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Apoptosis Occurs *via* the Ceramide Recycling Pathway in Human HaCaT Keratinocytes

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Keratinocytes contain abundant ceramides compared to other cells. However, studies on these cells have mainly focused on the barrier function of ceramide, while their other roles, such as those in apoptosis or cell cycle arrest, have not been well addressed. In this study, we investigated the apoptosis-inducing effect of exogenously added cell-permeable ceramides in HaCaT keratinocytes. We found that *N*-hexanoyl sphingosine (C6-ceramide) induced apoptosis efficiently through the accumulation of long chain ceramides. On the other hand, *N*-acetyl sphingosine (C2-ceramide) induced neither apoptosis nor accumulation of long chain ceramides. We also found that exogenously added C6-ceramide was hydrolyzed to sphingosine and then reacylated in long chain ceramides (ceramide recycling pathway), but that C2-ceramide was not hydrolyzed and thus not recycled. We propose that this is the basis for the chain length-specific heterogeneity observed in ceramide-induced apoptosis in these cells. These results also imply that keratinocytes utilize exogenous sphingolipids or ceramides to coordinate their own ceramide compositions.

Key words: apoptosis, ceramide, ceramide recycling pathway, human HaCaT keratinocytes, long chain ceramide accumulation.

Abbreviations: Cer, ceramide; DGK, *sn*-1,2-diacylglycerol kinase; DHC, dihydroceramide; FB1, Fumonisin B1; KC, keratinocyte; SM, sphingomyelin; SMase, sphingomyelinase; Sph, sphingosine; SPT, serine palmitoyltransferase; TLC, thin layer chromatography.

Ceramide (Cer) has emerged as an important mediator that affects cell differentiation and apoptosis in many cells (*1*). Over the past decade, elevated cellular Cer levels have been linked to cellular stress resulting from exposure to certain agents, such as cytokines, chemotherapeutic agents, irradiation, and exogenous lipopolysaccharides, or from increases in reactive oxygen species (*2–5*). Cer levels can increase through accelerated sphingomyelin (SM) hydrolysis, following the activation of sphingomyelinase (SMase) (*6–9*), and/or through accelerated *de novo* Cer synthesis, following activation of either dihydrosphingosine-N-acyltransferase (*10–13*) or serine palmitoyltransferase (SPT) (*14–16*).

Keratinocytes (KC) are one of several cell types that are susceptible to Cer challenge (*17*, *18*). In fact, studies on KC have demonstrated that after treatment with either exogenous cell-permeable Cer or bacterial SMase, alterations in cellular Cer levels inhibit DNA synthesis (*19*, *20*), initiate cell differentiation (*21*), and induce apoptosis (*22–24*). However, limited investigation has been performed regarding the mechanisms involved in apoptosis in KC.

When added to cultured cells in a permeable form such as C2-Cer (*N*-acetylsphingosine) or C6-Cer (*N*-hexanoylsphingosine), Cer is generally able to cause stress-induced apoptosis in a stereospecific manner (*25*). Additionally, inhibition of the formation of Cer has been shown, at least in some cases, to inhibit the progression of apoptosis (*26–29*). Exogenous Cer-induced apoptosis reportedly occurs following the release of cytochrome *c* from mitochondria into the cytoplasm (*30*) and the subsequent activation of caspases (*31*). The precise mechanism by which endogenous Cer causes cytochrome *c* release is not known. Furthermore, the comparative effectiveness of C2-Cer and C6-Cer differs depending on the cell type. For example, C2-Cer reportedly induces apoptosis more effectively in T cell lymphocyte cell lines HL-60 and Jurkat (*32*, *33*), whereas C6-Cer is more effective in human hepatoma cell line HepG2 (*34*).

In the study presented here, we examined the effects of C2-Cer and C6-Cer on apoptosis in HaCaT keratinocytes. We found that C6-Cer induced apoptosis easily, but that C2-Cer did not, indicating that these epidermal cells exhibit selective responses to the carbon length of the fatty acids of Cer. To understand this selectivity, we investigated the mechanism under lying the C6-Cer–induced apoptosis. Our studies revealed that the Cer recycling pathway contributes to the C6-Cer–induced apoptosis in HaCaT cells.

EXPERIMENTAL PROCEDURES

Materials—C2-Cer (C2:0, d18:1), C6-Cer (C6:0, d18:1), Fumonisin B1 (FB1), and ISP-1 (myriocin) were all purchased from Sigma (St. Louis, MO). *sn*-1,2-Diacylglycerol kinase (DGK) was from Calbiochem (San Diego, CA), and C6-dihydroceramide (DHC) (C6:0, d18:0) was from Biomol (Plymouth Meeting, PA). [^{32}P]ATP and [3-^{3}H]Sphingosine (Sph) were obtained from PerkinElmer Life Sciences

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(Wellesley, MA) and American Radiolabeled Chemicals (Newington, NH), respectively, and *N*-hexanoyl [3-^{3}H]-sphingosine was from PerkinElmer Life Sciences. All reagents were more than 98% pure, as judged on TLC analysis.

Cell Culture—Human keratinocyte cell line HaCaT (*35*), derived from human epidermis, was cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Iwaki, Chiba, Japan) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin, both from Sigma). The cultures were maintained at 37°C under 5% CO_2 in humidified air.

Flow Cytometry of Annexin V Binding—Cells were plated onto 6-well plates, grown to 80% confluence, and then treated with C2-Cer, C6-Cer or C6-DHC (the stock solution was 10 mM in 99.5% ethanol, with an equal volume of ethanol as a control). The cells were then trypsinized and washed twice with PBS. Apoptosis was determined by flow cytometric detection of phosphatidylserine translocation using fluorescein-labeled Annexin V and a MEBCYTO apoptosis kit™ (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions (*36*).

Measurement of Total Cer Levels—Total endogenous Cer levels were measured by the DGK method (*37*). In this *in vitro* method, all endogenous Cer is radiolabeled through phosphorylation by DGK and then separated for easy quantification. Briefly, total cellular lipids were extracted using the standard Bligh-Dyer protocol (*38*), dried, and then resuspended in micelle buffer (7.5% *n*-β-D-octyl glucopyranoside and 19.4 mg/ml α-dioleoyl-phosphatidylglycerol). This mixture was incubated with 0.1 units of *E. coli* DGK and 1 μCi [^{32}P]ATP for 1 h at 37°C. Lipids were separated on Silica Gel 60 thin layer chromatography (TLC) plates (Merck, Damstadt, Germany) with a solvent system of chloroform/methanol/15 mM $CaCl_2$ (7.5:4.4:1, v/v). The bands corresponding to C1P derived from C16- to C24-Cer (long chain Cer) were quantified using an imaging analyzer (BAS2500, Fuji Film, Tokyo, Japan).

N-*Acetylation of [3-^{3}H]Sph*—*N*-Acetyl [3-^{3}H]sphingosine ([^{3}H]C2-Cer) was prepared from [3-^{3}H]Sph by *N*-acetylation (*39*). Dried [3-^{3}H]Sph was dissolved in 0.008 N NaOH in methanol/acetic anhydride (1:1, v/v) by sonication. Acetylation reactions were allowed to proceed at 37°C for 2 h. The remaining anhydride was hydrolyzed by the addition of 10 volumes of 0.2 N NaOH in methanol. Following 1 h incubation at room temperature, the C2-Cer formed was extracted with 8.8 volumes of chloroform/methanol (98:78, v/v) and 4.5 volumes of 1 M KCl. The organic phase was washed twice with chloroform/methanol/water (3:48:47, v/v) and dried. The *N*-acetyl [3-^{3}H]sphingosine ([^{3}H]C2-Cer) was separated from [3-^{3}H]Sph by TLC with a solvent system of butanol/acetic acid/water (3:1:1, v/v), and then purified by re-extraction from the plates using a Sep-Pak plus Silica cartridge (Waters Corp., Milford, MA).

Cellular Metabolism of [3-^{3}H]Sph, C2-Cer, and C6-Cer—HaCaT cells were treated for 6 h with 25 μM [3-^{3}H]Sph (0.5 μCi/ml), 25 μM C2-Cer (0.5 μCi/ml), or 25 μM C6-Cer (0.5 μCi/ml), in the absence or presence of FB1 (50 μM). After the cells had been washed, total lipids were extracted and separated by TLC with a solvent system of chloroform/methanol/acetic acid (190:9:1, v/v). The TLC plates were then imaged on X-ray film by exposure at –80°C.

In Vitro *Ceramidase Assay*—After three washes with PBS, HaCaT cells were resuspended in Buffer A (25 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, and Complete™ protease inhibitor mixture (Roche Applied Science, Basel, Switzerland)), sonicated for 5 s, and then chilled on ice for 30 s. The process of sonication and chilling was performed twice more. The total cell lysates were centrifuged at 600 × *g* for 5 min. The supernatants were removed and centrifuged for 10 min at 8,000 × *g*. The resulting pellets were resuspended in Buffer A and used as the enzyme source.

Ceramidase activity was determined as the release of [3-^{3}H]Sph from a radiolabeled substrate, [^{3}H]C2-Cer or [^{3}H]C6-Cer. Briefly, 40 μl of a cell lysate (containing 10 μg total protein) in Buffer A was mixed with 40 μl of [^{3}H]C2-Cer or [^{3}H]C6-Cer (0.5 μCi per reaction) in Buffer A containing 0.5% Triton X-100 in a 1.5-ml tube. After 1 h incubation at 37°C, the reaction was terminated by adding 6.5 volumes of chloroform/methanol (1:1, v/v). The organic phase was dried, and the lipids were separated by TLC with a solvent system of chloroform/methanol/15 mM $CaCl_2$ (7.5:4.4:1, v/v). The TLC plates were then imaged on X-ray film following exposure at –80°C.

RESULTS

C6-Cer, but Not C2-Cer, Induces Apoptosis in HaCaT Cells—Using immortalized, nontransformed HaCaT cells, we examined the effects of exogenously added cell-permeable Cer, specifically C2-Cer and C6-Cer, in KC. Cells were analyzed by flow cytometry for Annexin V binding and PI dye exclusion as evidence of apoptosis. In cells treated for 24 h, C6-Cer induced apoptosis more efficiently compared to C2-Cer (Fig. 1). This result was similar to those of other studies, which showed that the effects of different Cer are cell type-specific (*32*, *33*). Such discrepancies suggest that different mechanisms are involved in the apoptosis induced by C6-Cer and C2-Cer.

C6-Cer Induces the Accumulation of Long Chain Cer, but C2-Cer Does Not—Some apoptotic agents, such as etoposide (*40*) and daunorubicin (*10*), have been shown to stimulate the generation of endogenous long chain Cer. Using HaCaT cells, we examined whether or not long chain Cer are accumulated during apoptosis induced with exogenous Cer. Cells were treated for 6 h with 25 μM C2-Cer or C6-Cer, and then the amount and species of Cer were determined by the DGK method. The levels of endogenous long chain Cer (C16- to C24-Cer), which are natural Cer, as opposed to the synthetic C2- and C6-Cer, increased significantly in response to C6-Cer treatment (approximately 6.5-fold). In contrast, a much lower increase (approximately 2-fold) was observed in response to C2-Cer treatment (Fig. 2). These results indicated that the C6-Cer–induced apoptosis was accompanied by the accumulation of long chain Cer, similar to the results obtained for other apoptotic agents (*39*, *10*), and raised the possibility that the apoptosis was a result of this accumulation.

Long Chain Cer Is Generated via *the Reacylation of Sph*—Next, we investigated the mechanism underlying the generation of the long chain Cer in HaCaT cells

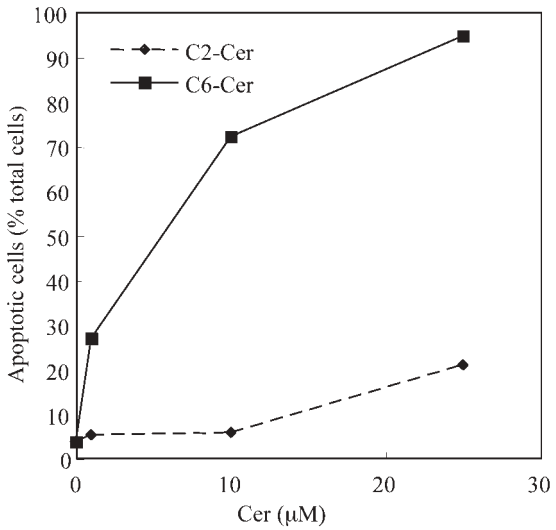

Fig. 1. **C6-Cer induces apoptosis in HaCaT cells efficiently, whereas C2-Cer does not.** HaCaT cells were incubated with the indicated concentrations of C2-Cer or C6-Cer for 24 h. Apoptotic cells were identified by means of Annexin V binding and PI dye exclusion detected by flow cytometry. Apoptotic cells (Annexin V^+/PI^+ and Annexin V^+/PI^- cells) are presented as a percentage of the 10,000 cells examined.

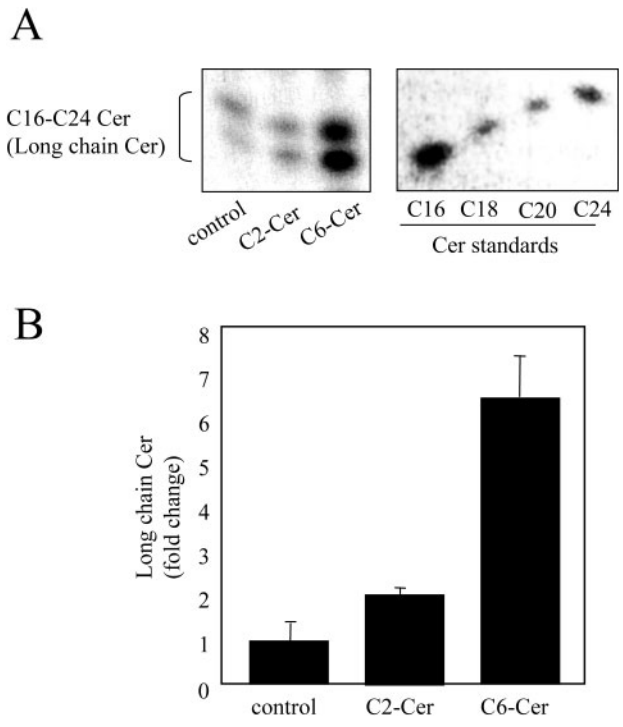

Fig. 2. **C6-Cer is a potent inducer of long chain Cer accumulation in HaCaT cells.** The levels of long chain Cer were examined in HaCaT cells before (control) or after 6 h treatment with 25 μM C2-Cer or C6-Cer. Total cellular Cer levels were measured by means of DGK assay as described under "EXPERIMENTAL PROCEDURES. (A) Long chain Cer are visible on TLC as C1P. The left panel shows the C1P derived from the C16- to C24-Cer (long chain Cer) from the HaCaT cells, and the right panel shows Cer standards. (B) The bands derived from long chain Cer in A were quantified, and the values are expressed as a ratio to the values for untreated cells. Data shown are the averages of three experiments.

treated with C6-Cer. To date, three possible Cer generation pathways are known, a *de novo* pathway that involves the catalytic action of SPT (*14*, *15*); the hydrolysis of SM, which is thought to be the main pathway involved in the SM cycle (*6–9*); and the Cer recycling pathway, in which sphingoid bases generated through the hydrolysis of complex sphingolipids are recycled via Cer synthesis (*41*). Both the *de novo* pathway and the recycling pathway involve dihydrosphingosine-*N*-acyltransferase, which catalyzes the *N*-acylation of dihydrosphingosine. To determine whether or not the accumulated long chain Cer associated with C6-Cer–induced apoptosis was generated through the action of this enzyme, we pretreated HaCaT cells with dihydrosphingosine-*N*-acyltransferase inhibitor FB1, incubated the cells overnight with C6-Cer, and then measured the Cer levels. As shown in Fig. 3A, FB1 blocked the C6-Cer–induced generation of long chain Cer significantly.

We also assessed whether or not inhibition of Cer synthesis could attenuate the C6-Cer–induced apoptosis. After overnight pretreatment with 50 μM FB1, HaCaT cells were treated with C6-Cer (10 μM) for 24 h, and then apoptotic cells were analyzed by flow cytometry using Annexin V binding and PI dye exclusion. When the generation of long chain Cer was inhibited by FB1, C6-Cer–induced apoptosis was significantly attenuated (Fig. 3B), demonstrating that C6-Cer–induced apoptosis does involve dihydrosphingosine-*N*-acyltransferase. Moreover, these results confirm that C6-Cer–induced apoptosis in HaCaT cells results from long chain Cer accumulation.

Since SPT is reportedly up-regulated by apoptotic inducers like UV radiation (*42*) and endotoxin (*43*), we examined its involvement in the long chain Cer accumulation observed here. HaCaT cells were treated for 6 h with 25 μM C6-Cer in the presence or absence of 50 nM ISP-1 (myriocin), a specific inhibitor of SPT. ISP-1 did not have any effect on the long chain Cer generation induced by C6-Cer (Fig. 3C). These results raised the possibility that the Cer recycling pathway, in which C6-Cer would be hydrolyzed to Sph and then acylated into a long chain Cer, might contribute to the accumulation and the resulting apoptosis.

To investigate this possibility, HaCaT cells were grown for 6 h in the presence of 25 μM *N*-hexanoyl [3-^{3}H]sphingosine ([^{3}H]C6-Cer; 0.5 μCi/ml), and then the labeled lipids were analyzed. Incorporation of the [^{3}H]label into long chain Cer was detected (Fig. 3D), indicating that the [^{3}H]Sph backbone was supplied by the labeled C6-Cer. These studies provide evidence that the long chain Cer accumulated in response to C6-Cer treatment are generated via hydrolysis of C6-Cer by ceramidases and subsequent reacylation of Sph by dihydrosphingosine-*N*-acyltransferase.

Exogenously Added Sph Induces Long Chain Cer Accumulation and Apoptosis in HaCaT Cells—Since the acylation of Sph appeared to be crucial for C6-Cer–induced apoptosis and long chain Cer accumulation, we further examined whether or not treatment with Sph has similar consequences. Sph treatment (25 μM) for 6 h stimulated long chain Cer accumulation, by approximately 4.5-fold (Fig. 4A). Moreover, pretreatment of the cells overnight with FB1 (50 μM) remarkably inhibited this accumulation. This result suggested that the generation

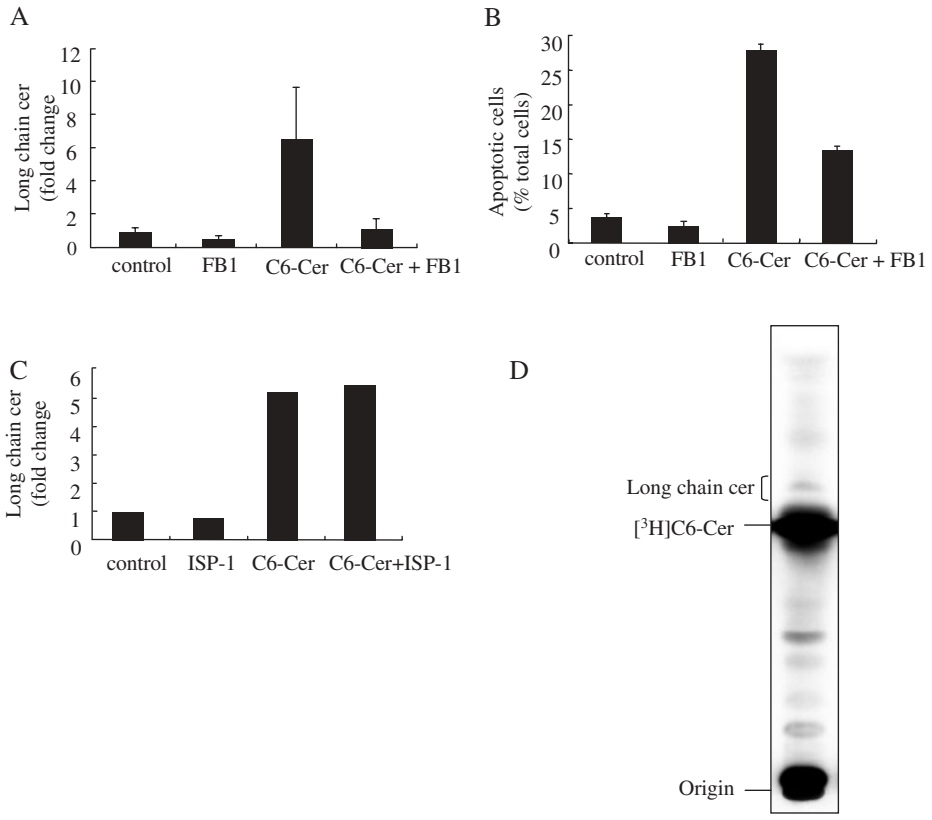

Fig. 3. **C6-Cer–induced long chain Cer generation occurs through Cer recycling.** (A–C) HaCaT cells were preincubated overnight with or without 50 μM FB1 (A, B) or 50 nM ISP-1 (C). (A, C) Cells were treated for 6 h with 25 μM C6-Cer, and then the levels of long chain Cer were measured and are presented as in Fig. 2. (B) Cells were treated for 24 h with 10 μM C6-Cer and then the number of apoptotic cells was determined as in Fig. 1. (A, B) Data shown are the averages of three experiments. (C) The result of a single experiment is shown. (D) HaCaT cells were treated for 6 h with 25 μM [^{3}H]C6-Cer (*N*-hexanoyl [3-^{3}H]sphingosine; 0.5 μCi/ml), and then the labeled lipids were extracted and analyzed by TLC as described under "EXPERIMENTAL PROCEDURES."

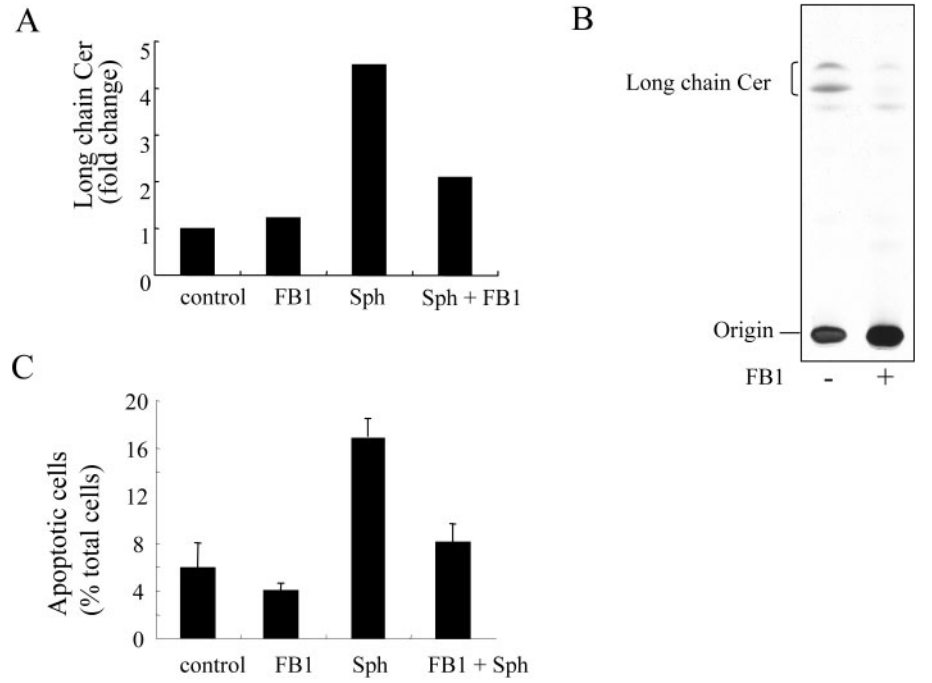

Fig. 4. **Exogenous Sph induces long chain Cer accumulation and apoptosis in HaCaT cells.** HaCaT cells were preincubated overnight with or without 50 μM FB1. (A) Cells were treated for 6 h with 25 μM, and then the levels of long chain Cer were measured and are presented as in Fig. 2. The result of a single experiment is shown in Fig. 4A. (B) Cells were treated for 6 h with [3-^{3}H]Sph 5 μCi/ml), and then the labeled lipids were extracted by the Bligh and Dyer method and analyzed by TLC as described under "EXPERIMENTAL PROCEDURES." (C) Cells were treated with 10 μM C6-Cer, and then the number of apoptotic cells was determined by means of Annexin V binding and PI dye exclusion as in Fig. 1. Data shown in (C) are the averages of three experiments.

of Sph following treatment with C6-Cer was important for the long chain Cer accumulation.

Next, to investigate the metabolism of Sph, FB1-pretreated HaCaT cells were treated for 6 h with 0.5 μCi/ml [3-^{3}H]Sph (25 μM). The [3-^{3}H]Sph was incorporated into long chain Cer, and this incorporation was inhibited significantly by the FB1 (Fig. 4B). We also investigated the effect of Sph on apoptosis in HaCaT cells, and found that apoptosis was induced in response to Sph treatment (10 μM, 24 h). FB1 also inhibited the Sph-induced apoptosis significantly, indicating that inhibition of dihydrosphingosine-*N*-acyltransferase attenuated the apoptosis (Fig. 4C). These results constitute further evidence that long chain Cer accumulation

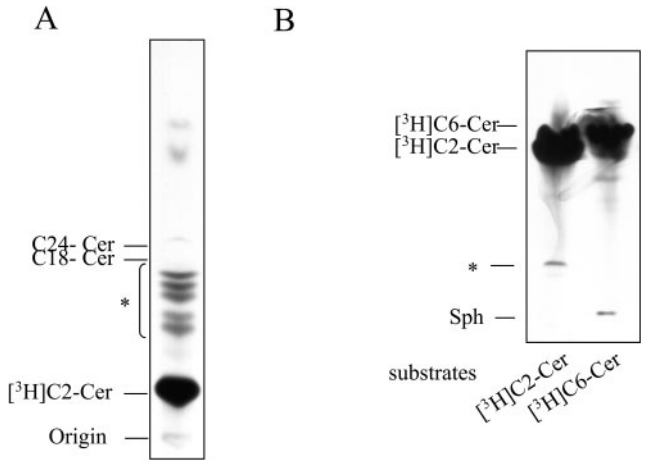

Fig. 5. **HaCaT cells can hydrolyze C6-Cer, but not C2-Cer.** (A) Metabolism of [^{3}H]C2-Cer (*N*-acetyl [3-^{3}H]sphingosine) in HaCaT cells. Cells were incubated with 25 μM [^{3}H]C2-Cer (0.5 μCi/ml) for 6 h. The labeled lipids were extracted and analyzed by TLC as described under "EXPERIMENTAL PROCEDURES. The asterisk indicates metabolites of impurities included in [^{3}H]C2-Cer. (B) Cell lysates (10 μg protein), removed nucleus and lysosome fractions were assayed at pH 7.4 for ceramidase activity using 1 μM [^{3}H]C2-Cer or [^{3}H]C6-Cer (0.5 μCi per reaction) as the substrate, as described under "EXPERIMENTAL PROCEDURES." The released product, [3-^{3}H]Sph, was separated by TLC and then imaged on X-ray film by exposure at –80°C. The asterisk indicates metabolites of impurities included in [^{3}H]C2-Cer.

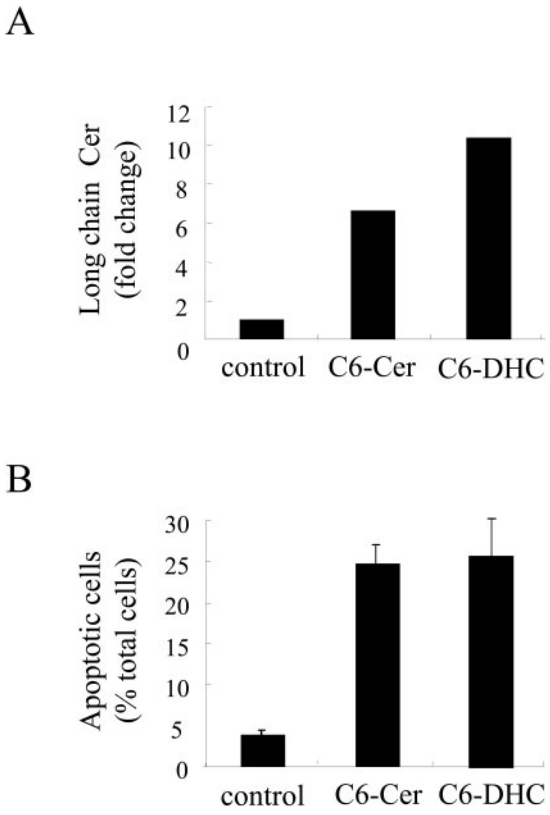

Fig. 6. **C6-DHC induces long chain Cer accumulation and apoptosis in HaCaT cells.** (A) Cells were treated for 6 h with 25 μM C6-Cer or C6-DHC, and then the levels of long chain Cer were measured and are presented as in Fig. 2. The result of a single experiment is shown. (B) Cells were treated for 24 h with 10 μM C6-Cer or C6-DHC and then the number of apoptotic cells was determined as in Fig. 1. Data shown are the averages of three experiments.

occurring through the acylation of Sph plays roles in certain apoptosis pathways, including those responding to C6-Cer.

HaCaT Cells Hydrolyze C6-Cer but Not C2-Cer—As noted above, C6-Cer induced apoptosis in HaCaT cells, as a result of C6-Cer recycling, but C2-Cer did not. We therefore studied the metabolism of exogenously added C2-Cer in HaCaT cells. Cells were treated with 0.5 μCi/ml *N*-acetyl [3-^{3}H]sphingosine ([^{3}H]C2-Cer; 25 μM) for 6 h, and then labeled lipids were analyzed by TLC. As shown in Fig. 5A, the [^{3}H]Sph backbone from the C2-Cer was not incorporated into the newly synthesized long chain Cer form.

One possibility for the discrepancy between the responses to the two short chain Cer, is that C6-Cer is recognized as a substrate by ceramidases, but C2-Cer is not. To assess this possibility, we investigated the hydrolysis of C2-Cer and C6-Cer by endogenous ceramidases from HaCaT cells. Cell lysates were prepared from HaCaT cells, and the nucleus and lysosome fractions were removed. Ceramidase activity was assayed at pH 7.4 using [^{3}H]C2-Cer and [^{3}H]C6-Cer as substrates. C6-Cer was hydrolyzed to Sph by the ceramidases in the HaCaT cells, but C2-Cer was not (Fig. 5B).

It is well known that exogenous ceramides, but not dihydroceramides, induces apoptosis in various cell types (*25*). We examined whether or not exogenously added C6-dihydroceramide (DHC) induces long chain Cer accumulation and apoptosis in HaCaT cells. Interestingly, similar results were obtained with C6-Cer (Fig. 6A and B). C6-DHC might be hydrolyzed to dihydro-Sph and converted to long chain DHC, and then long chain Cer. Both C6-Cer and C6-DHC might be converted to the same molecules. These results indicate that exogenously added Cer does not act as a direct inducer of apoptosis, but that the long chain Cer reacylated *via* the recycling pathway is crucial for apoptosis in HaCaT cells.

DISCUSSION

The effects of C2-Cer and C6-Cer on apoptosis are cell type–specific, although both have often been used to induce apoptosis in many types of cells. Few studies, however, have examined C2-Cer and C6-Cer use in KC. In this study, we observed a remarkable difference between C2-Cer and C6-Cer in the ability to induce apoptosis in HaCaT cells, namely, C6-Cer induced apoptosis efficiently, but C2-Cer did not (Fig. 1). We demonstrated that C6-Cer is incorporated, hydrolyzed to Sph, and then reacylated into long chain Cer (the Cer recycling pathway), and that the accumulation of long chain Cer contributed to the induction of apoptosis. This scenario is summarized in Fig. 7. Such a Cer recycling pathway was recently reported in A549 cells (*41*), and we found it to be crucial for the C6-Cer–induced apoptosis in KC. We also found that although exogenously added SMase increased the endogenous Cer level in HaCaT cells, apoptosis was not induced (data not shown), suggesting that the Cer

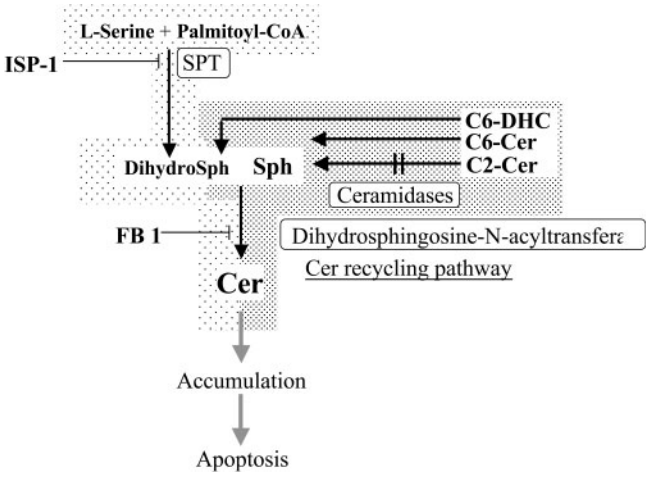

Fig. 7. **Cer recycling is crucial for C6-Cer–induced apoptosis in HaCaT cells.** C6-Cer is incorporated, hydrolyzed to Sph, and reacylated into long chain Cer, and the long chain Cer accumulation contributes to the induction of apoptosis. C6-DHC induces long chain Cer accumulation and apoptosis in HaCat cells.

generated through that pathway does not contribute to apoptosis in these cells. Figure 6 shows that C6-DHC induced apoptosis via the accumulation of long chain Cer. These results also indicate that exogenously added Cer dose not act as a direct inducer of apoptosis in HaCaT cells.

In HaCaT cells, C6-Cer was hydrolyzed to Sph (Fig. 5). Additionally, Sph treatment induced long chain Cer accumulation in these cells and apoptosis via dihydrosphingosine-*N*-acyltransferase (Fig. 4). In contrast, C2-Cer could not be hydrolyzed or reacylated, because C2-Cer was not recognized as a substrate by cellular ceramidases, the initial step in the recycling. This, then, might account for the selectivity in the induction of apoptosis observed with C2- and C6-Cer.

In a recent study, skin-targeted conditional SPT knockout mice exhibited a normal Cer composition in the epidermis soon after birth (*44*), implying that epidermal cells have some ability to maintain a normal Cer composition without *de novo* Cer synthesis. Considering this finding, we can safely say that epidermal cells positively adjust their Cer composition by utilizing exogenous sphingolipids. In the study presented here, we demonstrated that C6-Cer in HaCaT cells was deacylated to Sph by ceramidases and then recycled into long chain Cer. These observations, for the first time, reveal that epidermal cells have a Cer recycling pathway capable of utilizing exogenous sphingolipids. Since the epidermis contains abundant Cer yet does not depend solely on its *de novo* synthesis for its supply, we expect that the Cer recycling pathway is physiologically important in this tissue.

In this study, we investigated an apoptotic pathway in HaCaT cells. When KC differentiate into cornified cells, cell death occurs. Therefore, we might say that terminal differentiation of KC causes one kind of programmed cell death. Furthermore, the Cer content in the epidermis significantly increases during cell differentiation (*45*). Although the molecular structures of epidermal glucosylceramide and Cer have been studied extensively (*46–48*), the mechanisms that regulate Cer synthesis are not yet known. Activation of Cer synthesis during cell differentiation might result not only from accelerated *de novo* Cer synthesis *via* SPT, but also from accelerated hydrolysis and reacylation involving exogenous sphingolipids. Ceramidases and dihydrosphingosine-*N*-acyltransferase are principally responsible for Cer recycling. We actually found that the expression of alkaline ceramidase, which is highly expressed in skin tissue, increases during cell differentiation (RT-PCR, data not shown). Thus we propose that the accumulation of Cer during cell differentiation might be regulated by enzymes involved in epidermal Cer recycling, such as alkaline and other ceramidases, and dihydrosphingosine-*N*-acyltransferase.

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