and are expressed as the percent of total NBD-labeled lipids. Relative values for total intracellular NBD-labeled lipids, expressed as a percentage of the control at 0 min of chase, were 100, 90, and 83 for controls, and 105, 93, and 81 for PPMP-treated cultures, after 0, 30, and 90 min, respectively. SM, sphingomyelin; GlcCer, glucosylceramide; Cer, ceramide.

dinal epidermal cell line. By contrast, the cultures had varying levels of glucocerebrosidase activity, with fibroblasts expressing 20 times that of undifferentiated keratinocyte cultures and ~5 times that of 10-day nKGM and cDMEM cultures. Although differentiation of monocyte-macrophage cultures by treatment with phorbol ester did not significantly affect transferase activity, glucocerebrosidase activity was stimulated, as has been shown for other lysosomal enzymes in U937 cultures (34). In contrast to the results in the more highly differentiated keratinocyte cultures, relatively low levels of CerGlC transferase activity were found in homogenates of intact foreskin tissue, dermis, and trypsin-isolated epidermal cells.

**DISCUSSION**

Numerous recent studies of the cell biology and biochemistry of sphingolipids have demonstrated the crucial involvement of ceramides and other sphingosine derivatives in the regulation of diverse cellular processes (reviewed in Refs. 35 and 36). In most cell types, the glucosylation of ceramide serves primarily to provide a precursor for complex glycolipid synthesis (37). In epidermis, collective evidence from lipid analyses, electron microscopy, and enzymology has indicated a quantitatively important role for GlcCer as precursors of ceramides, the major component of the lipids responsible for the cutaneous permeability barrier (2, 7). The mechanisms involved in epidermal lamellar granule formation and enrichment with respect to GlcCer, and the subsequent extrusion of their contents into the extracellular space between the viable layers of the epidermis and the nonviable stratum corneum are not understood. The crucial role for GlcCer in the process suggests that its synthesis may be under unique regulatory control in differentiating keratinocytes.

Results from the present study directly demonstrate CerGlC transferase activity for the first time in keratinocytes and show that the activity is induced during keratinocyte differentiation. Analyses of steady-state kinetic parameters and intracellular turnover of the transferase activity suggest that the mechanism for enhanced expression of the activity during differentiation involves synthesis of new transferase protein. To our knowledge, regulation of GlcCer transferase activity has not been examined previously with respect to cell differentiation in any epidermal culture model, although induction of the enzyme has been demonstrated in a nerve regeneration model (38). Our results indicate that CerGlC transferase induction correlates with keratinocyte differentiation as shown by the differential expression of the enzyme in two culture systems. KGM cultures have been studied extensively and previously shown to support specific differentiation-related events (39, 40). DMEM-based media have been used mainly in conjunction with more highly differentiated, organotypic cultures (32, 41, 42). We have shown that cDMEM cultures grown on plastic are more highly differentiated than nKGM cultures and exhibit much higher transferase activity.

Changes in transferase activity paralleled those in GlcCer content. Thus, the more highly differentiated cultures were more enriched with respect to GlcCer, and the time course of the GlcCer increase in cDMEM cultures, beginning at ~3 days

TABLE III
Glucosyltransferase and β-glucocerebrosidase activity of selected human cell cultures and foreskin tissue

| Cell line | Glucosyltransferase | β-glucocerebrosidase |
|-----------|---------------------|----------------------|
| Keratinocyte cultures | | |
| KGM, low calcium | 28 ± 16 | 25 ± 6 |
| KGM, normal calcium, 10 days | 23 ± 4 | 106 ± 31 |
| cDMEM, 10 days | 154 ± 48 | 161 ± 22 |
| Cell lines | Glucosyltransferase | β-glucocerebrosidase |
| Foreskin fibroblasts | | |
| Caco-2 intestinal epithelial | 51 ± 1.6 | 566 ± 32 |
| THP-1 monocyte-macrophage | 6.8 ± 0.1 | 25 ± 2 |
| THP-1 + phorbol ester | 4.1 ± 0.7 | 24 ± 2 |
| U937 monocyte-macrophage | 1.7 ± 0.4 | 73 ± 6 |
| U937 + phorbol ester | 1.5 ± 0.4 | 9 ± 3 |

**Fig. 6.** Effect of PPMP on NBD-sphingolipid synthesis from NBD-ceramide. Cultures were switched from low calcium KGM to cDMEM on day 0. On day 7, cultures were preincubated for 2 h with either 10 μM PPMP or 0.1% ethanol control, then labeled with NBD-ceramide for 1 h at 4 °C, rinsed, and placed at 37 °C for 30 or 90 min, with PPMP in the chase medium. Cultures were harvested and the lipids were extracted and analyzed. The results from determinations on duplicate cultures were averaged and are expressed as the percent of total NBD-labeled lipids. Relative values for total intracellular NBD-labeled lipids, expressed as a percentage of the control at 0 min of chase, were 100, 90, and 83 for controls, and 105, 93, and 81 for PPMP-treated cultures, after 0, 30, and 90 min, respectively. SM, sphingomyelin; GlcCer, glucosylceramide; Cer, ceramide.
and plateauing after ~7 days (Table I), was roughly comparable to the increase in transferase activity (Fig. 4). Ceramides also accumulated in culture during this period and continued to increase after 7 days. This is consistent with the known precursor-product relationship of GlcCer and ceramides, in which precursor GlcCer are packaged into lamellar granules to form a “steady-state pool” from which ceramide product is formed as the lamellar granule contents are extruded into a cell-associated extracellular compartment. The electron microscopic images shown in Fig. 2 illustrate this unique pathway, with lamellar granules becoming most prominent around 7 days and extruding their contents in the 7–10-day time period.

Correlation between culture GlcCer content and transferase activity was also apparent in experiments with PPMP. PPMP inhibited keratinocyte CerGlc transferase activity in keratinocyte lysates with roughly the potency reported in other cell types. When applied to keratinocyte cultures, PPMP blocked the synthesis of NBD-GlcCer from exogenously added NBD-ceramide in short-term experiments and prevented an increase in GlcCer mass over a period of several days. These effects of the inhibitor are consistent with many of the findings for PPMP and analogs in other cell systems (21).

In the short-term study, blocking the conversion of NBD-ceramide pulse label to NBD-glucosylceramide would be expected to result in its diversion to sphingomyelin synthesis (the major alternative pathway of ceramide metabolism) as well as a delayed decay of NBD-ceramide during the 90-min span of the chase period, results which were observed (Fig. 6). After long-term PPMP inhibition, in addition to a profound decrease in GlcCer content, there was also a decrease in culture ceramide content (Table II). Remembering that, in epidermis, the majority of measured ceramide is extracellular and is a product of GlcCer hydrolysis, long-term PPMP inhibition of GlcCer synthesis would be expected to ultimately result in a decrease in its extracellular ceramide product. The lack of change in sphingomyelin mass during long-term inhibition may reflect the fact that cellular sphingomyelin levels are tightly controlled (as are levels of free ceramide); homeostatic mechanisms likely operate to restore normal levels during long-term experiments. Although PPMP experiments need to be interpreted carefully in view of certain nonspecific effects that have been demonstrated for this class of inhibitors (43), taken together, our results indicate that regulation of CerGlc transferase activity is an important determinant of the GlcCer content of keratinocytes and, thus, would be expected to affect production of ceramides, as well as the barrier function of the epidermis.

Glucocerebrosidase activity, which is required for conversion of GlcCer to ceramide in the stratum corneum, has been studied previously with relation to epidermal differentiation. Enzyme activity was increased in the outermost layers of skin (44, 45) and has also been demonstrated in lamellar granule preparations (4). In our studies, induction of GlcCer transferase activity was shown to be more strongly correlated with the morphological and lipid properties that characterize culture differentiation than was glucocerebrosidase activity. Glucocerebrosidase activity increased earlier than transferase activity and reached nearly the same maximum level in cDMEM and nKGM cultures, indicating that the requirements for stimulation of the two enzymes are different.

The expression of other markers of epidermal differentiation have been studied, including the structural proteins, keratin (46), loricrin (47), involucrin (48), and filaggrin (49); transglutaminase (50), believed to mediate cross-linking of cornified envelope proteins; and cholesterol sulfotransferase (50, 51), required for the formation of cholesterol sulfate, a barrier lipid whose hydrolysis is necessary for normal desquamation (2). A distinctive time course for induction of the individual markers (1, 46, 47, and references therein) indicates a complex programmed sequence of events in the differentiation process which remains poorly understood. Insight may be provided in the near future from the results of numerous investigations centering on the profound effects of retinoids on epidermal differentiation (52, 53).

Low levels of transferase activity were found in other cultured cell types as compared with that in differentiated keratinocytes, suggesting that epidermal cells have the capacity for high activity when there is a demand for GlcCer. Apical plasma membranes of intestinal brush border cells have been demonstrated to be enriched with unusually high levels of glycosphingolipids, which are putatively required for membrane stabilization and protection of the luminal surface against digestion by phospholipase A2 (reviewed in Ref. 54). This observation suggested that intestinal cells, as well as Caco-2 intestinal epithelial cultures, which also exhibit a polarized membrane glycolipid distribution (55), might express a high level of CerGlc transferase activity, similar to that of differentiated keratinocytes. This was not supported by our results for Caco-2 cultures (Table III), although sufficient data on lipid quantitation in this system are not available to enable correlation of transferase activity with glycosphingolipid content. Interestingly, the glucocerebrosidase activity of Caco-2 cells is much lower than that of fibroblasts and the other differentiated cells that were examined, suggesting that the cultured intestinal cells may regulate their glycolipid content, in part, by minimizing GlcCer breakdown.

The low level of transferase activity in homogenates of intact foreskin, dermis, and trypsin-isolated epidermal cells, as compared to that in differentiated keratinocyte cultures, may reflect some instability of the enzyme with respect to the harsh conditions necessary for disruption of the whole tissue. On the other hand, cDMEM cultures may accentuate a period in the differentiation process when keratinocytes are actively growing and producing the lipids needed to establish barrier function. This most likely represents the situation in regenerating epidermis, such as following barrier disruption or during wound healing, rather than the steady state condition of intact epidermis. Disruption of the epidermal permeability barrier with organic solvent in an in vivo experimental murine model has been shown to transiently stimulate the synthesis of DNA and of several lipids important in barrier formation (56); values returned to baseline once the barrier was re-established. A recent study of murine fetal differentiation demonstrated a transient increase in cholesterol sulfotransferase followed by a return to baseline shortly after birth (50).

A number of diseases characterized by abnormalities of epidermal lipids are recognized (57, 58). The only disorder in this group with a demonstrated defect in the ceramide pathway is the severe, neuropathic type of Gaucher disease, resulting from deficient glucocerebrosidase activity (13, 59). Biochemical abnormalities in CerGlc transferase activity or in the other steps involved in the production and processing of stratum corneum ceramides, whether primary or secondary, would be expected to result in aberrant epidermal structure and function. Such abnormalities could possibly have a role in, for example, harlequin ichthyosis, in which lamellar granules are known to be defective (60), or multisystem triglyceride storage disease (61), inherited disorders affecting skin that have not yet been explained at the biochemical level. Epidermal ceramide abnormalities have been reported in such diverse conditions as psoriasis (62), atopic dermatitis (63, 64), and aging (64), but the biochemical basis for these abnormalities remains
unknown.

The current work has established conditions under which CERGlc transerase activity is expressed at a high level by cultured human keratinocytes, and our results indicate an important role for this enzyme during epidermal differentiation. The use of this system will facilitate studies of the regulation of CERGlc transerase activity and its impact on keratinocyte function. Study of the unique aspects of the ceramide pathway in keratinocytes should further understanding of the biochemical and cell biology of sphingolipids in general.

Note Added in Proof—Recently, Ichikawa et al. (65) cloned a cDNA encoding a 394 amino acid protein corresponding to human ceramide glucosyltransferase. The mRNA was widely expressed in human tissues, although the distribution in epidermis was not reported.
