Novel synthetic ceramide derivatives increase intracellular calcium levels and promote epidermal keratinocyte differentiation

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Abstract Ceramide is an important constituent of stratum corneum lipids, which act as both physical barriers and signal modulators. We synthesized several ceramide derivatives and investigated their effects on keratinocyte differentiation. RT-PCR and Western blotting showed that the novel synthetic ceramide derivatives K6PC-4 (N-ethanol-2-hexyl-3-hydroxy-decanamide), K6PC-5, (N-ethanol-3-oxo-2-tetradecyl/hexadecyl-octadecanamide/eicosanamide)and K6PC-9 [N-(1,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide] markedly increased keratin 1 and involucrin expression in normal human epidermal keratinocytes cultured in vitro. These ceramide derivatives elicited a rapid transient increase in intracellular calcium levels, which were measured using laser scanning confocal microscopy. In addition, K6PC-4, K6PC-5, and K6PC-9 stimulated the phosphorylation of p42/44 extracellular signal-regulated kinase and c-Jun N-terminal kinase. In a reconstituted epidermis model, K6PC-4, K6PC-5, and K6PC-9 significantly increased keratin 1 expression in the suprabasal layer. These results indicate that these novel synthetic ceramide derivatives have the potential to promote keratinocyte differentiation, suggesting that the lipid molecules are applicable for treating skin diseases involving abnormal keratinocyte differentiation.—Kwon, Y. B., C. D. Kim, J-K. Youm, H. S. Gwak, B. D. Park, S. H. Lee, S. Jeon, B. J. Kim, Y-J. Seo, J-K. Park, and J-H. Lee. **Novel synthetic ceramide derivatives increase intracellular calcium levels and promote epidermal keratinocyte differentiation. *J. Lipid Res.* 2007.** 48: **1936–1943.**

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Sphingolipids, which consist of a hydrophobic ceramide moiety and various hydrophilic head groups, are integral structural constituents of the plasma membrane of eukaryotic cells. In addition to structural roles, sphingolipids and their metabolites, including ceramides, sphingosine, and sphingosine-1-phosphate (S1P), play important roles in cellular signaling, such as in proliferation, apoptosis, cellular senescence, growth arrest, and differentiation (1). For example, C2-ceramide (or *N*-acetylsphingosine) activates phosphatidylinositol 3-kinase and conventional protein kinase C in cultured airway smooth muscle cells, contributing to cell survival (2), and is a critical mediator for triggering photoreceptor apoptosis in the mammalian retina (3). In keratinocytes cultured in vitro, C2-ceramide inhibits cell proliferation and promotes differentiation (4). S1P enhances the differentiation of cultured keratinocytes, has an antiproliferative effect that protects cells from programmed cell death (5), and inhibits keratinocyte proliferation via Akt/protein kinase B inactivation (6).

The extraordinarily low permeability of skin is caused by the unique structure of the extracellular membrane in the stratum corneum; this structure comprises ceramides, cholesterol, and free fatty acids (7). Many reports suggest that ceramides are required for the normal skin permeability barrier, which controls transcutaneous water movement and prevents the penetration of exogenous materials from the environment (8, 9). The replenishment of ceramide with other lipids results in the acceleration of the permeability barrier recovery rate (10, 11).

Synthetic ceramides, or pseudoceramides, have the important structural components of natural ceramides (i.e., hydroxyl groups, two alkyl groups, and an amide bond). Because of these structural similarities, several synthetic ceramides are reported to form the multilamellar struc-

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ture observed in the intercellular spaces of the stratum corneum (12). Moreover, the topical application of synthetic ceramide accelerates the repair of skin in which the permeability barrier has been disturbed (13, 14). Most studies of synthetic ceramides have focused on the barrier function of skin. Because ceramides are critical in cellular signaling, we hypothesized that synthetic ceramides also play a role in cellular signaling by either triggering cellular responses or acting as a second messengers.

Recently, we developed the synthetic ceramide PC-9S (N-(2,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide) and reported its effects on skin barrier function (15–18). During those studies, we postulated that PC-9S also has a biological effect on epidermal differentiation. Here, we synthesized new ceramide derivatives of PC-9S and investigated their effects as signaling molecules on cultured human keratinocytes. The changes in intracellular calcium concentration induced by the synthetic ceramide derivatives were measured, and the effects on terminal differentiation were evaluated in cultured keratinocytes and reconstituted epidermis.

MATERIALS AND METHODS

Skin samples

Neonatal foreskin samples were obtained with the informed consent of the donors' guardians, in accordance with the ethics committee approval process of Chungnam National University Hospital, Daejeon, Korea. The study was conducted according to the principles of the Declaration of Helsinki.

Cell culture

Specimens were sterilized briefly in 70% ethanol, minced, and then treated with dispase overnight at 4°C. The epidermis was separated and placed in a solution containing 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (Gibco BRL, Rockville, MD) at 37°C for 15 min. After vigorous pipetting, the cells were pelleted and resuspended in a keratinocyte serum-free medium supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Gibco BRL). Third passage keratinocytes (60–75% confluence) were used.

Methyl thiazolyl tetrazolium assay

The methyl thiazolyl tetrazolium (MTT) assay was used to test the cytotoxicity of the test materials. Briefly, keratinocytes (2×10^4) were seeded on 24-well culture plates and treated with test materials for 24 h. The cultures then received 1 mg/ml MTT solution and were incubated for another 4 h. The medium was removed, and the resulting formazan crystal was solubilized in 200 μl of DMSO. The optical density at 540 nm was determined using an ELISA reader.

RT-PCR

Reverse transcription of 2 μg of total RNA was performed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Aliquots of the RT mixture were subjected to PCR with specific primer sets designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and verified using the Basic Local Alignment Search Tool to ensure that each sequence was unique to the specific target gene. These primers were as follows: involucrin forward, 5′-TAGAGGAGCAGGAGGGACAA-3′, involucrin reverse, 5′-TCTGGGTGCTCTAGGTGCTT-3′; keratin 1 forward, 5′-CTGGCAGACATGGGGATAGT-3′, keratin 1 reverse, 5′-GCCATAGCTGCTACCTCCAG-3′.

Western blots

The cell extracts were prepared using Pro-prep protein extraction solution (Intron, Daejeon, Korea). Protein samples were run on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and incubated with anti-involucrin and anti-loricrin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-actin antibody (Sigma, St. Louis, MO) was used as an internal control. The blots were then incubated with appropriate peroxidase-conjugated secondary antibodies and developed using enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Anti-phosphorylated p42/44 extracellular signal-regulated kinase (ERK), anti-total p42/44, anti-phosphorylated c-Jun N-terminal kinase (JNK), and anti-total JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Intracellular calcium measurement

Cells were grown on cover slips for 24 h under serum-free conditions and then incubated with 5 μM Fura-3/AM for 30 min. After three washes with serum-free medium, the cover slips were mounted in slide chambers and subjected to confocal laser scanning microscopy (Olympus FV500; Olympus, Tokyo, Japan). Scanning was performed at 3 s intervals using a 488 nm excitation argon laser. The ceramide derivatives were added to the slide chamber directly. All images were processed to analyze the changes in Ca^{2+} concentration at the cellular level. The results were expressed as relative fluorescence intensity.

Reconstituted epidermis

Epidermis was reconstituted using a described method (19). Briefly, type I collagen solubilized in 0.1% acetic acid (Bioland, Cheonan, Korea) was mixed with primary cultured skin fibroblasts (2×10^4 cells/ml), neutralized with NaOH, poured into the Transwell (Corning, Corning, NY), and polymerized at 37°C for at least 1 h. After a 2 day culture, the keratinocytes were plated on the dermal matrix and incubated with FAD medium (three parts DMEM, one part F12 medium, supplemented with 10% FBS, 10^{-10} M cholera toxin, 0.4 μM hydrocortisone, and 50 μg/ml ascorbic acid). When the cells reached confluence, the cultures were lifted to the air-liquid interface and incubated for 2 weeks.

Immunohistochemistry

Paraffin sections of the reconstituted epidermis were dewaxed, rehydrated, and washed three times with PBS. After treatment with proteinase K at 37°C for 5 min, the sections were treated with H_2O_2 at room temperature for 10 min, blocked in 0.1% Tween 20 and 1% BSA in PBS for 20 min, and reacted with anti-keratin 1 antibody (Covance, Berkeley, CA) for 1 h. The sections were then incubated with peroxidase-conjugated secondary antibody and visualized using a ChemMate EnVision detection kit (Dako, Carpentaria, CA). Sections without primary antibody were used as negative controls.

RESULTS

To study the biological effect of the synthetic ceramides on keratinocyte differentiation, we synthesized several ceramide derivatives. These chemicals have the characteristic

backbone of ceramide, with hydroxyl groups, two alkyl groups, and an amide bond, and were designed with short-chain alkyls and different hydroxyl groups from PC-9S (**Fig. 1**). To synthesize these compounds, alkyl ketene dimer (AKD) was first synthesized from acyl chloride with toluene and triethylamine. After extracting the AKD from the first reaction, 3-amino-1,2-propanediol [for K6PC-4 (N-ethanol-2-hexyl-3-hydroxy-decanamide)], 2-amino-1,3-propanediol [for K6PC-5 (N-ethanol-3-oxo-2-tetradecyl/hexadecyl-octadecanamide/eicosanamide)], or monoethanolamine [for K6PC-9 (N-[1,3-dihydroxypropy]-2-hexyl-3-oxo-decanamide)] was added to the reactor, in which the AKD was agitated with ethanol. The final compounds were obtained from the second reaction, recrystallized with heptane, and dried to obtain an off-white powder. To study the effect of the alkyl chain length of ceramide, different alkyl groups were also synthesized.

To investigate the effects of these novel synthetic ceramide derivatives on differentiation, we used primary cultured normal human epidermal keratinocytes (NHEKs). First, we determined the cytotoxicity of each ceramide derivative using the MTT assay to obtain half-maximal lethal dose values (**Table 1**). Because most of the ceramide derivatives were not cytotoxic at doses of <10 μM, we used these doses in the subsequent experiments.

Next, we investigated the effects of these novel synthetic ceramide derivatives on intracellular calcium mobilization. NHEKs were preloaded with Fura-3/AM and then measured using laser scanning confocal microscopy. Two ceramide-related sphingolipids, S1P and sphingosylphosphorylcholine (SPC), which induce keratinocyte differentiation (20), were used as positive controls. As anticipated, S1P and SPC treatments led to a robust increase in intracellular calcium (**Fig. 2**). Similarly, treatment with K6PC-4 and K6PC-5 led to a quick, robust increase in calcium. K6PC-9 and PC-9S also increased the intracellular calcium level, although the cellular response was delayed significantly compared with the other two ceramide derivatives, suggesting that K6PC-9 and PC-9S have a different mode of action. K10PC-5 (N-(1,3-dihydroxypropyl)-2-decyl-3-oxo-tetradecanamide) and K16PC-5 [N-(1,3-dihydroxypropyl)-2- hexadecyl-3-oxo- hexadecanamide] failed to elicit an increase in intracellular calcium, whereas K12PC-5 [N-(1,3-dihydroxypropyl)-2-dodecxyl-3-oxo-hexadecanamide] increased the intracellular calcium at ~4 min after treatment (Fig. 2).

We then tested the effect of these novel synthetic ceramide derivatives on keratinocyte differentiation using RT-PCR analysis. Calcium, the best-characterized keratinocyte-differentiating agent (21, 22), was used as a positive control. Calcium, S1P, and SPC markedly increased the level of the early differentiation marker keratin 1 (**Fig. 3A**). These agents also increased the intermediate differentiation marker involucrin, although SPC was less potent than S1P. Many of the novel synthetic ceramide derivatives increased the levels of involucrin and keratin 1 in NHEKs cultured in vitro (Fig. 3A). From this preliminary screening, we selected three ceramide derivatives for further experiments: K6PC-4, K6PC-5, and K6PC-9. To confirm the effects of the ceramide derivatives on keratinocyte differentiation, we performed Western blot analysis. All of the selected ceramide derivatives increased the involucrin and

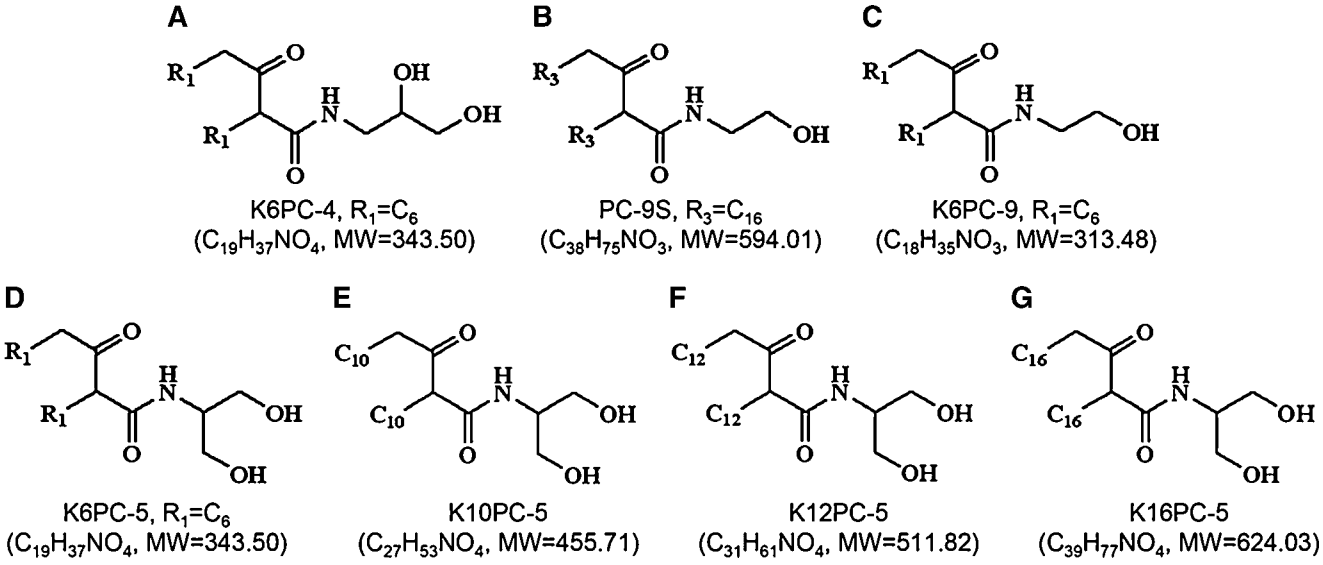

Fig. 1. Molecular structure of the ceramide derivatives. To synthesize N-ethanol-2-hexyl-3-hydroxy-decanamide (K6PC-4), K6PC-5, and K6PC-9, octanoyl chloride was used as the two alkyl group (C6). To examine the effect of alkyl chain length, the alkyl group of K6PC-5 was synthesized with lauroyl chloride (C10; K10PC-5), myristoyl chloride (C12; K12PC-5), or octyl chloride (C16; K16PC-5) instead of octanoyl chloride. The chemical names of each compound are as follows: K6PC-4 (A), *N*-(2,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide (PC-9S) (B), *N*-(1,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide (K6PC-9) (C), *N*-ethanol-3-oxo-2-tetradecyl/hexadecyl-octadecanamide/eicosanamide (K6PC-5) (D), *N*-(1,3-dihydroxypropyl)-2-decyl-3-oxo-tetradecanamide (K10PC-5) (E), *N*-(1,3-dihydroxypropyl)-2-dodecxyl-3-oxo-hexadecanamide (K12PC-5) (F), and *N*-(1,3-dihydroxypropyl)-2- hexadecyl-3-oxo- hexadecanamide (K12PC-5) (G).

TABLE 1. Cytotoxicity of ceramide derivatives. The half-maximal lethal dose (LD_{50}) was measured by MTT assay. Values represent the means and SEM from three independent measurements.

Ceramide	Chemical name	LD_{50} (μM)
K6PC-4	N-ethanol-2-hexyl-3-hydroxy-decanamide	38.4 ± 4.6
K6PC-9	N-(1,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide	58.7 ± 5.6
PC-9S	N-(2,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide	37.7 ± 4.5
K6PC-5	N-ethanol-3-oxo-2-tetradecyl/hexadecyl-octadecanamide/eicosanamide	110.5 ± 8.7
K10PC-5	N-(1,3-dihydroxypropyl)-2-decyl-3-oxo-tetradecanamide	12.3 ± 3.4
K12PC-5	N-(1,3-dihydroxypropyl)-2-dodecyl-3-oxo-hexadecanamide	45.3 ± 4.8
K16PC-5	N-(1,3-dihydroxypropyl)-2-hexadecyl-3-oxo-hexadecanamide	56.9 ± 5.2

loricrin levels in a dose-dependent manner in NHEKs cultured in vitro (Fig. 3B).

Because sphingolipid metabolites stimulate the mitogen-activated protein kinase system in various cell types, we examined the effects of the novel synthetic ceramide derivatives on the phosphorylation activation of ERK and JNK. K6PC-4, K6PC-5, and K6PC-9 stimulated the phosphorylation of p42/44 ERK and JNK (**Fig. 4**).

To demonstrate that blocking the increase in intracellular calcium levels inhibits p42/44 ERK phosphorylation, we preloaded the cells with the calcium chelator BAPTA-AM (50 μM) and Fura-3/AM for 40 min and then added the ceramide derivatives. There was no increase in intracellular calcium (**Fig. 5A**). As anticipated, in this condition, p42/44 ERK was not phosphorylated on treatment with the ceramide derivatives (Fig. 5B). We then investigated

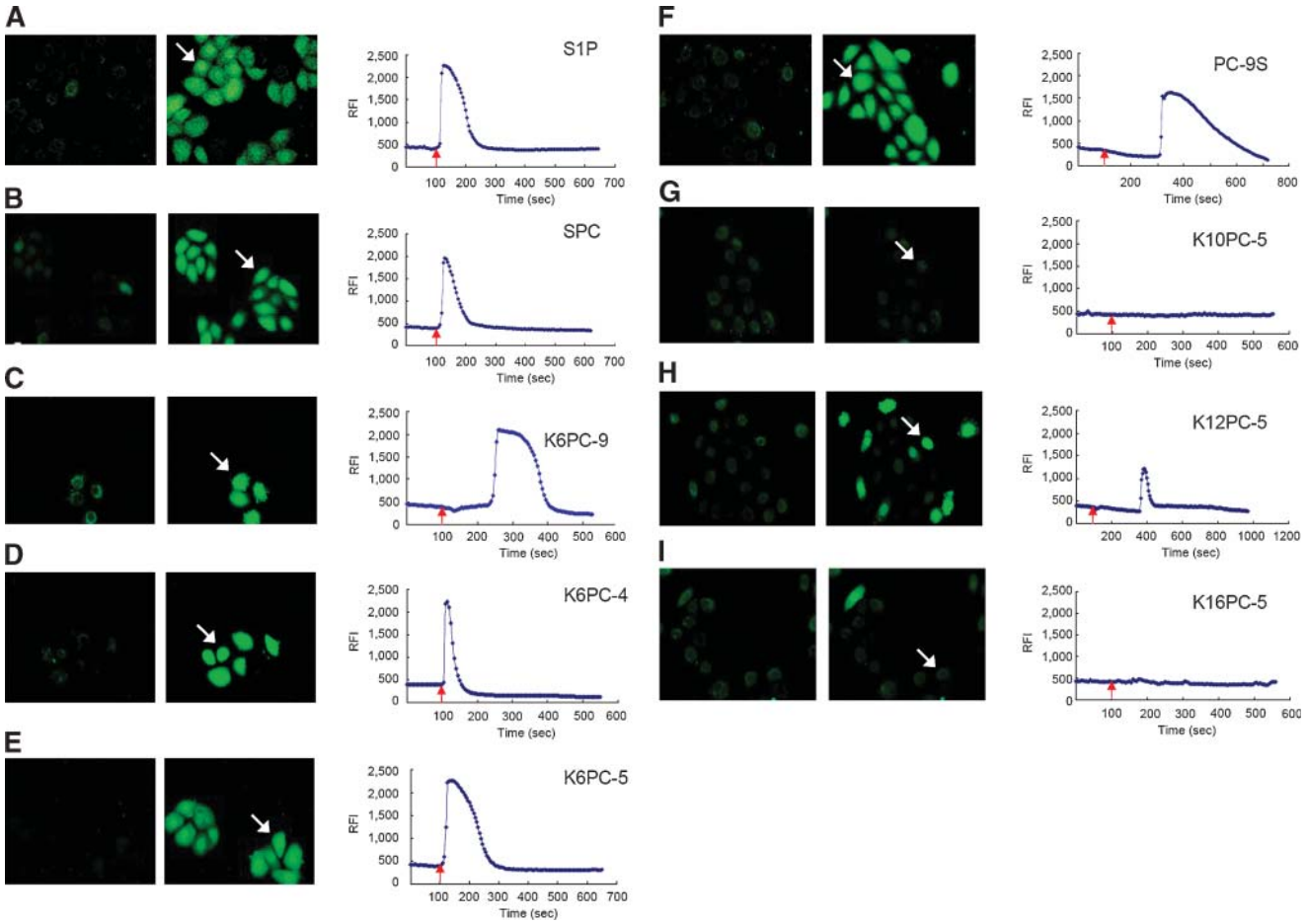

Fig. 2. Measurement of intracellular calcium levels. Keratinocytes were loaded with 4 μM Fura-3/AM for 30 min and then subjected to confocal laser scanning microscopy. Test materials were prepared in DMSO as the 1,000× concentrates and then added to the medium (final concentration, 5 μM) at 100 s (red arrows). Photograph pairs were taken before treatment with the test materials (left) and at the peak calcium levels (right). Cells indicated by white arrows were selected from each experimental group, and the fluorescence intensity was monitored in real time and plotted. The results are presented as relative fluorescence intensity (RFI). A: Sphingosine-1-phosphate (S1P). B: Sphingosylphosphorylcholine (SPC). C: K6PC-9. D: K6PC-4. E: K6PC-5. F: PC-9S. G: K10PC-5. H: K12PC-5. I: K16PC-5.

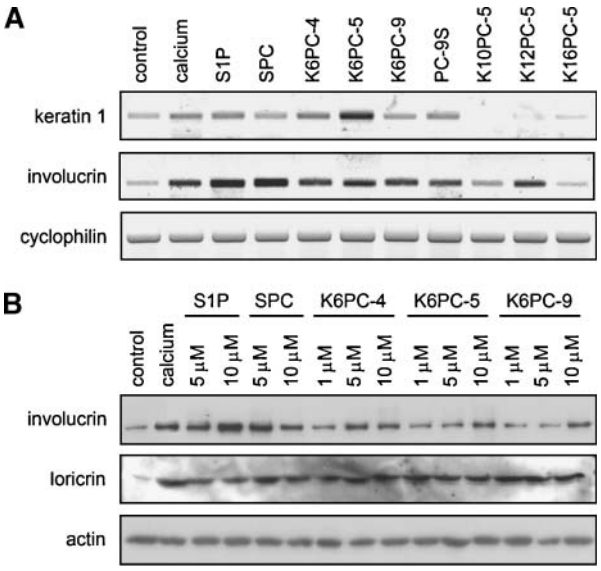

Fig. 3. Effects of ceramide derivatives on keratinocyte differentiation. A: Cells were treated with calcium (1.8 mM) and the ceramide derivatives (5 μM) for 24 h. The levels of involucrin and keratin 1 mRNA were determined using RT-PCR. B: Cells were treated with calcium (1.8 mM) and the ceramide derivatives at the indicated concentrations for 24 h. The levels of involucrin and loricrin protein were analyzed using Western blots. All of the experiments were performed at least three times with similar results.

whether the ceramide derivatives affected the calcium levels in the absence of extracellular calcium. We pretreated cells with 100 μM EGTA for 1 h and then added the ceramide derivatives. All of the lipid molecules induced the phosphorylation of p42/44 ERK (Fig. 5C), suggesting that the increase in cytosolic calcium levels results from the release of calcium from intracellular stores.

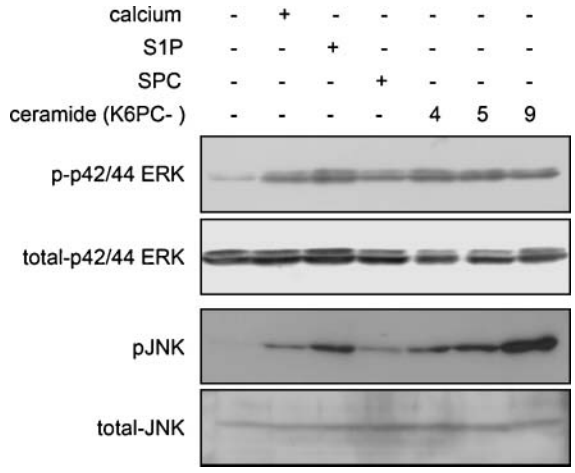

Fig. 4. Effects of ceramide derivatives on the phosphorylation of p42/44 extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). Keratinocytes were treated with calcium (1.8 mM) and the ceramide derivatives (5 μM) for 20 min, and cellular extracts were then prepared. The cellular proteins (30 μg/lane) were separated on duplicate 10% polyacrylamide gels and transferred onto nitrocellulose membranes. Each membrane was reacted with the appropriate anti-phosphorylated p42/44 ERK and JNK antibodies. Anti-total p42/44 ERK and JNK antibodies were used as loading controls.

We investigated the effects of ceramide derivatives on keratinocyte differentiation using a reconstituted epidermis model. Gross morphology revealed no significant difference between the control and ceramide-treated reconstituted epidermis, except for SPC. In the SPC-treated reconstituted epidermis, an irregular pattern and thickening of the stratum corneum with parakeratosis were observed (**Fig. 6A**). To examine the effects of the ceramides in more detail, we performed immunohistochemistry using keratin 1 antibody. S1P treatment resulted in greater keratin 1 expression in the upper layer of the epidermis than did other treatments (Fig. 6B). Interestingly, SPC treatment led to a decrease of keratin 1 expression, especially in the region with the irregular, thickened stratum corneum. Similar to S1P, K6PC-4 and K6PC-5 increased keratin 1 expression in the reconstituted epidermis. Although K6PC-9 resulted in higher keratin 1 expression at a dose of 10 μM, it was slightly less potent than K6PC-4 and K6PC-5. Thus, these novel synthetic ceramide derivatives have the potential to promote keratinocyte differentiation.

DISCUSSION

The most important function of the stratum corneum is to serve as a physical barrier, and corneocytes and intercellular lipids are key components in this barrier. In addition to their primary role as structural components, intercellular lipids possess other properties such as intercellular or intracellular modulation of cell signaling (23). Sphingolipids, especially ceramide, are the major components of the intercellular materials in the stratum corneum, and a decrease in some of these molecules may be the cause of several skin diseases related to abnormal keratinocyte differentiation (24). We have studied synthetic ceramides and the multilamellar emulsion expected to reconstitute the abnormal skin barrier that results from the decrease in ceramide biosynthesis, such as in atopic dermatitis and psoriasis, and the barrier impairment induced by glucocorticoid-epidermal atrophy (15–18). Here, we studied another possible use of these synthetic ceramides: as a bioactive lipid mediator of keratinocyte differentiation.

We synthesized several ceramide derivatives designed from the ceramide PC-9S and examined their effects on keratinocyte differentiation. We synthesized three ceramide derivatives with short-chain alkyl groups and different hydroxyl groups: K6PC-4, K6PC-5, and K6PC-9. To confirm the effect of long-chain alkyl groups, we synthesized long-chain ceramides of K6PC-5. These lipid molecules increased the expression of differentiation markers such as involucrin and keratin 1, likely through an increase in the intracellular calcium level. Interestingly, the increase in intracellular calcium was very quick and transient in response to K6PC-4 and K6PC-5, similar to the response to S1P and SPC. S1P and SPC increase the intracellular calcium quickly and transiently via G-protein-coupled recep-

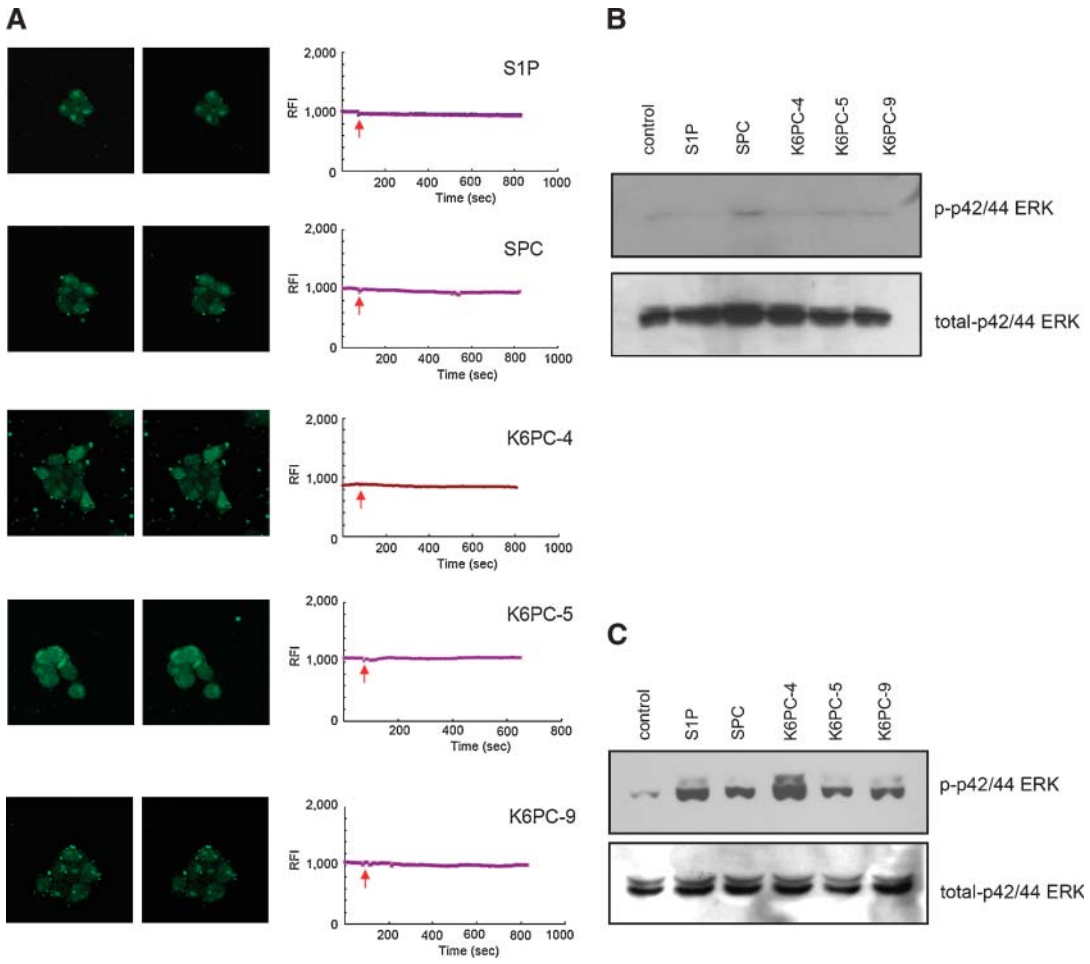

Fig. 5. A: Intracellular calcium chelation. Cells were preloaded with 50 μM BAPTA-AM and 4 μM Fura-3/AM for 40 min and subjected to confocal laser scanning microscopy. Photographs were taken before treatment with the test materials (left) and at 30 s after treatment with test materials (right). Test materials (5 μM) were added at 75 s (red arrows). The results are presented as relative fluorescence intensity (RFI). B: Effects of intracellular calcium chelation on ceramide-induced p42/44 ERK phosphorylation. The cells were preloaded with 50 μM BAPTA-AM for 40 min and treated with the ceramide derivatives (5 μM) for 20 min. The cellular proteins were separated and subjected to Western blotting with anti-phosphorylated p42/44 ERK antibody. C: Effects of extracellular calcium chelation on ceramide-induced p42/44 ERK phosphorylation. The cells were pretreated with 100 μM EGTA for 1 h and then treated with the ceramide derivatives. The cellular proteins were separated and subjected to Western blotting with anti-phosphorylated p42/44 ERK antibody.

tors on the membrane (25–27), and it is possible that K6PC-4 and K6PC-5 act via a similar mechanism. Considering their hydrophobicity and penetrability, it is also possible that the ceramide derivatives act as intracellular second messengers. The elucidation of the precise mechanism of these lipid molecules will be an interesting future study.

In preliminary screening, the other ceramide derivatives, K10PC-5, K12PC-5, and K16PC-5, also increased the involucrin expression in NHEKs cultured in vitro. However, they were considerably less potent than K6PC-5 (Fig. 3A). These four ceramide derivatives share the same backbone and differ in alkyl chain length. K10PC-5 and K16PC-5 failed to elicit the increase in intracellular calcium, whereas K12PC-5 increased the intracellular calcium at ~4 min after treatment. PC-9S, a long-chain derivative of K6PC-9, also increased involucrin expression and intracellular calcium in NHEKs, although it was no more potent than K6PC-9. Because these ceramide derivatives have longer alkyl chain groups, their hydrophobicity is increased. Paradoxically, it is likely that these long alkyl chain ceramide derivatives penetrate the cell membrane more easily than do K6PC-5 and K6PC-9. However, these long alkyl chain ceramide derivatives failed to increase the calcium concentrations, increasing the importance of hydrophobicity for their biological activity.

The increased intracellular calcium level activates numerous downstream signaling targets, including the mitogen-activated protein kinase pathway. For example, SPC-induced p42/44 ERK activation is critically dependent on increases in intracellular calcium in porcine aortic smooth muscle cells (28). Consistent with this, our novel synthetic ceramide derivatives increased the intracellular calcium rapidly, and the phosphorylation of p42/44 ERK and JNK was observed slightly later in keratinocytes cultured in vitro. Therefore, we speculate that the activation

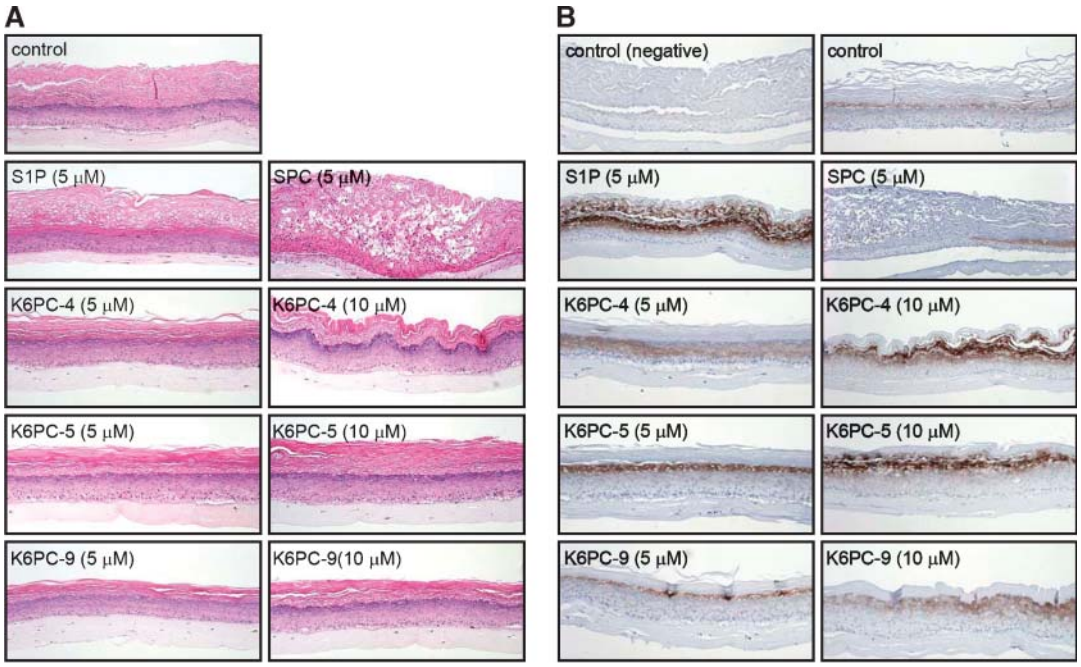

Fig. 6. Effects of ceramide derivatives on the reconstituted epidermis. The dermal matrices were prepared using type I collagen and skin fibroblasts. The keratinocytes were plated on the dermal matrices and incubated with FAD medium (see Materials and Methods). When the cells reached confluence, the cultures were lifted to the air-liquid interface and incubated with ceramide-containing medium for 2 weeks. Test materials were prepared in DMSO as the 1,000× concentrates and then diluted with the medium. Control reconstituted epidermis received the same amount of DMSO only. A: Paraffin sections were stained with hematoxylin and eosin to examine the morphology. B: Immunohistochemical staining was carried out with anti-keratin 1 antibody. The negative control was processed without primary antibody.

of p42/44 ERK and JNK are functional downstream signaling events that follow the ceramide-induced increase in intracellular calcium. This idea was supported by the experiments in which buffering the increase in intracellular calcium levels with a calcium chelator blocked the ceramide analog-induced phosphorylation of ERK and JNK. The putative relationship between these signaling cascades must be investigated further.

We also used a reconstituted epidermis model to determine the effects of ceramide derivatives on keratinocyte differentiation. Our results clearly indicate that the three representative ceramide derivatives, K6PC-4, K6PC-5, and K6PC-9, promote keratinocyte differentiation. One interesting point concerning these ceramides is that the number and location of the hydroxyl groups affect the status of keratinocyte differentiation in organotypic culture. That is, the expression of keratin 1 with K6PC-9 was slightly lower than that with K6PC-4 and K6PC-5 (Fig. 6B). Therefore, the hydroxyl groups of synthetic ceramide should be considered in designing the hydrophilic head groups of more potent ceramides.

In summary, we synthesized novel ceramide derivatives that have the potential to induce keratinocyte differentiation. These lipid molecules with short-chain alkyl groups had a potent biological effect on epidermal differentiation via increased intracellular calcium, suggesting that they may be useful in treating skin diseases arising from abnormal keratinocyte differentiation.

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ERRATA

In the article "Novel synthetic ceramide derivatives increase intracellular calcium levels and promote epidermal keratinocyte differentiation" by Kwon et al., published in the September 2007 issue of the *Journal of Lipid Research* (Volume 48, pages 1936–1943), the authors would like to note the following changes:

Page 1936, Abstract section, line 6: K6PC-4 (N-ethanol-2-hexyl-3-hydroxy-decanamide), K6PC-5, (N-ethanol-3-oxo-2-tetradecyl/hexadecyl-octadecanamide/eicosanamide) and K6PC-9 [N-(1,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide], should read: K6PC-4 [N-(2,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide], K6PC-5 [N-(1,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide] and K6PC-9 (N-ethanol-2-hexyl-3-oxo-decanamide).

Page 1937, Introduction section, fourth paragraph, line 1: PC-9S (N-(2,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide), should read: PC-9S (N-ethanol-3-oxo-2-tetradecyl/hexadecyl-octadecanamide/eicosanamide).

Page 1938, Results section, first paragraph, line 10: [for K6PC-4 (N-ethanol-2-hexyl-3-hydroxy-decanamide)], 2-amino-1,3-propanediol [for K6PC-5 (N-ethanol-3-oxo-2-tetradecyl/hexadecyl-octadecanamide/eicosanamide)], or monoethanolamine [for K6PC-9 (N-[1,3-dihydroxypropy]-2-hexyl-3-oxo-decanamide)], should read: K6PC-4 [N-(2,3-dihydroxypropyl)2-dexyl-3-oxo-decanamide], 2-amino-1,3-propanediol {for K6PC-5 [N-(1,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide]}, or monoethanolamine [for K6PC-9 (N-ethanol-2-hexyl-3-oxo-decanamide)].

Page 1938, Results section, third paragraph, line 16: K16PC-5 [N-(1,3-dihydroxypropyl)-2-hexadecyl-3-oxo-hexadecanamide], should read: K16PC-5 [N-(1,3-dihydroxypropyl)-2-hexadecyl-3-oxo-eicosanamide].

Page 1938, Figure 1 and its legend should read:

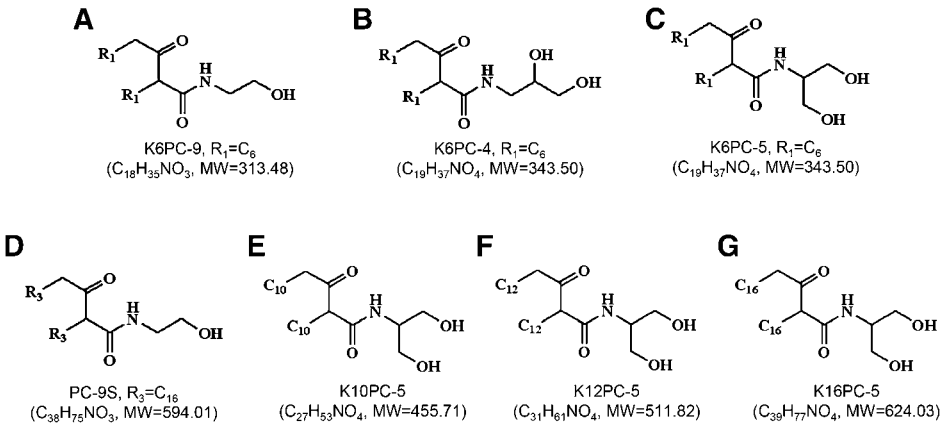

Fig. 1. The molecular structure of ceramide derivatives. To synthesize N-ethanol-2-hexyl-3-oxo-decanamide (K6PC-9), K6PC-4, and K6PC-5, octanoyl chloride was used as two alkyl group (C6). To examine the effect of alkyl chain length, the alkyl group of K6PC-5 was synthesized with lauroyl chloride (C10; K10PC-5), myristoyl chloride (C12; K12PC-5), and hexadecanoyl chloride (C16; K16PC-5) instead of octanoyl chloride. The chemical names of each compound are as follows: K6PC-9 (A), N-(2,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide (K6PC-4) (B), N-(1,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide (K6PC-5) (C), (N-ethanol-3-oxo-2-tetradecyl/hexadecyl-octadecanamide/eicosanamide) (PC-9S) (D), N-(1,3-dihydroxypropyl)-2-decyl-3-oxo-tetradecanamide (K10PC-5) (E), N-(1,3-dihydroxypropyl)-2-dodecxyl-3-oxo-hexadecanamide (K12PC-5) (F), and N-(1,3-dihydroxypropyl)-2-hexadecyl-3-oxo-eicosanamide (K16PC-5) (G).

Page 1939, Table 1 should be replaced by the following table:

TABLE 1

Ceramide	Chemical name	LD_{50} (μM)
K6PC-4	N-(2,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide	38.4 ± 4.6
K6PC-9	N-ethanol-2-hexyl-3-oxo-decanamide	58.7 ± 5.6
PC-9S	N-ethanol-3-oxo-2-tetradecyl/dexadecyl-octadecanamide/eicosanamide	37.7 ± 4.5
K6PC-5	N-(1,3-dihydroxypropyl)-2-hexyl-3-oxo-decanamide	110.5 ± 8.7
K10PC-5	N-(1,3-dihydroxypropyl)-2-decyl-3-oxo-tetradecanamide	12.3 ± 3.4
K12PC-5	N-(1,3-dihydroxypropyl)-2-dodecyl-3-oxo-hexadecanamide	45.3 ± 4.8
K16PC-5	N-(1,3-dihydroxypropyl)-2-dexadecyl-3-oxo-eicosanamide	56.9 ± 5.2