THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 18, pp. 13211–13219, May 4, 2007
Printed in the U.S.A.

Fatty Acid 2-Hydroxylase, Encoded by *FA2H*, Accounts for Differentiation-associated Increase in 2-OH Ceramides during Keratinocyte Differentiation*

Received for publication, December 18, 2006, and in revised form, March 2, 2007 Published, JBC Papers in Press, March 12, 2007, DOI 10.1074/jbc.M611562200

Yoshikazu Uchida[‡1], **Hiroko Hama**[§], **Nathan L. Alderson**[§], **Sounthala Douangpanya**[‡], **Yu Wang**[‡], **Debra A. Crumrine**[‡], **Peter M. Elias**[‡], **and Walter M. Holleran**[‡¶]

From the [‡]*Department of Dermatology, School of Medicine, University of California, San Francisco, and Veterans Affairs Medical Center, San Francisco, and the* [¶]*Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94121, and the* [§]*Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425*

Ceramides in mammalian stratum corneum comprise a heterogeneous mixture of molecular species that subserve the epidermal permeability barrier, an essential function for survival in a terrestrial environment. In addition to a variation of sphingol species, hydroxylation of the amide-linked fatty acids contributes to the diversity of epidermal ceramides. Fatty acid 2-hydroxylase, encoded by the gene *FA2H*, the mammalian homologue of *FAH1* in yeast, catalyzes the synthesis of 2-hydroxy fatty acid-containing sphingolipids. We assessed here whether *FA2H* accounts for 2-hydroxyceramide/2-hydroxyglucosylceramide synthesis in epidermis. Reverse transcription-PCR and Western immunoblots demonstrated that FA2H is expressed in cultured human keratinocytes and human epidermis, with FA2H expression and fatty acid 2-hydroxylase activity increased with differentiation. FA2H-siRNA suppressed 2-hydroxylase activity and decreased 2-hydroxyceramide/2-hydroxyglucosylceramide levels, demonstrating that *FA2H* accounts for synthesis of these sphingolipids in keratinocytes. Whereas FA2H expression and 2-hydroxy free fatty acid production increased early in keratinocyte differentiation, production of 2-hydroxyceramides/2-hydroxyglucosylceramides with longer chain amide-linked fatty acids (≥C24) increased later. Keratinocytes transduced with FA2H-siRNA contained abnormal epidermal lamellar bodies and did not form the normal extracellular lamellar membranes required for the epidermal permeability barrier. These results reveal that 1) differentiation-dependent up-regulation of ceramide synthesis and fatty acid elongation is accompanied by up-regulation of FA2H; 2) 2-hydroxylation of fatty acid by FA2H occurs prior to generation of ceramides/glucosylceramides; and 3) 2-hydroxyceramides/2-hydroxyglucosylceramides are required for epidermal lamellar membrane formation. Thus, late differentiation-linked increases in FA2H expression are essential for epidermal permeability barrier homeostasis.

Sphingolipids are ubiquitously distributed in eukaryotic cellular membranes, primarily in glycosylated and phosphorylated forms, which are involved in cell-cell recognition, signal transduction, and intercellular adhesion (see review in Refs. 1 and 2). The ceramide (Cer)[2] backbone of sphingolipids further serves as an intracellular signal of cell cycle arrest, cellular senescence, and apoptosis in a variety of cell types, including keratinocytes (see review in Refs. 3, 4). In addition to these ubiquitous bioregulatory functions, Cer are abundant bulk components of the extracellular lamellar membranes in the outermost layers of the epidermis, *i.e.* the stratum corneum (SC), where they subserve permeability barrier function (5, 6). Newly synthesized Cer are converted to glucosylceramide (GlcCer) and sphingomyelin in the suprabasal, nucleated cell layers of the epidermis, but following secretion at the stratum granulosum-SC interface, these polar precursors are hydrolyzed, again generating Cer (7–10). To fulfill this barrier requirement, Cer synthesis increases during epidermal differentiation under basal conditions and accelerates still further in response to acute barrier perturbations (11).

Galactosylceramides and sulfatides containing amide-linked 2-hydroxy fatty acids (2-OH FA) are plentiful in the central and peripheral nervous systems of mammals (12, 13), whereas Cer containing 2-OH FA (*N*-2-hydroxy-acylsphingosine or 2-OH Cer) and *N*-ω-hydroxy-acylsphingosine (ω-OH Cer), as well as their glucosylated counterparts, 2-OH GlcCer and ω-OH GlcCer, are also abundant in epidermis (10, 14, 15). Whereas ω-OH Cer and ω-OH GlcCer are found only in the differentiated layers of mammalian epidermis, where they are known to be important for epidermal permeability barrier function (16, 17), the roles of 2-OH Cer in epidermal function are not clear. In model lipid systems, the 2-OH moiety stabilizes multilam-

* This work was supported by National Institutes of Health Grants AR19098, AR39448, AR050629, and RR17677 and by the Medical Research Service of the Department of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dermatology Service and Research Unit (190), Veterans Affairs Medical Center, 4150 Clement St., San Francisco, CA 94121. Tel.: 415-750-2091; Fax: 415-751-3927; E-mail: uchiday@derm.ucsf.edu.

[2] The abbreviations used are: Cer, ceramide(s); CerS, Cer synthase; CHK, cultured human keratinocyte(s); GlcCer, glucosylceramide(s); FA, fatty acid(s); FFA, free FA; 2-OH Cer, N-2-hydroxyacylsphingosine; 2-OH GlcCer, β-glucosyl-N-2-hydroxyacylsphingosine; q-rtPCR, quantitative reverse transcription-PCR; SC, stratum corneum; AS (Cer 5), *N*-2-hydroxy-acylsphingosine; AP (Cer 6), *N*-2-hydroxy-acylphytosphingosine; AH (Cer 7), *N*-2-hydroxyacyl-6-hydroxysphingosine; EOS (Cer 1), *N*-(ω-*O*-acyl)-acylsphingosine; EOH (Cer 4), *N*-(ω-O-acyl)-acyl-6-hydroxysphingosine; NS (Cer 2), non-*N*-hydroxy-acylsphingosine; NP (Cer 3), non-*N*-hydroxy-acylphytosphingosine; siRNA, small interfering RNA; acylCer, *N*-(ω-*O*-acyl)-acylsphingosine; acylGlcCer, ω-*O*-acylGlcCer.

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melar structures by increasing intermolecular hydrogen bonding (18, 19). In addition to the contribution of *N*-(*ω*-*O*-acyl)-acylsphingosine (acylCer) to the long periodicity phase in SC lamellar bilayer membranes (20), 2-OH Cer appears to play a supportive role in the formation of this phase (21). Thus, 2-OH Cer could be important for permeability barrier function. Additionally, because exogenous 2-OH Cer are less pro-apoptotic than nonhydroxylated Cer (22), conversion to 2-OH Cer could protect the epidermis from apoptosis, as bulk Cer synthesis increases during epidermal differentiation.

Prior studies have demonstrated that a non-P-450 protein is responsible for the 2-hydroxylation of fatty acid (FA) in murine brain (12). Subsequently, the gene that encodes the fatty acid 2-hydroxylase, *FAH1* (SCS7), was identified in yeast (23, 24). The mammalian homologue of *FAH1*, *FA2H*, was recently identified and found to encode a 43-kDa transmembrane protein (25). As in yeast (24), mammalian FA2H contains a cytochrome b_5 domain (25), which accounts for the redox activity of FA2H, including its ability to hydroxylate FA (25). It is likely that electrons are transferred from NADH or NADPH to the heme iron in the N-terminal cytochrome b_5 domain and then to the putative catalytic non-heme di-iron in the C-terminal domain (26).

FA2H is expressed at high levels in brain, apparently accounting for the abundance of 2-OH galactosylceramide and 2-OH sulfatides in this tissue (25, 27). FA2H gene expression is up-regulated during myelination (28), which likely accounts for the progressive incorporation of 2-OH galactosylceramide into myelin (29).

We and others have demonstrated the molecular heterogeneity of both Cer and GlcCer in epidermis, including a progressive enrichment in 2-OH Cer and 2-OH GlcCer species during keratinocyte differentiation (30, 31). Although these results suggest that 2-OH FA synthesis could be linked to keratinocyte differentiation, neither the regulation of FA2H in relation to keratinocyte differentiation nor its role in 2-OH Cer/2-OH GlcCer synthesis in epidermis has been assessed systematically. Here, we demonstrate that FA2H: 1) is expressed in cultured human keratinocytes (CHK) and in human epidermis in a differentiation-associated manner; 2) accounts for 2-OH Cer/2-OH GlcCer generation in keratinocytes; 3) utilizes FA as a substrate; and 4) is required for epidermal permeability barrier formation.

EXPERIMENTAL PROCEDURES

Materials—Cer and GlcCer were purchased from Sigma and Matreya (Pleasant Gap, PA), respectively. High performance thin layer chromatography plates (Silica Gel 60) were purchased from Merck.

Cell Culture—Normal human keratinocytes were isolated from neonatal foreskins by a modification of the method of Pittelkow and Scott (32) under an Institutional Review Board-approval protocol (University of California, San Francisco). The cells were grown in keratinocyte growth medium supplemented with bovine epidermal growth factor, bovine pituitary extract, insulin, hydrocortisone, and 0.07 mM calcium chloride (Cascade Biologics, Portland, OR). CHK at three subsequent stages of differentiation were employed for these studies. To generate undifferentiated CHK, the cells were grown in a low Ca^{2+} (0.07 mM)-containing and serum-free medium and harvested at 80–90% confluence. For early stage differentiated CHK, a Ca^{2+} concentration in the medium was raised to 1.2 mM Ca^{2+}, when the cells were at 90% confluence, and harvested 2 days later. Advanced stage differentiated CHK, which generate both epidermal lamellar bodies and the full spectrum of Cer species (33), were obtained by post-confluent growth of CHK in Dulbecco's and Ham F-12 media (2:1, v/v), containing ≥1.2 mM calcium, supplemented with 10% fetal bovine serum, insulin (10 μg/ml), hydrocortisone (0.4 μg/ml) (34), and ascorbic acid (50 μg/ml) for 9 days described as previously (advanced differentiation medium) (33, 35). The cultures were maintained at 37 °C under 5% CO_2 in air. Because we use primary human cultured keratinocytes isolated from neonatal foreskin from various donors, we prepared analytical samples from two or three different cell preparations to minimize possible variation between sources of skin and combined these samples for analysis. In addition, we combined multiple culture dishes (five to ten) in each experiment to further minimize differences between dishes.

Assay of Fatty Acid 2-Hydroxylation Activity—Fatty acid 2-hydroxylase activity was measured as described previously (26). Briefly, microsomal fractions (25 μg of protein) or cell lysate (50 μg of protein) were incubated with 2.7 mM Tris-HCl, pH 7.6, containing 1.28 mM NADP, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$, 0.2 unit of glucose 6-phosphate dehydrogenase, 1 μg of human NADPH:cytochrome P-450 reductase, with the substrate, 1 μg (2.7 nmol) of [3,3,5,5-D4]tetracosanoic acid (C24) at 37 °C for 120 min. At the end of incubations, 1 pmol of tricosanoic acid (C23) was added to each sample as an internal standard, and FA were extracted immediately with 100% diethyl ether following acidification by glacial acetic acid. FA were converted to *O*-trimethylsilyl FA methyl esters, and then the derivatives were analyzed by gas liquid chromatography-mass spectrometry (GCMS-QP2010; Shimadzu Scientific, Columbia, MD) on a Restek RTX-5 column (30 m × 0.25 mm I.D., 0.25 μm D.F., Restek Corporation Bellefonte, PA). The initial temperature was 110 °C with 10 °C increases each min up to 300 °C.

Blockade of FA2H Activity Using siRNA—CHK, cultured in a low Ca^{2+} (0.07 mM) and serum-free medium, were incubated with 30 nM of siRNAs for FA2H (Ambion, Austin, TX or B-Bridge International, Inc., Sunnyvale, CA), using X-tremeGEN siRNA transfection reagent (Roche Applied Science) for 24 h. The mixtures of siRNA duplex (1:1:1) with the following sense and antisense sequences were used: 5′-GGAUUUGGUAUCAGCACUA-3′ (sense) and 5′-UAGUGCUGAUACCAAAUCC-3′ (antisense); 5′-GGCUAAAGAGAAGCAGUUU-3′ (sense) and 5′-AAACUGCUUCUCUUUAGCC-3′ (antisense); and 5′-CCACGGUUCAAAGUGGUGG-3′ (sense) and 5′-CCACCACUUUGAACCGUGG-3′ (antisense). siRNA for green fluorescence protein (B-Bridge International, Inc.) was used as control siRNA control. The Ca^{2+} concentration in the medium was then raised to 1.2 mM and cultured for 48 h. Alternatively, siRNA were transfected into CHK twice. The medium was switched to advanced differentiation medium (as above) 24 h following the first transfection and cultured for an

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additional 24 h. CHK then were incubated with siRNA mixtures for second time (as above) for 48 h and cultured for an additional 3 days.

Reverse Transcription-PCR Analysis—Reverse transcription-PCR or quantitative reverse transcription-PCR (q-rtPCR) was performed using cDNA prepared from total RNA, as described previously (35, 36). The following primer sets were used for q-rtPCR 5′-AGCCTGTAGCCCTTGAGGAAAC-3′ and 5′-TTCACCACCTAACCCTGTTCC-3′ (77 bp). For q-rtPCR, 10 ng of cDNA was mixed with sets of primer pairs (final concentration, 200 pM) and SYBR Green PCR mix containing *Taq* DNA polymerase and SYBR Green I dye (Applied Biosystems, Foster City, CA). The thermal cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by 95 °C for 15 s, and 60 °C for 1 min, repeated for 40 times on ABI Prism 7700 (Applied Biosystems). The values shown represent the means (±S.D.) for three independent assays. mRNA expression was normalized to levels of 18 S ribosomal RNA and G3PDH.

Western Immunoblot Analysis—Western blot analysis was performed using a previously described procedure (36). Briefly, the cell lysates (prepared as described above) were suspended in sample buffer (60 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.005% bromphenol blue) and resolved by electrophoresis on 10% SDS-polyacrylamide gel (20 μg of proteins/lane). The resultant bands were electrophoretically transferred to a nitrocellulose membrane, probed with anti-human FA2H polyclonal antibody (25), and detected using an enhanced chemiluminescence system (Amersham Biosciences).

Analysis of Amide-linked FA of Cer and GlcCer—Total lipids were isolated from CHK by the method of Bligh and Dyer (37), and Cer/GlcCer fractions were fractionated as described previously (10, 33). Briefly, total lipid extracts were first applied to aminopropyl silica gel column (Varian Inc., Palo Alto, CA), equilibrated with *n*-hexane to separate the Cer and GlcCer containing fraction from free FA (FFA). After washing the column with *n*-hexane, the fraction containing Cer and GlcCer was eluted with chloroform-isopropanol (2:1, v/v). FFA fraction was then eluted with 2% acetic acid in diethylether. The Cer- and GlcCer-containing fraction was subjected to mild alkaline hydrolysis and then enriched on an aminopropyl silica gel column, as above. To quantify the amide-linked FA of Cer and GlcCer, a mixture of odd chain FA (C15-C25) was added to each sample as internal standards. Following methanolysis the resulting FA methylesters were derivatized to *O*-trimethylsilyl ethers and then were analyzed by gas liquid chromatography-mass spectrometry, as above.

Electron Microscopy—CHK samples were fixed *in situ* by the addition of modified Karnovsky's fixative to the Petri dish for 1 h (room temperature), transferred to a glass tube, and fixed further overnight (4 °C); the samples were divided and post-fixed in either ruthenium tetroxide or 2% aqueous osmium tetroxide, both containing 1.5% potassium ferrocyanide, as previously described (33, 38). After fixation, all of the samples were dehydrated in graded ethanol solutions and embedded in an Epon-epoxy mixture. Ultrathin sections were examined, with or without further contrasting with lead citrate, in an electron microscope (Zeiss 10A; Carl Zeiss, Thornwood, NY) operated at 60 kV.

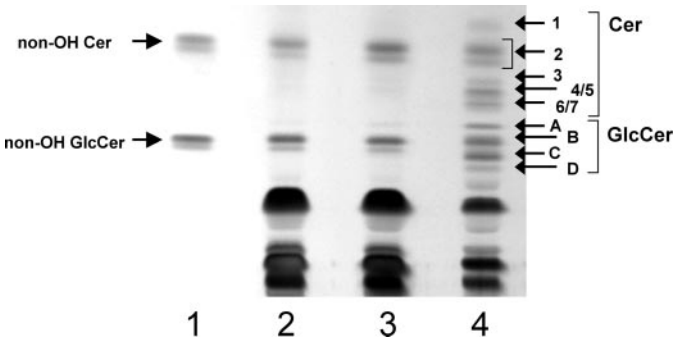

FIGURE 1. **Molecular heterogeneity of Cer and GlcCer becomes evident in advanced stage differentiated CHK.** Equivalent of lipid extracts of cells corresponding to 210 μg of protein were applied on a plate and separated by high performance thin layer chromatography plate (10). *Lane 1 N*-non-hydroxyacylsphingosine (*upper band*) and β-glucosyl-non-OH acylsphingosine (*lower band*); *lane 2*, undifferentiated CHK; *lane 3*, early stage differentiated CHK; *lane 4*, advanced stage differentiated CHK. The abbreviations for Cer structures are according to Robson *et al.* (61) and Motta *et al.* (41). *Cer 1*, EOS; *Cer 2*, NS; *Cer 3*, NP; *Cer 4*, EOH; *Cer 5*, AS; *Cer 6*, AP; *Cer 7*, AH. *GlcCer A*, acylGlcCer (glucosylated forms of EOS and EOH); *GlcCer B*, glucosylated forms of NS and NP; *GlcCer C*, glucosylated forms NS and AS; *GlcCer D*, of glucosylated forms of NS, AP, and AH (31). See details under "Experimental Procedures."

Statistical Analysis—Statistical analyses were performed using an unpaired Student's *t* test.

RESULTS

Increased FA 2-Hydroxylase Activity Parallels Increased 2-OH Cer/2-OH GlcCer Generation during Keratinocyte Differentiation—The molecular heterogeneity of Cer and GlcCer, including the appearance of species containing amide-linked 2-OH FA, becomes evident late in keratinocyte differentiation (33). Under these conditions, keratinocytes further 2-hydroxylate some FFA, generating 2-OH Cer and 2-OH GlcCer (33). Hence, we first compared FA 2-hydroxylase expression in relation to the generation of 2-OH Cer/2-OH GlcCer as CHK progressively differentiate. Three different stages of differentiation were assessed: 1) undifferentiated, proliferative CHK (undifferentiated CHK); 2) early stage differentiated CHK; and 3) advanced stage differentiated CHK) (for details see "Experimental Procedures"). Cellular proliferation declines, and differentiation initiates by 2 days following an increase in medium Ca^{2+} concentration from 0.07 to 1.2 mM (39), but late differentiation markers, *e.g.* loricrin (40) and the full spectrum of Cer species, characteristic of SC, appear only after CHK stratify with prolonged growth in high Ca^{2+} plus vitamin C and 10% serum (33). As noted previously (33), analysis of Cer and GlcCer profiles by thin layer chromatography demonstrated not only an increase in total Cer and GlcCer content but also in the 2-OH Cer fraction in advanced stage differentiated CHK (Cer 5 or AS; Cer 6 or AP; and Cer 7 or AH) (41, 42) and 2-OH GlcCer (GlcCer B-D) (10) *versus* both undifferentiated CHK and early stage differentiated CHK (Fig. 1). Moreover, gas liquid chromatography-mass spectrometry analysis of amide-linked FA in the Cer and GlcCer fractions indicates increased levels of molecular species containing 2-OH FA in advanced stage differentiated CHK (359% of undifferentiated CHK and 333% of early stage differentiated CHK) (Table 1).

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TABLE 1

Amide-linked FA composition of Cer and GlcCer of CHK

O-Trimethylsilyl FA methyl esters prepared from Cer and GlcCer fraction were analyzed by gas liquid chromatography-mass spectrometry. The cells were combined from ten, eight, and six individual treatment groups for undifferentiated, early stage differentiated, and advanced stage differentiated CHK, respectively. The details are described under "Experimental Procedures."

	FA composition		
	Undifferentiated CHK	Early stage differentiated CHK	Advanced stage differentiated CHK
	nmol/mg of protein (% total FA)		
Total FA	208.7 (100)	216.4 (100)	246.3 (100)
Total non-OH FA	170.4 (81.7)	175.1 (80.9)	108.7 (44.2)
16:0	78.9 (37.8)	80.0 (37.0)	50.0 (20.3)
18:0	90.0 (43.1)	92.5 (42.7)	49.8 (20.2)
20:0	0.51 (0.2)	0.71 (0.3)	0.34 (0.1)
22:0	0.73 (0.3)	0.99 (0.5)	5.65 (2.3)
24:0	0.29 (0.1)	0.81 (0.4)	2.59 (1.1)
26:0	0.04 (0.02)	0.13 (0.1)	0.36 (0.1)
Total 2-OH	38.3 (18.3)	41.2 (19.1)	137.5 (55.8)
2-OH 16:0	37.1 (17.8)	40.2 (18.6)	86.2 (35.0)
2-OH 18:0	0.29 (0.1)	0.41 (0.2)	8.03 (3.3)
2-OH 20:0	0.11 (0.03)	0.20 (0.1)	2.96 (1.2)
2-OH 22:0	0.06 (0.2)	0.07 (0.03)	2.81 (1.1)
2-OH 24:0	0.47 (0.2)	0.30 (0.1)	29.2 (11.9)
2-OH 26:0	0.25 (0.1)	0.06 (0.03)	8.33 (3.4)

TABLE 2

Increased FA 2-hydroxylase activity and FA2H mRNA levels in differentiated CHK

FA2H activity was determined in microsomal fractions (25 μg of protein) prepared from CHK, as described under "Experimental Procedures." The cells were combined from ten, eight, and six individual treatment groups for undifferentiated, early stage differentiated, and advanced stage differentiated CHK, respectively. The values represent the means of duplicate determinations. Experiments 1 and 2 were from separate experiments from different primary cultured cells. FA2H mRNA expression was normalized to levels of 18 S ribosomal RNA expression, with expression in undifferentiated CHK to 18 S ribosomal RNA set to 1.00. Further details of q-rtPCR methods are provided under "Experimental Procedures." The values represent the means of triplicate determinations (±S.D.).

	FA2H activity		mRNA expression relative to undifferentiated CHK
	Experiment 1	Experiment 2	
	nmol/mg of protein/min		
Undifferentiated CHK	0.31	0.21	1.00 ± 0.21
Early stage differentiated CHK	4.87	4.65	4.91 ± 0.79
Advanced stage differentiated CHK	4.38	4.54	6.58 ± 1.03

We next compared FA 2-hydroxylase activity in the CHK in parallel cultures, *i.e.* three stages of differentiation. Enzyme activity significantly increased in both early (Experiment 1, 1571% and Experiment 2, 2214%) and advanced stage (Experiment 1, 1412% and Experiment 2, 2162%) differentiated CHK in comparison with undifferentiated CHK (Table 2). Yet there was no further increase in enzyme activity in advanced *versus* early stage differentiated CHK. Together, these results show that FA 2-hydroxylase activity is up-regulated during keratinocyte differentiation in parallel with the appearance of increased levels of 2-OH Cer/2-OH GlcCer.

FA2H mRNA and Protein Expression Increase with Keratinocyte Differentiation—To investigate whether the differentiation-associated increase in enzyme activity is regulated at a transcriptional level, we next compared mRNA levels for FA2H under the same conditions. Specific PCR products of FA2H were evident in CHK at all three stages of differentiation (Fig. 2*A*, *lane 2*, undifferentiated; *lane 3*, early stage of differentiation; *lanes 5* and *6*, advanced stage of differentiation), as well as for human epidermis (*lane 4*). Yet, consistent with the differentiation-associated increase in 2-hydoxylase activity (Table 2), mRNA levels for FA2H, assessed by q-rtPCR, increased in early stage differentiated in comparison with undifferentiated CHK, with a further increase in mRNA levels in advanced stage differentiated CHK (Table 2).

We next investigated FA2H protein expression in relation to differentiation by Western immunoblot analysis. A single

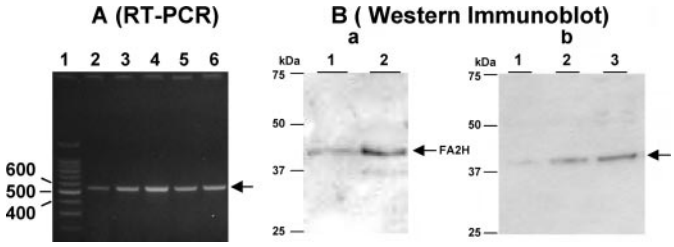

FIGURE 2. **mRNA for FA2H and its coded protein are expressed in CHK and human epidermis.** *A*, FA2H expression. Reverse transcription-PCR was performed using 50 ng of DNaseI-treated total RNA from undifferentiated CHK, early stage differentiated, advanced stage differentiated CHK, or human epidermis isolated from neonatal foreskin. *Lane 1*, molecular weight markers; *lane 2*, undifferentiated CHK; *lane 3*, early stage differentiated CHK; *lane 4*, epidermis; *lanes 5* and *6*, advanced stage differentiated CHK. *B*, Western immunoblot analysis: FA2H protein levels increased in differentiated CHK. The cells were combined from six individual treatment groups for undifferentiated CHK, early stage differentiated CHK, and advanced stage differentiated CHK. The cell lysates were resolved by electrophoresis on 10% SDS-ployacrylamide gels and probed with anti-FA2H antiserum. *Panel a*, *lane 1*, HeLa cells with pcDNA human FA2H; *lane 2*, murine brain. *Panel b*, *lane 1*, undifferentiated CHK; *lane 2*, early stage differentiated CHK; *lane 3*, advanced stage differentiated CHK. The *arrow* indicates FA2H.

43-kDa band, consistent with the predicted molecular size of FA2H protein from murine brain (Fig. 2*B*, *panel a*, *lane 2*) and HeLa cells transfected with pcDNA-human FA2H (Fig. 2*B*, *panel a*, *lane 1*) (25), was present in CHK (Fig. 2*B*, *panel b*). Changes in the expression levels of this 43-kDa protein paralleled increased differentiation of CHK (early stage differentiated CHK, 269% (Fig. 2*B*, *panel b*, *lane 2*) and advanced stage

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differentiated CHK, 488% (Fig. 2*B*, *panel b*, *lane 3*) *versus* undifferentiated CHK, (Fig. 2*B*, *panel b*, *lane 1*). Because these results demonstrate concurrent changes in the mRNA and protein for FA2H, they suggest that the differentiation-associated changes in enzyme activity are regulated at a transcriptional level.

FA2H Accounts for FA 2-Hydroxylase Activity in CHK—As a direct test of the hypothesis that FA2H accounts for FA 2-hydroxylase in CHK, we next blocked FA2H gene expression in CHK with siRNA against FA2H. q-rtPCR analysis revealed significant (75–80%) reduction of FA2H mRNA in siRNA-treated CHK *versus* either control siRNA-treated or sham transfected CHK (data not shown). Consistent with the decrease in mRNA of FA2H, FA 2-hydroxylase activity diminished significantly in siRNA-transfected cells compared with control cells, *i.e.* decreased by 57% at 48 h and 80% at 72 h *versus* sham-transfected cells and decreased by 63% at 48 h and 77% at 72 h *versus* control siRNA-transfected cells (Table 3).

To assess whether reduced FA2H activity leads to decreased formation of 2-OH Cer/2-OH GlcCer, the FA composition of Cer/GlcCer-enriched fractions were determined in CHK treated with FA2H siRNA (Table 4). Although total Cer/GlcCer levels were not affected by prior siRNA treatment, the 2-OH FA contents within Cer/GlcCer were reduced in CHK that had been transfected with FA2H siRNA in comparison with sham-transfected cells (by 24%) or control siRNA-transfected cells (by 25%) (Table 4). It should be noted that the Cer/GlcCer content measured in this assay represents both preformed and newly formed lipids, explaining the less dramatic effect of FA2H siRNA on steady-state levels of 2-OH Cer/2-OH GlcCer than on FA2H activity in these cells (Table 4). Together, these results strongly suggest that FA2H is responsible both for keratinocyte FA 2-hydroxylase activity and for 2-OH Cer/2-OH GlcCer synthesis in CHK.

2-Hydroxylation of FFA Increases Early in Differentiation—We next investigated the sequence in which FFA and amide-linked FA become 2-hydroxylated during differentiation. The total FFA content increased by 152 and 162% in early and advanced stage differentiated CHK, respectively, in comparison with undifferentiated CHK (Table 5). Likewise, the levels of both 2-OH FFA, as well as non-OH FFA, increased in both stages of differentiated CHK. In contrast, the FFA composition at all stages of differentiation was comparable, *i.e.* hexadecanoic acid (C16), 2-OH hexadecanoic acid, and 2-OH octadecanoic acid (C18) predominated. These studies suggest that 2-OH FFA generation occurs during the initial stages of differentiation.

2-OH Cer/2-OH GlcCer Increase Late Stages in Differentiation—We next determined the changes in the composition of amide-linked FA, including 2-hydroxylated FA, nonhydroxylated FA species in Cer and GlcCer, in early *versus* late stages of keratinocyte differentiation. Because prior studies have shown that very long chain ω-OH Cer and ω-OH GlcCer (>C24) appear only in advanced stage differentiated CHK (30, 31), we did not quantitate sphingolipid species containing ω-OH FA. Instead, we show that the total FA content of amide-

TABLE 3

siRNA for FA2H decreases FA 2-hydroxylase activity in CHK

The activity was determined in cell lysates prepared from CHK as described under "Experimental Procedures." The cells were combined from five individual treatment groups. Sham transfected, transfection reagent alone; Control siRNA, siRNA for green fluorescent protein.

	Activity	
	48 h	72 h
	nmol/mg of protein/min (% sham transfected control)	
Sham transfected	0.14 (100)	0.15 (100)
Control siRNA	0.16 (117)	0.13 (83.0)
FA2H siRNA	0.06 (42.9)	0.03 (17.6)

TABLE 4

siRNA for FA2H decreases 2-OH Cer/GlcCer production of CHK

O-Trimethylsilyl FA methyl esters prepared from Cer/GlcCer fraction were analyzed by GC-MS. Cells were combined from eight individual treatment groups. Details are described under "Experimental Procedures." Sham transfected = transfection reagent alone; Control *si*RNA = *si*RNA for green fluorescent protein.

	Content		
	Sham transfected	Control siRNA	FA2H siRNA
	nmol/mg of protein (% total amide-linked FA)		
Total FA	236.9 (100)	215.7 (100)	219.4 (100)
Total non-OH FA	142.4 (60.1)	119.1 (55.2)	146.8 (66.9)
Total 2-OH	94.5 (39.9)	96.6 (44.8)	72.6 (33.1)

TABLE 5

FFA composition of CHK

O-Trimethylsilyl FA methyl esters prepared from FFA fraction were analyzed by gas liquid chromatography-mass spectrometry. The cells were combined from ten, eight, and six individual treatment groups for undifferentiated, early stage differentiated, and advanced stage differentiated CHK, respectively. The details are described under "Experimental Procedures."

	FFA composition		
	Undifferentiated CHK	Early stage differentiated CHK	Advanced stage differentiated CHK
		nmol/mg of protein (% total FFA)	
Total FFA	207.1 (100)	521.9 (100)	553.2 (100)
Total non-OH FFA	195.5 (94.4)	473.4 (90.7)	515.6 (93.2)
16:0	130.2 (62.8)	259.5 (49.7)	306.8 (55.5)
18:0	54.6 (26.4)	184.2 (35.3)	182.5 (33.0)
20:0	1.6 (0.8)	3.8 (0.7)	3.3 (0.6)
22:0	0.5 (0.2)	0.8 (0.1)	0.9 (0.2)
24:0	7.8 (3.8)	22.8 (3.5)	19.4 (3.5)
26:0	0.7 (0.4)	2.4 (0.5)	2.6 (0.5)
Total 2-OH FFA	11.6 (5.6)	48.4 (9.3)	37.6 (6.8)
2-OH 16:0	4.3 (2.1)	19.4 (3.7)	18.9 (3.4)
2-OH 18:0	3.3 (1.6)	18.0 (3.4)	6.5 (1.2)
2-OH 20:0	0.4 (0.2)	0.7 (0.1)	0.8 (0.1)
2-OH 22:0	0.1 (0.03)	0.1 (0.03)	0.2 (0.03)
2-OH 24:0	3.0 (1.5)	9.1 (1.7)	9.9 (1.8)
2-OH 26:0	0.5 (0.3)	0.2 (1.3)	1.3 (0.2)

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linked FA in Cer and GlcCer modestly increased in advanced stage differentiated CHK in comparison with both undifferentiated CHK and early stage differentiated CHK (Table 1). The total non-OH FA content of *N*-acylated FA in Cer and GlcCer decreased by 37–38% in advanced stage differentiated CHK in comparison with both undifferentiated or early stage differentiated CHK. In contrast, 2-OH FA content of *N*-acylated FA in Cer and GlcCer increased by 233–259% in advanced stage differentiated CHK *versus* undifferentiated CHK or early stage differentiated CHK. As with the FFA formation (Table 5), a major 2-OH FA in Cer and GlcCer was 2-OH hexadecanoic acid (C16) at all stages of differentiation in CHK. Moreover, the levels of amide-linked, very long chain 2-OH FA, particularly 2-OH tetracosanoic acid (C24) and 2-OH hexacosanoic acid (C26), increased only in advanced stage differentiated CHK (Table 1). Likewise, Cer/GlcCer species, containing longer chain non-OH FA, increased only in advanced stage differentiated CHK (Table 1). These results suggest first that FFA are 2-hydroxylated prior to Cer formation; second that 2-OH Cer/2-OH GlcCer increase in the advanced stage of keratinocyte differentiation; and finally that FA elongation increases in the early stage of keratinocyte differentiation.

Epidermal Permeability Barrier Formation Requires FA2H—The extracellular domains of outer epidermis, or stratum corneum, contain lamellar membranous structures that consist of primarily three lipids, Cer, cholesterol, and FFAs that mediate the epidermal permeability barrier. Precursors for these barrier lipids are stored in epidermal lamellar bodies, generated within the outermost nucleated cell layers of the epidermis. Secretion of lamellar bodies contents into the intercellular (or extracellular) spaces between the stratum granulosum and stratum corneum is followed by enzymatic processing of lipid precursors, to form the extracellular lamellar bilayers that provide barrier function (6). In a final series of studies, we investigated whether FA2H activity is required for the formation of these critical epidermal organelles. Whereas lamellar bodies showing lamellar membrane structures and their secretion were evident in CHK transduced with either siRNA for green fluorescence protein, control siRNA (Fig. 3*A*, *thick arrows*), or cells treated with transfection reagent alone (Fig. 3*B*), normal-appearing lamellar bodies were largely absent in cells treated with FA2H siRNA, with significant abnormalities in the contents of the few lamellar bodies found in these cells (Fig. 3*C*). In addition, although the secreted lamellar body contents were processed into normal-appearing elongated lamellar membrane structures in keratinocytes transfected with control siRNA (Fig. 4*A*, *solid arrows*), the scattered, secreted lamellar materials within the intercellular spaces in keratinocytes treated with siRNA against FA2H were not transformed into lamellar membrane structures (Fig. 4*B*, *open arrows*). These results indicate that blockade of FA2H expression diminishes lamellar body formation, leading to diminished secretion and defective processing of secreted lipids into lamellar membrane structures. Together, these studies suggest that FA2H is required for the formation of lamellar bodies leading to formation of the permeability barrier.

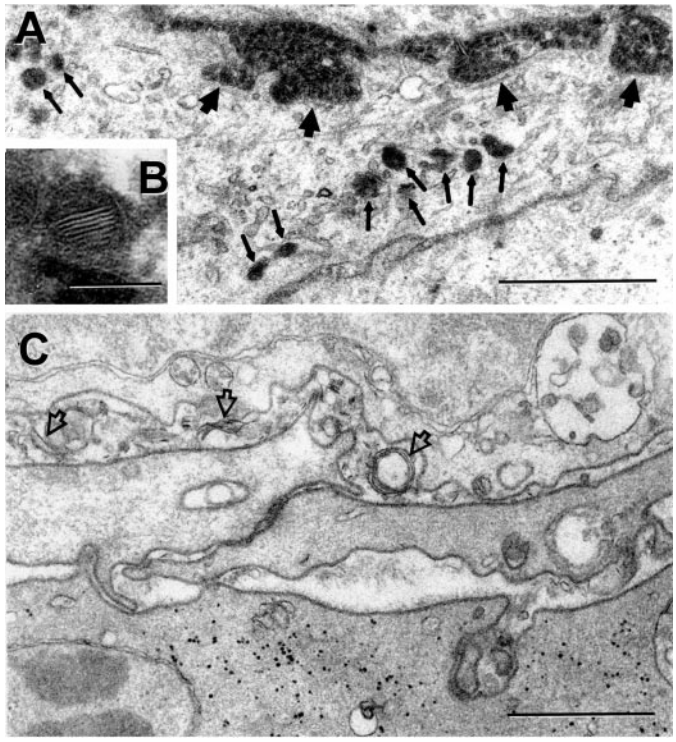

FIGURE 3. **Down-regulation of lamellar body formation and secretion with siRNA-induced blockade of FA2H.** *A*, quantity of lamellar bodies (*thin arrows*) and secretion (*thick arrows*) is normal in keratinocytes transfected with siRNA (30 nM) for green fluorescence protein (control siRNA). *B*, normal-appearing lamellar body in keratinocytes treated with transfection reagent alone. *C*, Near absence of lamellar bodies in keratinocytes transfected with siRNA (30 nM) against FA2H. The *open arrows* indicate small amounts of secreted lamellar materials. *A* and *C*, osmium tetroxide post-fixation; *B*, ruthenium tetroxide post-fixation. *A* and *C*, *bars*, 1 μm; *B*, *bar*, 0.1 μm.

DISCUSSION

Sphingolipids of the differentiated layers of epidermis display a unique heterogeneity of Cer and GlcCer molecular profiles not found in other tissues, including the undifferentiated layers of epidermis (30, 31). This molecular heterogeneity can be attributed to a combination of variations in the sphingol, *i.e.* sphingenine, sphinganine, 4-hydroxysphinganine (or phytosphingosine), and 6-hydroxy sphingenine, and in the composition of amide-linked FA, *i.e.* non-OH FA, 2-OH FA, and ω-*O*-acylated FA (10, 14, 15, 42, 43). acylCer/ω-*O*-acylGlcCer (acylGlcCer) represent another unique aspect of epidermal Cer and GlcCer, *i.e.* ω-*O*-acylation, primarily linoleation, and the very long chain length of amide-linked ω-OH FA (C24-C34) (10, 14, 15, 42, 43). The organization of lamellar membranes within the SC interstices requires not only these heterogeneous Cer but also two other characteristic SC lipids, *i.e.* cholesterol and primarily nonessential FFA, which subserve the epidermal permeability barrier, a prerequisite for mammalian survival in a desiccating terrestrial environment (6). The potential importance of Cer heterogeneity for permeability barrier function is further suggested by changes in their composition in certain skin diseases that alter Cer molecular distribution and show decreased Cer content, coincident with barrier defects, *e.g.* in inflammatory dermatoses such as atopic dermatitis, psoriasis, and the inherited ichthyosis (7, 41, 44–47). Pertinently, Cer containing 2-OH FA, which appear to have a supportive role in

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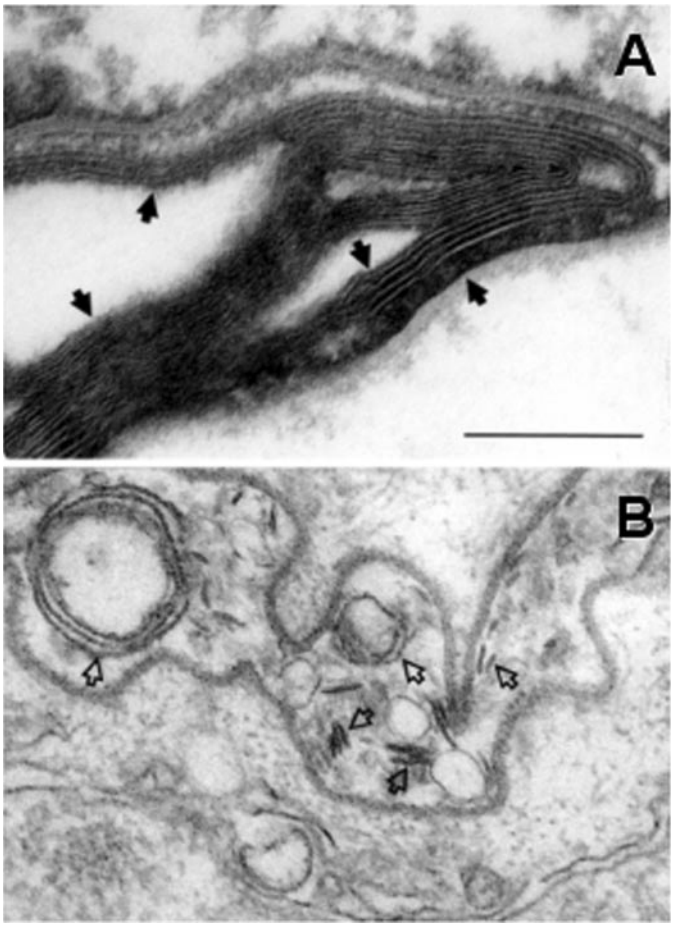

FIGURE 4. **Absence of post-secretory lipid processing with siRNA-induced blockade of FA2H.** *A*, extensive post-secretory processing of secreted lamellar into elongated membrane structures (*solid arrows*) in keratinocytes transfected with siRNA (30 nM) for green fluorescence protein. *B*, scattered secreted lamellar materials can be seen in intercellular spaces (*open arrows*) in keratinocytes treated with siRNA (30 nM) against FA2H. *A*, ruthenium tetroxide post-fixation; *B*, osmium tetroxide post-fixation. *Bars*, 0.2 μm.

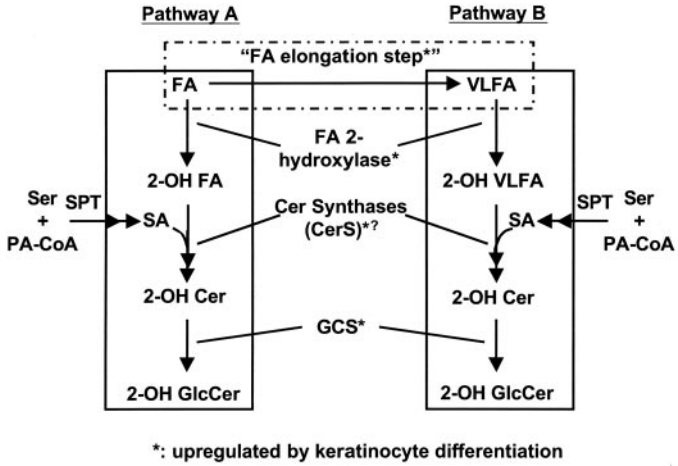

FIGURE 5. **2-OH Cer-2-OH GlcCer formation in different stages of CHK differentiation.** *Cer synthase*, ceramide synthase; *GCS*, GlcCer synthase; *PA-CoA*, palmitoyl-CoA; *SA*, sphinganine; *Ser*, L-serine; *SPT*, serine palmitoyl transferase; *VLFA*, very long chain FA. Whereas pathway A operates in all stages of differentiation, *i.e.* undifferentiated, early stage, and advanced stage differentiated CHK, 2-OH FA generation is active in both early stage and advanced stage differentiated CHK, and 2-OH Cer and 2-OH GlcCer synthesis is increased in advanced stage differentiated CHK. FA elongation followed by 2-OH very long chain fatty acid synthesis (in pathway B) are activated in both early stage and advanced stage differentiated CHK. However, 2-OH Cer and 2-OH GlcCer synthesis again increases only in advanced stage differentiated CHK in Pathway B.

the formation of the long periodicity phase of the lamellar membranes in the SC (21), are decreased in atopic dermatitis (21), potentially contributing to the barrier abnormality in this disorder.

Our present studies demonstrate first, that FA2H, which already has been shown to be responsible for the synthesis of 2-OH galactosylceramides and 2-OH sulfatides in neuronal tissues (27, 28), is expressed in human epidermis and in a differentiation-associated manner in cultured human keratinocytes. Second, using siRNA, we showed here that FA2H is responsible for 2-OH Cer and 2-OH GlcCer synthesis in keratinocytes. Third, our results provide insights about the likely pathways that lead to 2-OH Cer/GlcCer formation in epidermis; *i.e.* FA → 2-OH FA → 2OH Cer → 2-OH GlcCer. Finally, electron microscopy revealed that blockade of FA2H by siRNA causes deficiencies of normal epidermal lamellar body contents and lamellar membrane structures in the extracellular domain, which are required for epidermal permeability barrier formation (6), revealing that FA2H plays a critical role in epidermal permeability barrier function.

With regard to differentiation, both enzyme activity and mRNA levels of FA2H increased significantly in both early stage and advanced stage differentiated CHK in comparison with undifferentiated CHK (Table 2). Consistent with these changes, free 2-OH FA levels also increased in these early and advanced stages of differentiated CHK (Table 5). Whereas there are no further differences in the distribution of 2-OH FA, as well as non-OH FA, in FFA in early *versus* advanced stages of CHK differentiation (Table 5), Cer/GlcCer containing very long chain length 2-OH FA only appear in the advanced stages of differentiation (Table 1). These results suggest that CHK utilize two 2-OH Cer/2-OH GlcCer synthetic pathways. Pathway A generates 2-OH Cer/2-OH GlcCer, whose dominant chain length of FA (or amide-linked FA residues) are C16–C18 (Fig. 5), whereas pathway B produces 2-OH Cer/2-OH GlcCer containing very long chain 2-OH FA 2-OH FA (≥C20) (Fig. 5). Whereas pathway A operates in CHK at all stages of differentiation, this pathway is stimulated in advanced stage differentiated CHK but not in early stages of differentiated cells (Fig. 5). In contrast, pathway B operates only during the advanced stages of keratinocyte differentiation. Thus, whereas FA2H upregulation, accompanied by increased FA elongation, occurs in both early and advanced stage differentiated CHK (FA elongation step in Fig. 5), 2-OH Cer/2-OH GlcCer synthesis increases only in advanced stage differentiated CHK (both pathways A and B in Fig. 5 and Table 1).

Recent studies have shed light on the possible control points for the regulation of very long chain 2-OH FA metabolism into Cer/GlcCer late in keratinocyte differentiation. For example, expression(s) or activity(ies) of Cer synthase, *i.e.* *N*-acyltransferase of sphingol, could increase during the advanced stages of differentiation, allowing for formation of these ceramide species. Six homologues of the yeast longevity assurance genes (LAG1 and LAC1), LASS 1–6 (or Cer synthase (CerS)), have been identified whose gene products are responsible for acyl-

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CoA-dependent Cer synthesis in mammals (48–50). Interestingly, the different CerS enzymes preferentially utilize selected carbon chain lengths of acyl-CoA as substrates for Cer synthesis (48–50). Thus, it is possible that selective alterations in CerS isoform expression late in differentiation could account for the increase in Cer species containing very long chain amide-linked FA, including 2-OH FA. Although 2-OH FA have not yet been examined as substrates for CerS-dependent Cer formation, it also is possible that one or more of the CerS isoforms could preferentially utilize 2-OH FA-CoA as substrates.

We recently reported defects of acylCer/acylGlcCer and very long chain FA generation in transgenic mice that lack an enzyme for the elongation of very long chain FA (ELOVL4) (51). Animals with defective ELOVL4 have a severe epidermal permeability barrier abnormality that results in early neonatal death (51). Epidermal lamellar bodies display abnormal contents, and extracellular lamellar membrane structures are lacking in the stratum corneum in the epidermis of these transgenic mice. Moreover, transgenic mice with defective ELOVL4 display decreased very long chain 2-OH FA (≥C28) levels in both free FA and amide-linked FA of Cer/GlcCer (51). Here, we demonstrate that a near absence of lamellar bodies also occurs in CHK with siRNA-induced blockade of FA2H expression. Thus, in addition to epidermal acylCer/acylGlcCer, both 2-OH Cer/2-OH GlcCer appear to be critical for the formation of the epidermal lamellar bodies. Deficiency or abnormal lamellar body formation occurs in Harlequin ichthyosis, a hereditary skin disease with severe scaling ichthyosis, accompanied by abnormal lamellar membrane structures in the stratum corneum and abnormal epidermal permeability barrier function, despite near-normal levels of total Cer in the stratum corneum (52). Recent studies revealed that defects of lamellar body formation in Harlequin ichthyosis are associated with mutations of the ABCA12 gene, which normally encodes an ATP-binding cassette transporter protein (53, 54). These prior studies and the current results suggest that abnormal formation of lamellar bodies can be the primary cause for epidermal permeability barrier abnormality(ies). ABCA12 normally appears to transport precursor lipids to lamellar bodies (53). However, the roles of 2-OH Cer/2-OH GlcCer, as well as acylCer/acylGlcCer, in lamellar body formation or lamellar membrane organization remains unknown. In model lipid systems, intermolecular hydrogen bonding is increased by the 2-OH moiety of Cer (18, 19), which may contribute to the organization of lamellar bilayer structures. Yet, based on the current results, it remains unresolved as to how decreased hydrogen bonding alone could explain a failure to form lamellar body structure/contents. Nevertheless, our current study suggests that FA2H activity is required for epidermal permeability barrier function. As such, these results represent the first evidence of physiological relevance for FA2H in a mammalian tissue/organ.

In addition to their importance for barrier function, 2-hydroxylation could also protect keratinocytes from apoptosis. Cer are proapoptotic in a variety of cell types (see review in Ref. 55) including keratinocytes (56). Accordingly, previous studies demonstrate that 2-OH Cer are less potent as proapoptotic species than *N*-non-hydroxyacylsphingosine in human leukemia U937 cells (22). Interestingly, differentiated CHK are more resistant to apoptosis, induced either by ultraviolet irradiation (57) or by tumor necrosis factor-related apoptosis-inducing ligand (58). Because increased cellular Cer accounts for a part of the mechanism of ultraviolet irradiation (56, 59) or tumor necrosis factor-related apoptosis inducing ligand-induced apoptosis (see review in Ref. 60), changes in Cer molecular species may be an important protective mechanism. Taken together with the increase in 2-OH Cer generation that occurs during keratinocyte differentiation, the differentiated keratinocyte may render a pool of Cer less proapoptotic in response to oxidative stressors, such as ultraviolet irradiation.

In conclusion, we now demonstrate that FA2H is responsible for the differentiation-associated increase in 2-OH Cer and 2-OH GlcCer generation in CHK. Moreover, keratinocyte differentiation up-regulates the gene and protein expression of FA2H. Increases in sphingolipids containing 2-OH FA in keratinocytes likely accompany increases in fatty acid elongation, acyl-CoA-dependent Cer synthase activities and sphingol generation. Finally, these 2-OH Cer and 2-OH GlcCer species appear to have a critical role in the normal formation and function of the epidermal permeability barrier.

Acknowledgment—We gratefully acknowledge the expert technical support of Sally Pennypacker for cell culture.

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