

# Sphingosylphosphorylcholine is an activator of transglutaminase activity in human keratinocytes

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**Abstract** We characterize functional roles of a newly discovered chemical, sphingosylphosphorylcholine (SPC), in the epidermis by elucidating the biological effect of SPC on human keratinocytes in culture. The intracellular calcium level of human keratinocytes was increased by incubation with SPC, but not with sphingosine (SS) or sphingomyelin (SM). The addition of SPC, sphingosine 1-phosphate (SSP), or SS to human keratinocytes at 10  $\mu\text{M}$  concentrations also significantly suppressed DNA synthesis, and SPC, but not SSP, or SS increased the activities of membrane-bound and soluble transglutaminases (TGases). Reverse transcription polymerase chain reaction (RT-PCR) of TGase transcripts revealed that SPC treatment at 10  $\mu\text{M}$  concentrations increased the expression of TGase 1 mRNA. The increased activity of soluble TGase was accompanied by the concomitant activation of cathepsin D as revealed by the increased ratio of mature active form to inactive intermediate form of the protease. Pretreatment of human keratinocytes with pepstatin, a protease inhibitor, blocked the increase in soluble TGase activity induced by treatment with SPC. Consistently, SPC treatment at 1–10  $\mu\text{M}$  concentrations stimulated the cornified envelope formation. **These findings suggest that SPC plays an important role in the altered keratinization process of epidermis in skin diseases with high expression of sphingomyelin deacylase, such as atopic dermatitis.**—Higuchi, K., M. Kawashima, Y. Takagi, H. Kondo, Y. Yada, Y. Ichikawa, and G. Imokawa. **Sphingosylphosphorylcholine is an activator of transglutaminase activity in human keratinocytes.** *J. Lipid Res.* 2001. 42: 1562–1570.

**Supplementary key words** sphingomyelin deacylase • atopic dermatitis • sphingolipid • ceramide

In the skin, sphingomyelin (SM) to ceramide hydrolysis by sphingomyelinase (SMase) plays an essential role in maintenance of epidermal barrier function, providing ceramides in the intercellular space between the stratum corneum layers (1). In Niemann-Pick patients, who have a genetic defect in SMase, there is a distinct barrier disruption in the stratum corneum of the patients (1). Recently, we demonstrated that in the epidermis of patients with atopic dermatitis (AD) there is an abnormal expression of a hitherto undiscovered epidermal enzyme, termed sphin-

gomyelin deacylase. SM deacylase hydrolyzes SM to yield sphingosylphosphorylcholine (SPC), rather than ceramides, which are generated by the action of (SMase), and which lead to ceramide deficiency as an etiological factor in AD barrier disrupted and dry skin (2).

Sphingolipids and their metabolites have been implicated in diverse biological functions in various cellular processes (3–5). It has been suggested that this complex class of lipids may play an important role in the regulation of epidermal growth and differentiation because the epidermis is extremely rich in sphingolipids and metabolic enzymes such as SMase and ceramidase (6). Desai et al. (7) reported that SPC is a powerful mitogen that stimulates DNA synthesis and cellular proliferation of Swiss 3T3 fibroblasts and other cell types to a greater extent than do other known growth factors or structurally related molecules, such as sphingosine (SS) and sphingosine 1-phosphate (SSP). In a parallel study (8), SPC was shown to release calcium from inositol trisphosphate-sensitive or insensitive intracellular pools in permeabilized smooth muscle cells. Because an increase in intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) appears to be an early and general response to stimuli of proliferation and differentiation in epidermal cells, such as keratinocytes (9), it would be of considerable interest to determine how the biologic function of keratinocytes is affected by SPC relevant to the excess formation of SPC in the epidermis of AD patients and their high susceptibility to inflammation. In the previous study, we demonstrated that SPC is a potent inducer of intercellular adhesion molecule-1 expression in human keratinocytes, partly through the stimulated secretion of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and the activation of mitogen activated protein kinase (10). We report here that in contrast to previous reports, which used fibroblasts to demonstrate

Abbreviations: AD, atopic dermatitis; RT-PCR, reverse transcription polymerase chain reaction; SM, sphingomyelin; SMase, sphingomyelinase; SPC, sphingosylphosphorylcholine; SS, sphingosine; SSP, sphingosine 1-phosphate; TGase, transglutaminase

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the mitogenic effect (7), SPC inhibits DNA synthesis in human keratinocytes with a rapid rise in intracellular calcium level, which is accompanied by an activation of soluble and membrane-bound transglutaminases (TGases). This activation is associated with a marked expression of TGase 1 mRNA and an activation of cathepsin D, which is one of possible proteases for either releasing soluble forms from the membrane-anchored TGase 1 or processing the cytosolic form leading to the activation, suggesting that the ability of SPC to act as a cellular effectant may affect the predisposition of the skin to altered keratinization.

## MATERIALS AND METHODS

### Materials

Normal human keratinocytes were obtained from Kyokuto Pharmaceutical Industrial Co. Ltd. (Tokyo, Japan). Serum-free keratinocyte growth medium (SFM) containing low calcium (0.09 mM Ca<sup>2+</sup> modified MCDB 153), bovine pituitary extract (BPE), and epidermal growth factor (EGF), were obtained from GIBCO BRL (Gaithersburg, MD). SPC and SSP were purchased from Matreya Inc. (Pleasant Gap, PA) and BIOMOL Research Laboratories, Inc. (Plymouth, PA), respectively. Other chemicals were of reagent grade.

### Cell cultures

Human keratinocytes were seeded at a density of 5–8 × 10<sup>4</sup> cells/ml in SFM supplemented with 5 ng/ml EGF and 0.5% BPE, and were cultured at 37°C under a 5% CO<sub>2</sub> atmosphere. In experiments to measure effects of SPC, human keratinocytes were cultured in SFM without EGF and BPE.

### Measurements of intracellular free Ca<sup>2+</sup>

Cells were loaded with the calcium indicator Fura-2/AM (2 μM) by incubation for 30 min at 37°C in SFM. The cells were washed twice with fresh SFM without EGF and BPE and exposed to SS derivatives at 37°C. Fluorescence images were obtained at alternating excitation wavelengths of 340 and 380 nm through a SIT vidicon camera, and processed using an ARGUS-200 image analyzer (Hamamatsu Photonics Corp., Hamamatsu, Japan). The fluorescence signal was calibrated in terms of [Ca<sup>2+</sup>]<sub>i</sub> using a digital imaging microscope, as previously described (11). The average curve was obtained by averaging the curves from seven cells in computer system attached in an ARGUS-200 image analyzer.

### Assay of DNA synthesis

After keratinocytes were cultured in 24-well culture dishes for 24 h in SFM without EGF and BPE, SPC and other SS-related lipids were added to the culture at various concentrations. Twenty hours later, the cells were labeled for 4 h with 1.0 mCi/ml [<sup>3</sup>H]-thymidine (New England Nuclear, Boston, MA, 2 Ci/mmol). After three washes with PBS (20 mM Na-phosphate and 150 mM NaCl, pH 7.0), the cells were lysed with 2 N NaOH at 37°C for 15 min and neutralized with 2 N HCl. Acid insoluble materials were precipitated by adding four volumes of 10% ice-cold TCA, collected on a glass microfiber filters, washed three times with 10% TCA, once with ethanol, and then dried in air.

### Measurement of cornified cell envelope formation

Three days after seeding in 6-well culture dishes, SPC was incubated at various concentrations with keratinocytes cultured in SFM as described above, and the cells were cultured for another

9 days in SFM with 0.5% BPE. Then, cells were trypsinized and total cells were counted in an aliquot. The cell suspension was centrifuged and resuspended in 45 μl of PBS containing 1% sodium dodecylsulphate (SDS) and 20 mM dithiothreitol. The suspensions were boiled for 10 min, and then 5 μl of 10 mg/ml DNase (Sigma, St. Louis, MO) was added. Detergent- and reducing agent-resistant cornified cell envelopes (CEs) were counted in a hemocytometer under phase optics. The ratio of resistant cells to total cells was calculated.

### Preparation of cellular fractions

Keratinocytes grown in 100 mm dishes were rinsed three times with PBS containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Basel, Switzerland) and were frozen at –80°C until assayed. For total cell activity of TGases, cells were resuspended in 0.25 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM EDTA, 1% Triton X-100, and the cocktail of protease inhibitors. The suspensions were sonicated three times for 30 s each in a Bioruptor sonicator (OLYMPUS Corp., Tokyo, Japan) and then incubated at 37°C for 10 min. The suspensions were centrifuged at 100,000 g for 30 min at 4°C. The final supernatants were used as the total cellular activity of TGases. For activities in soluble or particulate extracts, cells obtained after thawing were scraped into 0.25 ml 10 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM EDTA and the protease inhibitor cocktail. The suspensions were sonicated three times for 30 s each in a Bioruptor sonicator and then centrifuged at 100,000 g for 30 min at 4°C. The resultant supernatants were used as the soluble fractions. The precipitates were then sonicated three times for 30 s, each time with the same volume of 10 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM EDTA, 1% Triton X-100, and the cocktail of protease inhibitors. The suspension was then incubated at 37°C for 10 min to solubilize the particulate TGase, then centrifuged at 100,000 g for 30 min at 4°C, and the supernatants were used as the particulate fractions.

### Measurement of TGase

Enzymatic activity of TGase was measured by a modification of the method of Lichti et al. (12). Briefly, the standard reaction mixture (total volume, 0.2 ml) contained 0.4 mg dimethyl-casein, 50 mM sodium borate buffer, pH 9.0, 0.5 mM EDTA, 2.5 mM dithiothreitol, 0.25% Triton X-100 or distilled water (if not contained in the cell extract), 5 mM CaCl<sub>2</sub>, 0.75 mM putrescine, 5 μCi [1,4-<sup>14</sup>C]putrescine (New England Nuclear, 80–120 mCi/mmol), and the enzyme preparation. Sample and blanks, containing the buffer used for scraping cells instead of cellular fractions, were incubated for 1 h at 37°C and terminated by addition of 1 ml of ice cold 10% TCA. The TCA-insoluble precipitates were collected by centrifugation and washed twice with cold 10% TCA. TCA-insoluble material was lysed with 50 μl of 1N NaOH, and centrifuged after precipitation with 1 ml of 10% TCA. The final precipitate was dissolved in 0.1 ml 1N NaOH and then neutralized with 40 μl 2N perchloric acid for measurement of radioactivity. Total protein in the fraction was determined by BCA protein assay reagent (Pierce, Rockford, IL). All experiments were performed in duplicate and done at least twice.

### Western blotting

For Western blotting analysis, aliquots of soluble fractions from keratinocytes were electrophoresed on 10% acrylamide gels, and electroblotted on polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA) in 25 mM phosphate buffer, pH 6.8, containing 0.5% methanol at 16 V for 15 h. Membranes were then blocked in PBS, containing 0.05% Tween 20 (PBS-T), and 3% nonfat milk overnight. Cathepsin D

protein was detected by incubation for 2 h with a monoclonal mouse antihuman cathepsin D antibody (Oncogene research products, Cambridge, MA) at a dilution of 1:100 in PBS-T. The blots were then washed in PBS-T three times and subsequently incubated for 60 min with anti-mouse Ig secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, England) diluted 1:1000 in PBS-T. The membranes were again washed with PBS-T and the blots were developed using a chemiluminescent detection system (ECL, Amersham Pharmacia Biotech), with subsequent exposure to X-ray film. The appropriate bands on the resulting autoradiographs were quantified by densitometry.

### Treatment with pepstatin

Pepstatin dissolved in DMSO was added to SFM without EGF and BPE, and 10 ml of the pepstatin-containing SFM was added to each dish (100 mm in diameter). After incubation for 12 h, SPC was added to the dishes. After exposure to SPC for 48 h, medium was removed and cells were fractionated as described above.

### RT-PCR

Total cellular RNAs were extracted from cultured human keratinocytes by the standard acid guanidinium phenol-chloroform method, and were quantified by measuring their optical densities at 260 nm. Before synthesis of oligo(dT<sub>18</sub>)-primed cDNAs, total RNAs were heated to 65°C for 3 min, then chilled on ice to remove secondary structure of template RNA. Reverse transcription was performed in a 20 µl reaction mixture containing 2 µl of 10× PCR buffer II (PE Applied Biosystems, Foster City, CA); 4 µl MgCl<sub>2</sub> (25 mM); 2 µl oligo(dT<sub>18</sub>) (25 µM); 8 µl dNTPs (2.5 mM each); 1 µl RNase inhibitor (20 U/ml, Takara Shuzo Co. Ltd., Shiga, Japan); 1 µl AMV reverse transcriptase (5 U/µl); and 2 µl RNA (200 ng in DEPC-treated water). The reactions were incubated at 42°C for 60 min, then at 52°C for 30 min, and then heated to 99°C for 5 min to inactivate the reverse transcriptase. For PCR amplification of the cDNAs, 3 µl of the cDNA reaction mixtures were added to 12 µl of 80 µl PCR mixture containing 4 µl MgCl<sub>2</sub> (25 mM), 8 µl of 10× PCR buffer II, 0.5 µl of both 5' and 3' primers (100 µM each), and 0.5 µl Taq polymerase (PE Applied Biosystems). The mixtures were amplified using a thermal cycler (MJ Research, Inc., Watertown, MA). The PCR cycle conditions were denaturation for 1 min at 94°C, annealing for 2 min at 65°C, and extension for 1.5 min at 72°C. Reaction products (5 µl) were resolved on 1.5% agarose gels, visualized by ethidium bromide staining, and analyzed with a digital fluorodensitometer (FM-BIO100, Hitachi Software Engineering Co., Kanagawa, Japan). Primers were synthesized on a DNA synthesizer (PE Applied Biosystems). The sequences of the 5' and 3' primer pairs (13–16) used in this study are shown in **Table 1**. To quantitate the expression of the transcripts, the intensities of PCR bands were measured by densitometry and are expressed as relative intensities to glyceraldehyde-3-phosphate dehydrogenase (G3PDH).

### Northern blotting

For Northern blotting of TGase 1, poly A(+) RNA was prepared from the total RNA using oligo dT-conjugated latex particles (Oligotex-dT30, Takara Shuzo). Northern blotting was conducted using NorthernMax kit (Ambion, St. Austin, Texas), following the manufacturer's instruction. Briefly, 20 µg of the poly A(+) RNA was electrophoresed on formaldehyde-denaturing 1% agarose gel in MOPS running buffer followed by capillary-transfer to positively charged nylon membrane. After blocking with a prehybridization solution, the membrane was hybridized for 16 h at 55°C with a biotin-labeled TGase 1 probe, which was generated by PCR using the primers listed in Table 1 and by subsequent labeling reaction with psoralen-biotin (Ambion). The blot was developed using an alkaline phosphatase-based chemiluminescence detection system (BrightStar BioDetect, Ambion), with subsequent exposure to X-ray film. After stripping the TGase 1 probe, the same membrane was re-hybridized with a similarly generated biotin-labeled G3PDH probe and the blot was developed in the same manner.

## RESULTS

### SPC induces calcium influx

Recently, SPC was shown to elicit the release of calcium in viable, quiescent cultures of Swiss 3T3 fibroblasts (8). Using digital imaging fluorescence microscopy, we have now studied the effect of SPC on [Ca<sup>2+</sup>]<sub>i</sub> in human keratinocytes. Application of SPC at a concentration of 10 µM induced a change in [Ca<sup>2+</sup>]<sub>i</sub> that peaked within 60 s and then gradually returned to the basal level over the following 10–15 min (**Figs. 1** and **2C**). In contrast, SM or SS had no effect at the same concentration (Fig. 2A and B). The SPC-induced response disappeared in the absence of extracellular calcium (Fig. 2D), suggesting that the SPC-induced changes in [Ca<sup>2+</sup>]<sub>i</sub> may be due to an influx of calcium from the extracellular milieu.

### SPC suppresses DNA synthesis

SPC caused a dose-dependent decrease in [<sup>3</sup>H]thymidine incorporation into DNA, with an IC<sub>50</sub> of 3 µM (**Fig. 3**). It was of interest to compare the potential antimetabolic properties of these structurally related compounds. As can be seen in Fig. 3, while SSP induced the most marked inhibition of DNA synthesis, SPC suppressed DNA synthesis to a greater extent than did SS or SM.

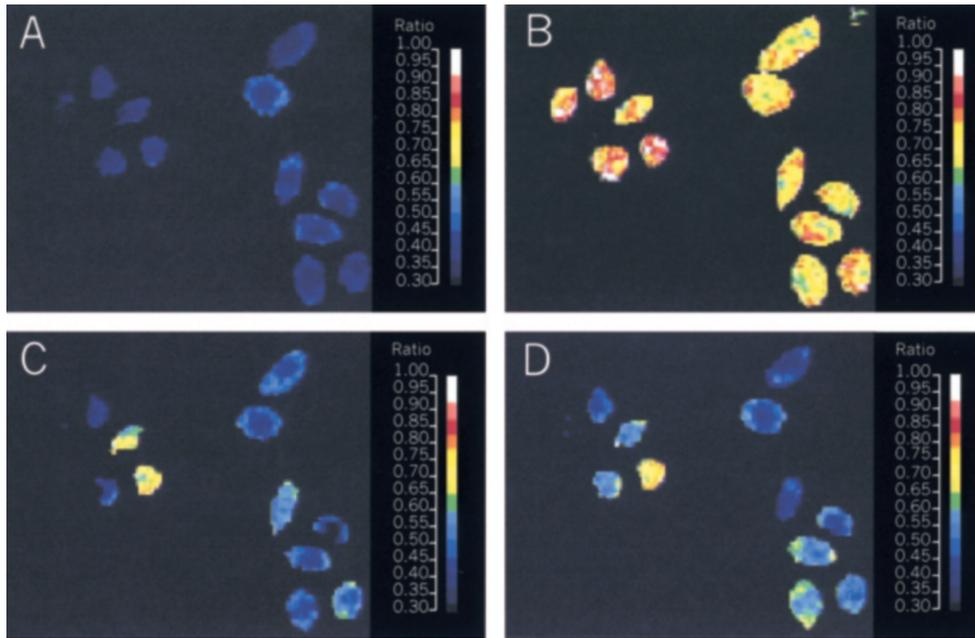
### SPC stimulates TGase activity

TGase is expressed in abundance by cultured human keratinocytes and is an enzyme that catalyzes the forma-

TABLE 1. Oligonucleotides used for PCR analysis in this study

Molecule	Primer	Sequence (5' to 3')	Product Size	Reference
TGase 1	5'	TGAATAGTGACAAGGTGTACTGGCA	524 bp	(14)
	3'	GTGGCCTGAGACATTGAGCAGCAT		
TGase 2	5'	TCACCCACACCTACAAATACCCAG	453 bp	(15)
	3'	TGATTTCTGGATTCTCCAGGTAGAG		
TGase 3	5'	TGAACATGACAGCCTGGACCAT	468 bp	(16)
	3'	CGAGCAGTTGCTTGGTGCCACT		
G3PDH	5'	GAAGGTGAAGGTCCGAGTCAACCGA	516 bp	(13)
	3'	AGTCCTTCCACGATACCAAAGTTG		

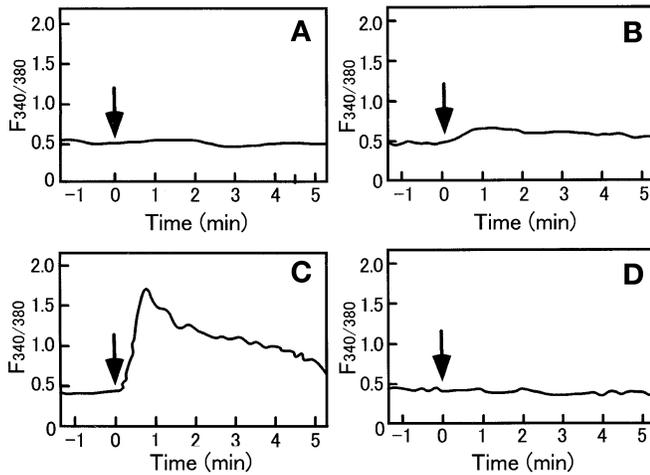
G3PDH, glyceraldehyde-3-phosphate dehydrogenase.



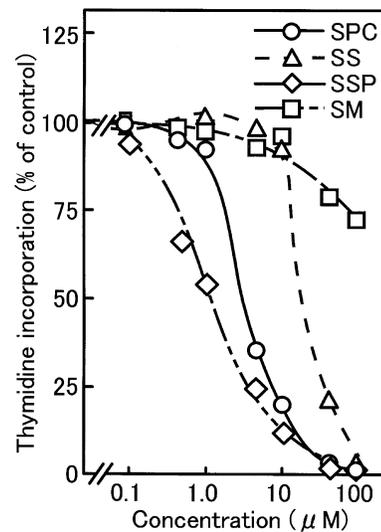
**Fig. 1.** Pseudocolor images of Fura-2 fluorescence intensity ratio imaging in human keratinocytes at various times after stimulation with SPC. Keratinocytes were loaded with the calcium indicator Fura-2/AM (2  $\mu$ M) for 30 min at 37°C in SFM. Fura-2/AM-loaded keratinocytes were stimulated by 10  $\mu$ M SPC. Fluorescence images were obtained at alternating excitation wavelengths of 340 and 380 nm through a SIT vidicon camera and were processed using an ARGUS-200 image analyzer. A: 0 s. B: 47 s. C: 1 min, 46 s. D: 2 min, 28 s.

tion of  $\epsilon$ -( $\gamma$ -glutamyl) lysine isopeptide bonds to make the cornified CE during the process of terminal differentiation in the epidermis. Because the expression of TGase activity is highly dependent upon intracellular and extracellular concentrations of calcium (17), it was of interest to

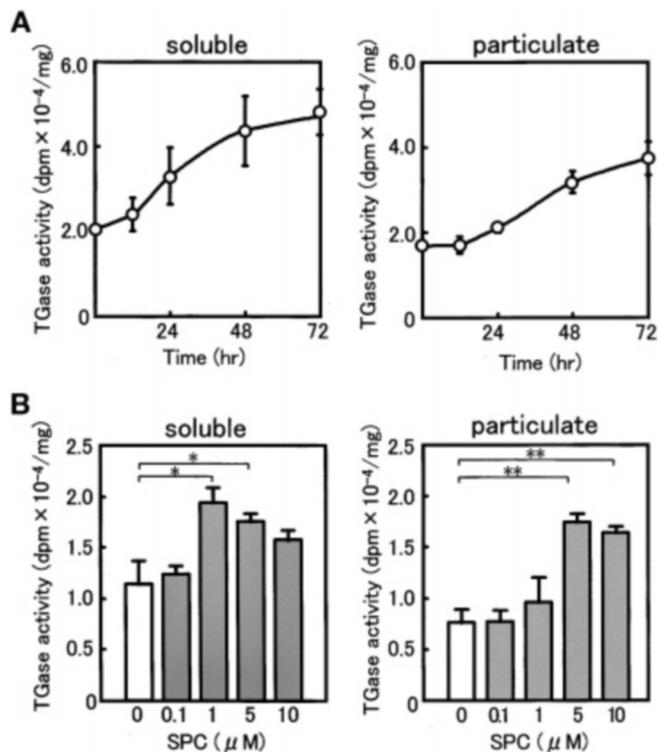
test the effect of SPC on the activity of TGase in human keratinocytes. When incubated with highly proliferating human keratinocytes in culture under low calcium conditions (<0.1 mM), SPC enhanced TGase activities in the particulate and soluble fractions with a peak at 72 h incubation, reaching a plateau at concentrations of 5–10  $\mu$ M (Fig. 4A and B). At concentrations greater than 10  $\mu$ M,



**Fig. 2.** Effect of sphingolipid derivatives on intracellular calcium mobilization in cultured human keratinocytes. Keratinocytes were loaded with the calcium indicator Fura-2/AM (2  $\mu$ M) for 30 min at 37°C in SFM. The arrows indicate when the stimulators were added. Results are reported as the ratio of fluorescence emitted at 340 nm to that at 380 nm and represent the average curve from seven cells. A: 10  $\mu$ M SM; B: 10  $\mu$ M SS; C: 10  $\mu$ M SPC with 0.09 mM calcium; D: 10  $\mu$ M SPC in the absence of calcium. Extracellular calcium was depleted by adding 2 mM EDTA for 2 h to the medium or PBS (-) was used as  $\text{Ca}^{2+}$  medium.



**Fig. 3.** Effects of sphingolipid derivatives on DNA synthesis by cultured human keratinocytes. Human keratinocytes were incubated for 24 h with various concentrations of sphingolipid derivatives, and [ $^3\text{H}$ ]thymidine incorporation was measured as described in Materials and Methods. Data are expressed as % of the untreated control.



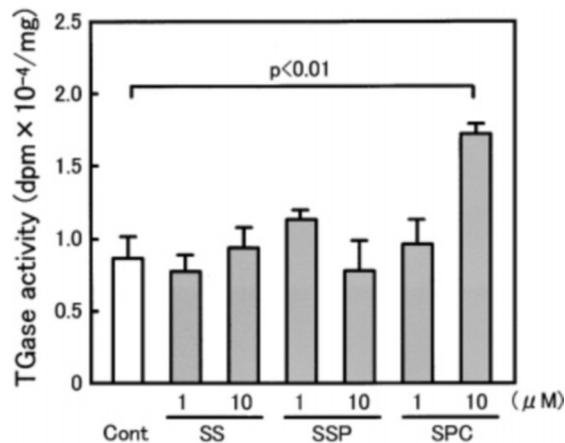
**Fig. 4.** Stimulatory effect of SPC on TGase activity in cultured human keratinocytes. Human keratinocytes were grown in medium containing 0.09 mM Ca<sup>2+</sup> and were exposed to SPC at various concentrations for 0–72 h. Triplicate samples were assayed for TGase activity as described in Materials and Methods. Bar, SD from three experiments. A: Time course study of TGase activity following treatment with 10 μM SPC. B: Dose-dependent study of TGase activity following 48 h treatment with SPC. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

SPC inhibited the TGase activity in the cytosolic fraction. To clarify an association of the inhibitory effect on DNA synthesis with the activation of TGase, we have compared the stimulatory effects on the total cell activity of TGase among SS, SSP, and SPC, which have been found to inhibit DNA synthesis in cultured human keratinocytes. Among those antimitotic lysosphingolipids, only SPC stimulated the total cell activity of TGase at a concentration of 10 μM (Fig. 5).

#### SPC stimulates expression of TGase 1 mRNA

To determine whether the enhanced activity of TGase in cultured human keratinocytes is associated with an increase in gene expression of specific TGase isoforms, the effects of SPC on mRNA levels of TGase was examined by RT-PCR. RT-PCR analysis of RNA obtained from cells exposed to SPC revealed that the addition of SPC at 10 μM concentrations increased the expression of mRNA encoding TGase 1, but not TGase 2 or 3 within the 24 h incubation period (Fig. 6A and B).

Expression of TGase 1 gene was examined further by Northern blotting analysis. As shown in Fig. 7, SPC increased the mRNA level of TGase 1 in human keratinocyte. An increase of mRNA of TGase 1 was evident in cells treated with 10 μM SPC for 24 h. By contrast, no expression of TGase 1 mRNA was detected in cells exposed to ethanol as a vehicle control.



**Fig. 5.** Effects of SSP, SS, and SPC on total cellular TGase activity in cultured human keratinocytes. Human keratinocytes were grown in medium containing 0.09 mM Ca<sup>2+</sup> and were exposed to reagents at various concentrations for 72 h. The cell lysate was subjected to the measurement of TGase activity. Triplicate samples were assayed for TGase activity as described in the Materials and Methods. Bar, SD from three experiments.

#### SPC may solubilize TGase by stimulating the processing of cathepsin D

Because it is known that some proteases are involved in solubilizing TGase 1, which is anchored to membrane (18), and because cathepsin D was reported to be involved in the activation of TGase during the terminal keratinization process (19), we determined whether treatment with SPC causes the activation of cathepsin D as one of the possible proteases. Western blotting of cytosolic fractions of human keratinocytes following treatment with 1 μM SPC demonstrated that SPC induces an increase in the mature active form of cathepsin D (31 kDa molecular mass) in an incubation time-dependent manner (Fig. 8A and B).

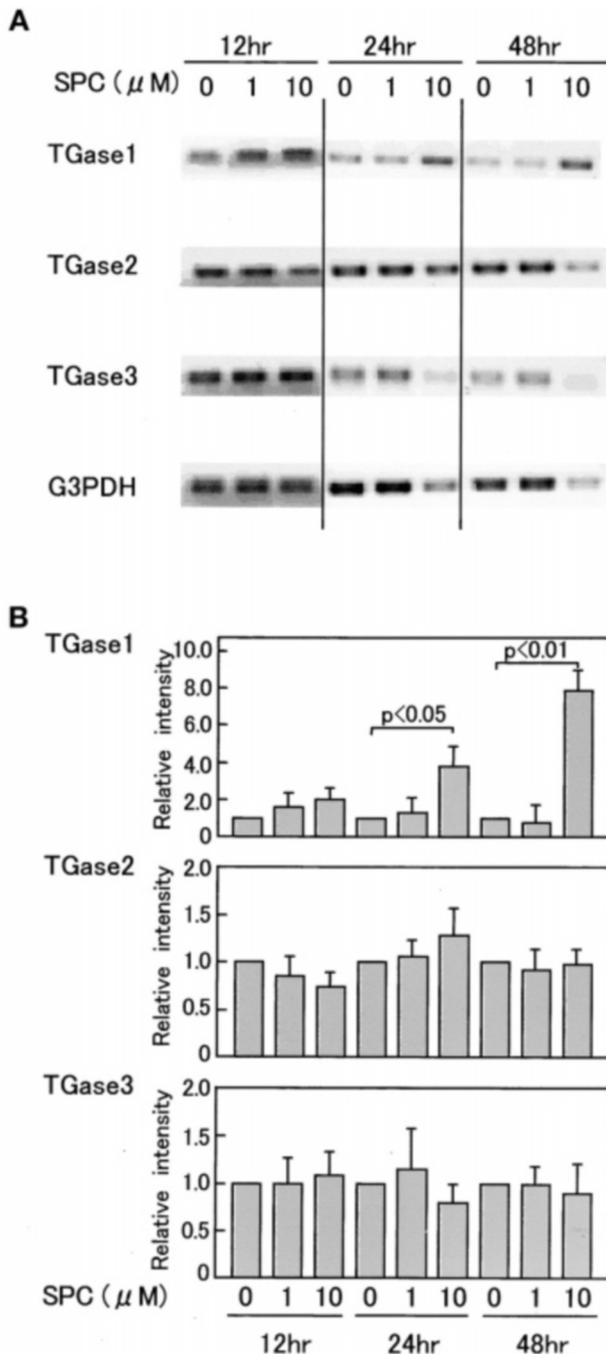
Additional study was performed to determine whether activation of cathepsin D was associated with enhanced soluble TGase activity induced by treatment with 10 μM SPC. As shown in Fig. 9, 12 h preincubation with pepstatin (0.5 mM) suppressed SPC induction of soluble TGase activity. In contrast to that effect, pepstatin had no remarkable effect on particulate TGase activity in human keratinocytes (data not shown).

#### SPC stimulates cornified CE formation

To determine whether the increased activity of TGase has a functional significance in cultured human keratinocytes, the effects of SPC on cornified CE formation were examined by hemocytometer. Counting of detergent- and reduced agent-resistant CEs revealed that the application of SPC for 9 days to cultured human keratinocytes stimulated the CE formation by approximately 170% at more than a concentration of 1 μM (Fig. 10).

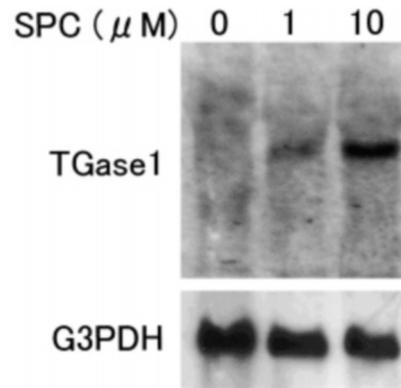
## DISCUSSION

Increased activities of SM deacylase, which may result in unusually high levels of SPC, have been found in the epi-



**Fig. 6.** Effects of SPC on the expression of TGase transcripts. Human keratinocytes were grown in medium with 0.09 mM  $\text{Ca}^{2+}$  and exposed to SPC at the concentrations indicated for various times. Total RNAs extracted from treated or control keratinocytes were reverse-transcribed using oligo(dT18) as a primer. The cDNAs synthesized were PCR amplified with TGase 1, 2, and 3 specific primer sets (and the G3PDH primer set as the positive control) using the sequences described in Table 1. A: Fluorograms are shown using 30 cycles of PCR for TGase 1, 2, and 3, or 20 cycles for G3PDH. B: Densitometric comparison of the expression of TGase 1, 2, and 3 transcripts. Bar, SD from three experiments.

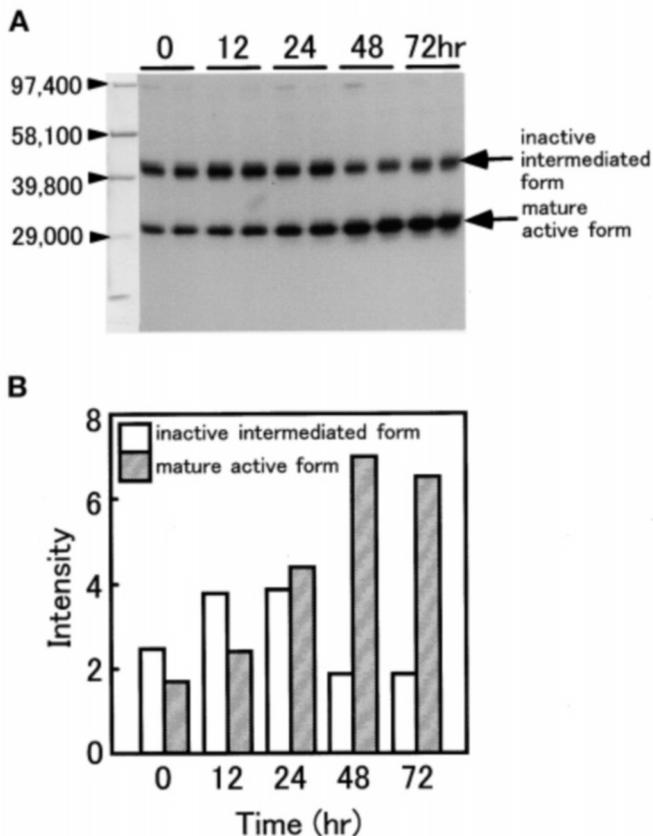
dermis of patients with AD (2). The potential role of SM deacylase in the pathophysiology of AD was suggested by its deleterious effects on the function of the stratum corneum as a permeability barrier due to the interruption of



**Fig. 7.** Stimulatory effect of SPC on the expression of TGase 1 gene. Human keratinocytes were grown in medium with 0.09 mM  $\text{Ca}^{2+}$  and exposed to SPC at the concentrations indicated for 24 h. Poly A(+) RNA was prepared from the total RNA using oligo dT-conjugated latex particles. The poly A(+) RNA was electrophoresed on formaldehyde-denaturing agarose gel followed by capillary-transfer to positively charged nylon membrane. After blocking with a prehybridization solution, the membrane was hybridized with a biotin-labeled TGase 1 probe and by subsequent labeling reaction with psoralen-biotin. The blot was developed using an alkaline phosphatase-based chemiluminescence detection system.

ceramide production (2). Because data are available on increased levels of SPC in the epidermis of patients with AD (20), it would be predicted that SPC may play different roles in various cutaneous symptoms during the elicitation and continuation of AD. Increased levels of SPC have been found in patients with Niemann-Pick disease, a form of sphingolipidosis that results from a deficiency of SMase activity, although there have been no reports on SM deacylase expression in this disease (21). The pathophysiological role of SPC in Niemann-Pick disease has been suggested to result from its deleterious effects on mitochondrial function and calcium uptake (22). It is well established that SPC stimulates cellular proliferation of quiescent Swiss 3T3 fibroblasts to a greater extent than other known growth factors or structurally related molecules, including SS and SSP (8). The mitogenic effect of SPC was accompanied by a rapid rise in intracellular free calcium in viable 3T3 fibroblasts and by a release of arachidonic acid, but not with increases in phosphatidic acid levels or changes in cAMP levels. Therefore, the ability of SPC to act as an extremely potent mitogen for fibroblasts has been suggested to result from the activation of signaling pathways distinct from those used by SS or SSP (8).

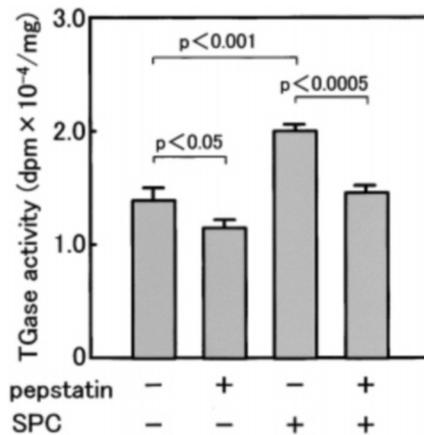
In contrast to previous reports that used fibroblasts to demonstrate the mitogenic effect of SPC (which was maximized at 10  $\mu\text{M}$ ) (8), we show in this study that treatment of human keratinocytes with 1–10  $\mu\text{M}$  SPC is inhibitory for DNA synthesis. This antimitogenic effect is accompanied by the activation of TGase, a hallmark of the keratinization. However, because other sphingolipids, SS, and SSP have no capacity to activate TGases despite their distinctive inhibitory effects on DNA synthesis, it is conceivable that the effect of SPC on TGase is not secondary to an inhibition of proliferation. Using a digital imaging system to



**Fig. 8.** Western blotting of cathepsin D following SPC treatment with human keratinocytes. Human keratinocytes in culture were treated with 1  $\mu$ M SPC for indicated periods of time and aliquots of the soluble fractions from keratinocytes were subjected to 10% acrylamide electrophoresis, followed by electroblotting and then immunostaining with anti-human cathepsin D. A: Western blotting of cathepsin D. B: Densitometric comparison of inactive intermediated form and mature active form of cathepsin D.

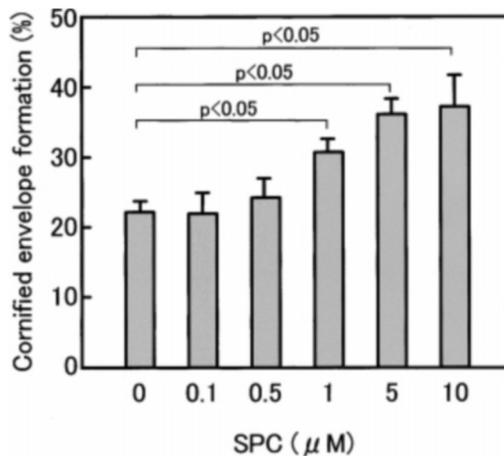
measure cytosolic free calcium in human keratinocytes, we observed that SPC induced a transient increase in  $[Ca^{2+}]_i$ . The rapid rise in  $[Ca^{2+}]_i$  was partially abolished by depletion of extracellular calcium, indicating that the response was in part due to calcium influx from the external milieu.

In keratinocytes, intracellular calcium mobilization is coupled to an increase in TGase activity (17). Extracellular calcium levels are very potent regulatory signals for switching from proliferation to differentiation in keratinocytes (9); at a low concentration of calcium (0.01 mM), human keratinocytes undergo proliferation, whereas at higher concentrations (0.1 mM), they become differentiated and begin the keratinization process. Very few chemicals, including PMA or retinoic acid, can stimulate the activity of TGases in keratinocytes at micromolar levels with a maximum effect at 6 h and 24 h, respectively, after the onset of incubation (23). Of components known to stimulate TGase activity, SPC is the only naturally occurring one that acts as a calcium-mobilizing agonist in human keratinocytes. Because the SPC enhancement of TGase activity was accompanied by increased expression of TGase 1 mRNA, it is



**Fig. 9.** Suppression of SPC induction of soluble TGase activity by treatment with pepstatin. Pepstatin was added to keratinocyte culture at a concentration of 0.5 mM. After incubation for 12 h, cells were treated with 10  $\mu$ M SPC and cultured another 48 h. Soluble TGase activities were assayed as described in Materials and Methods.

likely that mechanisms involved in TGase activation are identical to those induced by PMA where increased expression of TGase transcripts occurs in concert with the enhanced activities. In addition to this, we found that the activation of soluble TGase by SPC is accompanied by an increase in the expression of TGase 1, but not TGase 2 or 3. In keratinocytes, TGase 1 exists as multiple soluble forms, either intact or proteolytically processed (24). Moreover, TGase 1, which is constitutively anchored to membrane, is released to the cytosolic fraction by trypsin treatment (18). In this study, SPC induced an increase in mature active form of cathepsin D, which is one of typical proteases implicated in the keratinization (25). In addition, pepstatin, a protease inhibitor, suppressed SPC induction of soluble TGase activity. Thus, it is likely that SPC stimulates soluble TGase activity in some parts by activating the pepstatin-sensitive proteases, including cathepsin D.



**Fig. 10.** Cornified envelope formation by SPC. Human keratinocytes in culture were treated with SPC at various concentrations for 9 days and the cell pellet was subjected to cell envelope formation analysis in a hemocytometer under phase optics as described in Materials and Methods.

TGase 1 and 3 are involved in the formation of the cornified CE of the epidermis and hair follicle. The induction of TGase activation by SPC was also corroborated by the subsequent increase in cornified envelope formation. In pathophysiologic studies of AD, some data are available that describe altered keratinization in the epidermis of AD (26–29), although there are no reports on the status of TGase in the epidermis or on any abnormality of TGase-dependent products, cornified CE, in the stratum corneum. Therefore, it would be intriguing to know if the cornified CE becomes thickened in the stratum corneum of AD patients. In addition, we recently found that SPC stimulates the expression of intercellular adhesion molecules-1 (ICAM-1) in human keratinocytes (10). This biologic effect is accompanied by the release of several inflammatory cytokines, such as TNF- $\alpha$  and activation of mitogen-activated protein (MAP) kinase (10). Although how the release of inflammatory cytokines or the activation of MAP kinase evoked by SPC is involved in intracellular signaling mechanisms underlying the observed TGase activation remains to be clarified, our results suggest that SPC play an important role in the inflammatory process of epidermis in skin disease with high expression of SM deacylase.

In summary, our results demonstrate that SPC inhibits proliferation of cultured human keratinocytes and stimulates the activities of TGase. These biological effects may be accompanied by the mobilization of intracellular calcium. More recently, we observed that skin from AD patients exhibits a high activity of SM deacylase, which catalyzes liberation of SPC from SM (30, 31), and that cultured normal human keratinocytes also contain significant levels of SM deacylase activity even under nonstimulated conditions (32). Because SPC itself was found at detectable levels in cultured normal human keratinocytes and in normal stratum corneum (32), SPC provides an attractive agonist to explore molecular mechanisms underlying the regulation of keratinization and proliferation in human epidermis. ■

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