

Sphingolipid metabolism during epidermal barrier development in mice

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Abstract In rodents, a competent skin barrier to water loss is formed within 2 or 3 days prior to birth. Acquisition of barrier function during rat gestation correlates with the formation of a stratum corneum enriched in ceramides, cholesterol, and fatty acids (Aszterbaum, M., G. K. Menon, K. R. Feingold, and M. L. Williams. 1992. Ontogeny of the epidermal barrier to water loss in the rat: correlation of function with stratum corneum structure and lipid content. *Pediatr. Res.* 31: 308–317). We analyzed the formation and epidermal localization of glucosylceramides during embryonic skin barrier development in Balb/c mice. Using immunohistochemistry, epidermal glucosylceramides were hardly detectable 3 days prior to birth. After further 24 h of gestation the level of glucosylceramides was maximal and decreased with increasing gestational age. In parallel, glucosylceramides were targeted to the apical side of the outermost granular keratinocyte layer. A spectrum of five distinct epidermal ceramides was present 2 days prior to birth. With ongoing gestation the composition of the ceramide fraction changed markedly. Most importantly, the level of ω -hydroxylated acylceramides decreased paralleled by the formation of the corneocyte lipid envelope. This structure consists of ω -hydroxylated ceramides and fatty acids bound to surface proteins of the corneocytes. The covalent attachment of ceramides or glucosylceramides correlated with the maturation of the stratum corneum and might contribute to its chemical and enzymatic resistance.—Doering, T., H. Brade, and K. Sandhoff. Sphingolipid metabolism during epidermal barrier development in mice. *J. Lipid Res.* 2002. 43: 1727–1733.

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Mammalian skin possesses a competent barrier to water loss at birth that is localized within the stratum corneum (SC). This outermost epidermal layer is composed of terminally differentiated keratinocytes (corneocytes) surrounded by a mixture of barrier lipids (i.e., ceramides,

cholesterol, and fatty acids) that are arranged in continuous multilamellar membranes (1). A low water permeability is observed for these extracellular membranes because the lipids are organized in multilamellar structures that are stabilized by long chain ceramides.

Probarrier lipids such as glucosylceramides (GlcCers) are delivered to the SC by secretion of lamellar bodies that contain lamellar stacks of the probarrier lipids colocalized with hydrolytic enzymes such as β -glucocerebrosidase (GlcCerase). Using transgenic mice it has been shown that this enzyme is required for skin barrier formation (2, 3). Postsecretory processing of GlcCers to a family of ceramides (Cers) requires GlcCerase to be assisted by a sphingolipid activator protein (4). Five different Cers and the corresponding set of GlcCers have been identified in mouse epidermis (4, 5). One of these Cers consists of ω -hydroxy acids linked by amide bonds to sphingosine. The predominant chain lengths of these ω -hydroxy fatty acids are 32-carbon saturated as well as 32- and 34-carbon monounsaturated species that are acylated with linoleic acid via the ω -OH position. This unique epidermal lipid has been shown to be important for both the lamellar organization of the barrier lipids (6, 7) and for the formation of a lipid monolayer covalently bound to surface proteins of the corneocytes (3, 8, 9).

The surface of the corneocytes is constituted by the cornified cell envelope (CE) formed by enzymatic crosslinking of various proteins such as loricrin and involucrin (10, 11). Very long-chain ω -hydroxylated Cers and fatty acids are ester-linked to glutamate side chains of the outermost CE proteins (12). This cornified lipid envelope is considered to be essential for the skin barrier because it might

Abbreviations: A, α -hydroxy fatty acid; CE, cornified cell envelope; Cer, ceramide; E, esterified; EGA, estimated gestational age; GlcCer, glucosylceramide; GlcCerase, β -glucocerebrosidase; MALDI, matrix assisted laser desorption ionisation; N, nonhydroxy fatty acid; O, ω -hydroxy fatty acid; P, phytosphingosine; S, sphingosine; SC, stratum corneum; SL, stratum lucidum.

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be required to maintain a weak adhesion between corneocytes (13, 14) or even to provide a template for the proper organization of the extracellular lipid bilayers.

The SC of murine and rat epidermis is formed 2 or 3 days prior to birth paralleled by the loss of the periderm. The periderm seals the developing epidermis from the amniotic fluid unless the SC is formed (15). A functional epidermal barrier to water loss is established within this final period of gestation (15–17). The acquisition of a competent epidermal barrier proceeds in a patterned manner in mice as demonstrated by analysis of dye penetration kinetics (17). Barrier competence is first observed at specific dorsal initiation sites after 16 days of gestation, then barrier function spreads across the epidermis although a regular SC is still lacking. Hardman et al. have shown that the permeability change is associated with early stages of SC formation (17). The impermeable SC-precursor is characterized by prominent cornified envelopes, dispersed keratohyalin granules, and extruded lamellar body contents that initially remain disorganized in the extracellular domains of the developing SC. The barrier function is further improved between 16 and 17 days of gestation and then remains constant until birth that occurs after 19 days of gestation (17). The sequence of lipid metabolic events required for the targeting of barrier lipids to the interstices of the SC and for the formation of the corneocyte lipid envelope have not been analyzed during the process of embryonic skin barrier development.

The metabolism of epidermal GlcCers has been shown to be regulated during rat gestation (18). Using a specific inhibitor for GlcCerases, these authors have demonstrated that the processing of GlcCer to Cer is required for the formation of a competent epidermal barrier to water loss. Therefore, we analyzed the formation and epidermal localization of GlcCers and their transformation to both free and protein-bound Cers during late mouse gestation.

METHODS

Embryos

Balb/c mice were time-mated within a 6 h mating window. The mid-point of the mating window designated gestational age zero and was used to calculate the estimated gestational age (EGA). Spontaneous delivery occurred at day 19.5 EGA. The skin was removed from decapitated mice. The epidermis was separated from dermis after floating the skin on Dispase (Roche, grade II) diluted 1:1 in Hank's buffer at 4°C overnight.

Lipid analysis

After washing in PBS, the epidermis was homogenized, lyophilized, and weighed. Unbound lipids were extracted and separated as described previously (4). For separation of polar lipids (i.e., GlcCers, sphingomyelin and glycerophospholipids), the thin-layer silica gel 60 plates (Merck Darmstadt, Germany) were developed with chloroform-methanol-water (70:30:5, vol/vol/vol). Cers were resolved twice using chloroform-methanol-acetic acid (190:9:1, vol/vol/vol) as developing solvent. Bound lipids were recovered and analyzed as described previously (4). Briefly, covalently bound lipids were released by incubation of pre-extracted samples with 1 M KOH in 95% methanol for 2 h at

60°C. The released lipids were recovered by extraction into chloroform and separated by TLC using twice chloroform-methanol-acetic acid (190:9:1, vol/vol/vol) as developing solvent. To ascertain that the recovered lipids indeed were covalently attached, each sample was checked to insure that there was no residual extractable lipids before undergoing base hydrolysis. For quantitative analytical TLC determination, increasing amounts of standard lipids (N-stearoyl-sphingosine (kind gift of Beiersdorf AG, Hamburg, Germany), GlcCer (purified from Gaucher spleen in our laboratory), and palmitic acid (Fluka Buchs, Switzerland) were applied. After development, plates were air-dried, sprayed with 8% (wt/vol) H₃PO₄ containing 10% (w/vol) CuSO₄ charred at 180°C for 10 min and lipids were quantified by photodensitometry (Shimadzu, Kyoto, Japan).

Immunohistochemistry

Skin samples were taken at autopsy from the back and cryo-fixed. Semithin sections (5 μm) were placed on glass coverslips, treated with normal goat serum (1% in PBS) for 1 h at room temperature, washed twice, and incubated with rabbit anti-GlcCer-antiserum (1:1,000, Glycobiotech GmbH Kueckels, Germany) for 1 h at room temperature. The samples were washed three times and incubated at room temperature with an Alexa 594 conjugated-goat anti-rabbit IgG secondary antibody (1:800). The samples were washed five times, air dried, and fixed with 25% (w/v) MOVIOL™ 4–88 (Calbiochem, San Diego) in PBS and photographed with an Axiovert 35 fluorescence microscope (Zeiss, Jena, Germany).

Statistical analysis

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

RESULTS

Development of epidermal GlcCer levels during mouse gestation

To characterize the sequence of lipid metabolic events during barrier formation we first analyzed the alterations in the profiles of lipids that are not covalently bound to the cornified envelope. Epidermal GlcCers were separated into three distinct fractions using TLC (Fig. 1A). The molecular structure of each GlcCer has previously been confirmed by matrix assisted laser desorption ionisation (MALDI) mass spectroscopy (4). In this work, structural data are presented in accordance with the epidermal Cer terminology proposed by Motta et al. (19). Briefly, the Cer structures are denoted by the composition of the sphingoid base [either sphingosine (S) or phytosphingosine (P)] and the N-acyl fatty acid by the presence of an α-hydroxy-group (A), an ω-hydroxy-group (O), or no hydroxy-group (N), and whether the omega position is further acylated [i.e., esterified (E)].

The epidermal level of GlcCer decreased significantly during development of the SC (Fig. 1A, B). The total GlcCer content was maximal at 17.5 days EGA with 6.6 ± 0.8 μg/mg dry epidermis. At 18.5 and 19.5 days EGA the GlcCer levels were significantly decreased by 39% to 4.0 ± 0.4 and by 61% to 2.6 ± 0.5 μg/mg dry epidermis, respectively. These alterations were seen for each distinct GlcCer fraction [i.e., GlcCer(EOS), GlcCer(C16-AS), and GlcCer a/b: complex mixture of GlcCer(NS), (NP), and (C24,26-AS)].

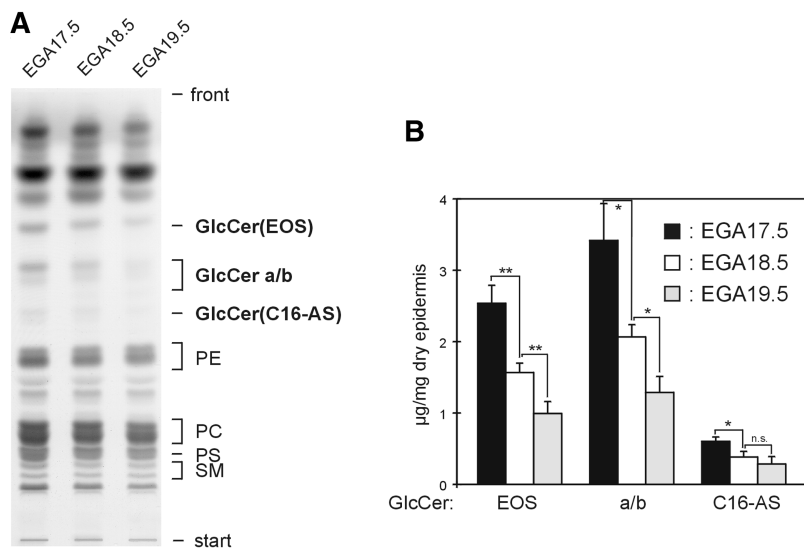


Fig. 1. Glucosylceramide (GlcCer) levels during epidermal organogenesis. The epidermis of murine Balb/c embryos of various gestational ages was prepared at autopsy. Spontaneous delivery occurred at 19.5 days estimated gestational age (EGA). Lipids were extracted and separated using TLC. A: Shows a representative TLC analysis of lipids extracted from 1.5 mg dry epidermis. Lipids were quantified by densitometric analysis (B). Data are presented as the mean \pm S.D. ($n = 4$). The lipid contents differ significantly at ** $P \leq 0.001$ and * $P \leq 0.005$. GlcCer a/b is a complex mixture of GlcCer(NS), (NP) and (C24,26-AS). n.s., not significant; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine, SM, sphingomyelin.

The epidermal level of sphingomyelin was decreased between 17.5 and 18.5 days EGA by $29.0 \pm 3.5\%$ and between 17.5 and 19.5 days EGA by $43.5 \pm 9.2\%$ (data not shown). This was also evident for the glycerophospholipids. The levels of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine decreased between 17.5 and 18.5 days EGA by $22.1 \pm 3.4\%$ and between 17.5 and 19.5 days EGA by $38.2 \pm 5.5\%$ (data not shown).

The localization of epidermal GlcCers during SC formation was studied using immunohistochemistry. The anti-GlcCer rabbit anti-serum has been shown to react with several epidermal GlcCer fractions [i.e., GlcCer(NS), (NP), (OS), (OH)] without any crossreactivity with either free Cer or galactosylceramide (20, 21). At 16.5 days EGA GlcCers were hardly detectable in murine skin (**Fig. 2A**). A very weak staining was restricted to a putative periderm layer that was not present after 16.5 days EGA. It was not possible to demonstrate this by quantitative lipid analysis because the very thin and frag-

ile epidermis of pups at 16.5 days EGA could not be isolated from dermis. After further 24 h of embryonic development, epidermal GlcCers were clearly detectable at 17.5 days EGA with a punctuate pattern accumulating at the apical side of a SC precursor that was composed of one or two distinct annucleated cell layer(s) (**Fig. 2B**). At 18.5 days EGA, a multilayered SC was seen and GlcCer was predominantly found within the stratum lucidum (SL) forming the interface between stratum granulosum and developing SC (**Fig. 2C**). Analysis of samples incubated without the primary anti-GlcCer anti-serum indicated that the staining of the SC but not of the SC precursor is non-specific. The epidermis of newborn mice (i.e., pups of 19.5 days EGA) showed GlcCer in a punctuate distribution predominantly at the apical side of the stratum granulosum (**Fig. 2D**). In accordance with quantitative lipid analysis (**Fig. 1B**) the relative amount of GlcCer appeared to be reduced when compared with the immunostaining intensity one day prior to birth.

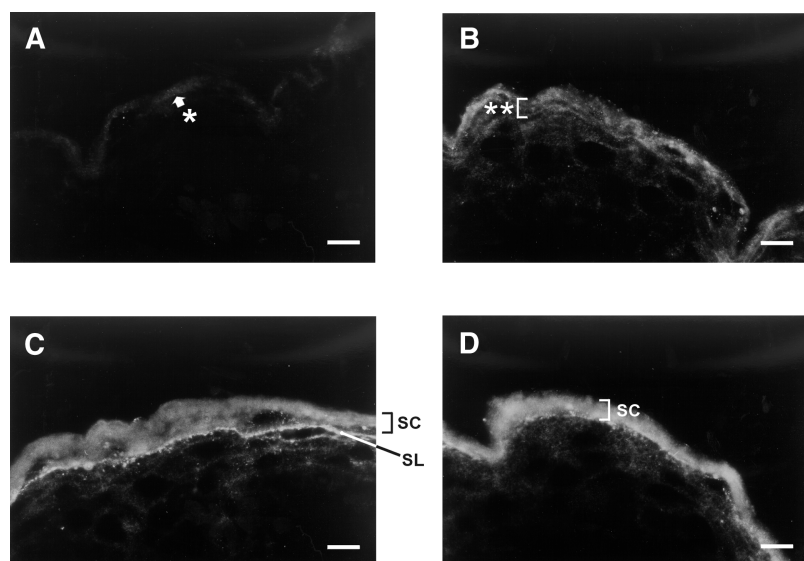


Fig. 2. Localization of epidermal GlcCer during gestational stratum corneum (SC) formation. Full thickness skin was prepared from the back of Balb/c murine embryos and kryofixed. Thin sections were incubated with rabbit anti-GlcCer and Alexa 594 conjugated-goat anti-rabbit IgG. Figures were photographed and printed under identical conditions. A: 16.5 days EGA; B: 17.5 days EGA; C: 18.5 days EGA; D: 19.5 days EGA (term pups). Note the increase of GlcCer between 16.5 and 17.5 days EGA and the redistribution of GlcCer to the apical side of the stratum granulosum at 18.5 days EGA (i.e., one day prior to birth). * Putative periderm layer; ** SC-precursor; SC, stratum corneum; SL, stratum lucidum; Scale bars, 10 μ M.

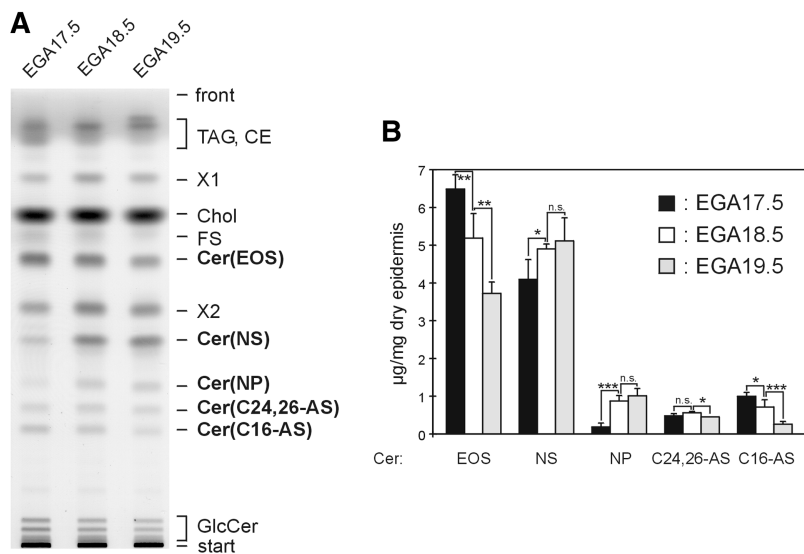


Fig. 3. Development of epidermal ceramide (Cer) profiles with increasing gestational age. Epidermal lipids were extracted and separated using TLC. Panel A shows a representative TLC analysis of lipids extracted from 1.5 mg dry epidermis. Lipids were quantified by densitometric analysis (B). Data are presented as the mean \pm S.D. ($n = 4$). The lipid contents differ significantly at *** $P \leq 0.005$; ** $P \leq 0.01$; * $P \leq 0.05$. n.s., not significant; X1 and X2, not identified.

Alterations in Cer profiles during embryonic SC formation

Epidermal Cers were separated into five distinct fractions using TLC (Fig. 3A). The molecular structure of each Cer has previously been confirmed by MALDI mass spectroscopy (4). The epidermal Cer profile changed markedly during the final period of mouse gestation. The levels of Cer(NS) and Cer(NP) increased between 17.5 and 19.5 days EGA by 25% and 440%, respectively (Fig. 3B), paralleled by a decrease of the corresponding GlcCer a/b fraction (Fig. 1B). In contrast, the levels of α -hydroxylated Cer(C24,26-AS) or Cer(16-AS) either remained constant or decreased by 74%, respectively. Interestingly, although GlcCer(EOS) was found to decrease during the 2-day period prior to birth this was also true for Cer(EOS), which decreased by 43% between 17.5 and 19.5 days EGA (Fig. 3B). This finding indicates that the recruitment of either GlcCer(EOS) or Cer(EOS) for the formation of the

corneocyte lipid envelope occurs during this period of epidermal barrier formation.

Formation of the corneocyte lipid envelope during mouse gestation

To analyze the development of the corneocyte lipid envelope, the extractable lipids were removed from the epidermis and the samples were subjected to alkaline hydrolysis and re-extracted. Lipids recovered by this procedure were separated by TLC into three distinct fractions (Fig. 4A), which have previously been identified as *i*) non-hydroxylated fatty acids, *ii*) Cer(OS), and *iii*) ω -hydroxylated fatty acids with a predominating chain length of 32-carbon and 34-carbon that are also found to predominate in Cer(OS) (4). Between 17.5 and 19.5 days EGA the levels of Cer(OS) and ω -OH-fatty acids recovered from the CE by alkaline hydrolysis increased dramatically by 340% and 540%, respectively (Fig. 4B). The increase of these two

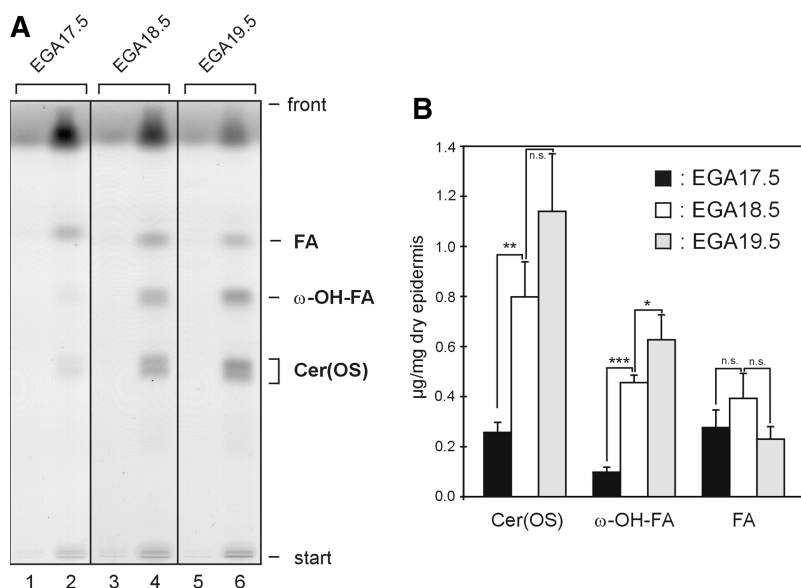


Fig. 4. Biogenesis of the corneocyte lipid envelope during embryonic development. Ester linked epidermal lipids were released by alkaline hydrolysis and analyzed by TLC. Panel A: Representative TLC analysis of lipids recovered from mice of different gestational ages (lanes 2, 4, 6). Note that the samples were devoid of unbound lipids before undergoing base hydrolysis (lanes 1, 3 and 5). B: Individual lipid levels quantified by densitometric analysis. Data are presented as the mean \pm S.D. ($n = 4$). The lipid contents differ significantly at *** $P \leq 0.001$; ** $P \leq 0.005$; * $P \leq 0.05$. n.s., not significant. FA, fatty acids.

constituents of the corneocyte lipid envelope was most prominent between 17.5 and 18.5 days EGA. In contrast, the levels of non-hydroxylated fatty acids did not change significantly during the two-day period prior to birth (Fig. 4B). Because it was not possible to isolate the epidermis of pups at 16.5 days EGA, full thickness skin was processed for analysis of protein-bound lipids. Neither Cer(OS) nor ω -OH-fatty acids could be detected after alkaline hydrolysis of skin prepared from pups delivered at 16.5 days EGA. Only trace amounts of protein-bound GlcCer were detected in the epidermis of term pups (19.5 days EGA) and pups delivered at either 17.5 or 18.5 days EGA.

DISCUSSION

The SC of rodent epidermis is formed 2 or 3 days prior to birth immediately after loss of the periderm. Within this final period of gestation, a functional barrier is established as measured by transepidermal water loss and dye penetration kinetics (16, 17). In this study, we characterized the development of epidermal lipid profiles during embryonic SC formation underlying the acquisition of a competent barrier to water loss. It has been shown that the processing of epidermal GlcCer to Cer is a prerequisite for barrier formation (2–4). In these studies, transgenic mice were used that were deficient in either GlcCerase or sphingolipid activator proteins required for enzymatic deglycosylation of GlcCer. Here, we demonstrate that this metabolic event correlates with the development of the SC during normal mouse gestation.

Three days prior to birth, GlcCer was hardly detectable in murine skin using immunohistochemistry. After further 24 h of gestation the GlcCer level peaked indicating that the synthesis of GlcCer required for barrier competence at birth is sharply initiated. The signal triggering the onset of GlcCer biosynthesis in fetal epidermis remains to be determined. At 17.5 days EGA GlcCer was localized predominantly in vesicular structures. These putative epidermal lamellar bodies were concentrated in a SC precursor composed of one or two annucleated cell layer(s). The GlcCer level decreased with ongoing gestation from 17.5 days EGA to birth. During this period GlcCer accumulated at the interface between the stratum granulosum and the developing SC. The immunohistochemical localization of GlcCer at 18.5 days EGA suggests that the bulk hydrolysis of GlcCer to Cer occurs within the extracellular domains at the apical side of the stratum granulosum. The decrease of GlcCer levels between 17.5 and 19.5 days EGA is most likely due to increased enzymatic hydrolysis of GlcCer to Cer together with decreased de novo synthesis. It has been shown that Cer synthesis decreases during late rat gestation reaching a minimum when barrier competence is fully established at birth (22). Moreover, GlcCerase mRNA and protein levels increase during rat barrier ontogenesis (18).

The epidermal Cer profile changed during late mouse gestation. In this work, we focused on alterations in lipid profiles rather than on the increase of mass levels of barrier

lipids per surface area that is self-evident for the developing SC. This has previously been reported by Aszterbaum et al. (16), who analyzed the SC lipids of fetal rats of various EGA. Most interestingly, we found a decrease of Cer(EOS) among the total Cer during late mouse gestation. Up to this point, the free lipids were analyzed that were isolated by solvent extraction. Corneocytes are known to be coated by a monolayer of Cer(OS) and ω -hydroxylated fatty acids that are covalently bound (i.e., ester-linked) to CE proteins such as involucrin (8, 12). These lipids are ester-linked to glutamate side chains of the proteins by their ω -hydroxyl group (23). This corneocyte lipid envelope is thought to be essential for the interaction of highly cross-linked proteins of the CE with the extracellular lipid matrix (13). The formation of the corneocyte lipid envelope requires GlcCer(EOS), which is transacylated either prior or after deglycosylation to CE proteins (3, 4). Most interestingly, not only the epidermal level of GlcCer(EOS) but also of corresponding Cer(EOS) decreased during gestational barrier formation indicating that a pool of both GlcCer(EOS) and Cer(EOS) is recruited for the formation of the corneocyte lipid envelope. At least the recruitment of GlcCer(EOS) is strongly supported by the accumulation of protein-bound GlcCer in GlcCerase knock in mice (3). Only traces of protein-bound GlcCer were detected not only in term pups (19.5 days EGA) but also in pups delivered at either 17.5 or 18.5 days EGA. This finding indicates that GlcCer is rapidly deglycosylated after attachment to CE proteins. However, since epidermal GlcCerase expression is upregulated during late rat gestation (18), a pool of protein-bound Cer was expected to remain glycosylated at 17.5 days of mouse gestation. On the one hand, the expression of GlcCerase at 17.5 days EGA might be sufficient for rapid and complete deglycosylation of protein-bound GlcCer. On the other hand, it might be predominantly the free Cer(EOS), which is recruited for the formation of the corneocyte lipid envelope when sufficient GlcCerase activity is present.

The corneocyte lipid envelope is formed within the final 2 day-period prior to birth. The levels of bound Cer(OS) and ω -OH-fatty acids are very low at 17.5 days EGA and increase dramatically within the following 48 h of gestation until birth. The bound ω -OH-fatty acids derive most probably from Cer(OS) by cleavage from the sphingoid base mediated by acidic ceramidase acting on either the bound or the free Cer(OS). In contrast, the levels of non-hydroxylated fatty acids released by base hydrolysis remained constant during SC formation. These fatty acids were most likely recovered from non-CE structures such as GPI anchored proteins.

It is widely recognized that the CE in conjunction with the attached corneocyte lipid envelope is of critical importance for the skin barrier. Hardman et al. reported that the murine skin possesses a partial barrier status at 16.5 days EGA and complete barrier competence at 17.5 days EGA (17). This has been demonstrated by measuring both transepidermal water loss and dye penetration kinetics. Interestingly, at 16.5 days of gestation, a multilayered SC is not present in mouse embryos and the corneocyte

lipid envelope is hardly detectable even in those pups delivered at 17.5 days EGA. Extrusion of lamellar body contents was first observed after 16 days of mouse gestation with extruded lipid material remaining disorganized in the interstices of the developing SC (17). This has also been observed for rat embryos 3 days prior to spontaneous delivery (16). At this time point rat SC-lipids were not enriched in GlcCers, indicating that the failure of extruded lipid discs to form broad continuous lamellar bilayers is not based on insufficient postsecretory processing of GlcCer to Cer (16). In this study, we show that the corneocyte lipid envelope is not yet present after 16.5 days of mouse gestation. Therefore, this structure might provide a template that is of critical importance for the postsecretory dispersion and organization of SC-lipids. Other major functions of the corneocyte lipid envelope might be to improve the chemical and enzymatic resistance of the SC and to contribute to the integrity of the barrier against environmental insults. In addition, the hydrophobic interaction between the extracellular lipid bilayers and the corneocyte lipid envelope may restrict swelling of intercellular domains between adjacent desmosomes when the SC becomes hydrated.

It has been shown that pharmacologic inhibition of the biosynthesis of ω -hydroxylated Cers results in impaired barrier recovery after stripping the SC by adhesive tape (9). However, from these data it is not possible to deduce a specific role for protein-bound Cers in the skin barrier because the level of Cer(EOS) is also reduced. The same holds for a recent study showing a correlation between covalently bound Cers and transepidermal water loss in rats that received a diet deficient in essential fatty acids (25). The significance of the corneocyte lipid envelope for the epidermal barrier is not clear at present. The bulk of ω -hydroxylated Cers and fatty acids is attached to CE proteins between 17.5 and 18.5 days of gestation. It is likely that the barrier is nearly complete in Balb/c mice 2 days prior to birth (i.e., at 17.5 days EGA). This has been demonstrated, at least, for the ICE mouse strain used by Hardman et al. (17) and for Sprague-Dawley rats (16). Therefore, the formation of the corneocyte lipid envelope might not be required for the initiation of barrier function during epidermal development. However, protein-bound lipids present in small quantities at 17.5 days of gestation might be restricted to the lowermost layer of the developing SC. In this scenario the corneocyte lipid envelope might be relevant for SC-lipid organization and barrier maturation. The later accumulation of measurable amounts of Cer(OS) simply may require the presence of a number of corneocyte layers. Recently, it has been reported that epidermal tight junctions play a significant role in the skin barrier function (24). It is likely that tight junctions provide a partial barrier sealing the stratum granulosum from the developing SC unless the maturation of the CE is completed.

In conclusion, we have characterized distinct lipid biochemical alterations in the epidermis during mouse gestation. GlcCer is targeted to the apical side of the stratum granulosum and the enzymatic processing of these pro-

barrier lipids provides Cers required for the formation of the corneocyte lipid envelope. The assembly of the corneocyte lipid envelope correlates with the organization of SC-lipids into continuous lamellar bilayers but appears to be preceded by acquisition of a partial barrier status. Further studies are required to clarify the role of the corneocyte lipid envelope for the homeostasis and integrity of the epidermal permeability barrier. ■

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