**Upregulation of gene expression levels of ceramide metabolic enzymes after application of sphingomyelin-based liposomes to a three-dimensional cultured human epidermis model**

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***Abbreviations:*** β-GCase, β-glucocerebrosidase; CBE, conduritol B epoxide; GlcCer, glucosylceramide; SM, sphingomyelin; SM-L, sphingomyelin-based liposomes; SMase, sphingomyelinase;

**Abstract**

***Background/Aims:*** We have previously reported that the application of sphingomyelin-based liposomes (SM-L) to a three-dimensional cultured skin model increase the content of ceramides NS, NP, AS and AP. However, the mechanism responsible for these increased ceramide levels was not identified.

***Methods:*** SM-L and sphingomyelinase (SMase) were combined and incubated at 37 °C for 24 h. SM-L were also applied to three-dimensional cultured skin for 24 h and quantification of SMase and β-glucocerebrosidase (β-GCase) mRNA expression levels performed using real-time PCR. Additionally, three dimensional cultured skin was incubated with SM-L and the β-GCase inhibitor conduritol B epoxide (CBE) and the ceramide content determined by high performance thin layer chromatography.

***Results:*** We observed generation of ceramide NS after reaction of SM-L and SMase. However, the other ceramide classes were not detected. Notably, SMase and β-GCase mRNA expression levels were significantly increased in cells of the skin model following application of SM-L. The levels of ceramides NS, NP, AS and AP were decreased by treatment with CBE. However, only ceramide NS was significantly increased by treatment with CBE and SM-L in combination.

***Conclusion:*** These findings indicate that application of SM-L to cultured skin upregulates the expression of SMase and β-GCase and increases ceramide content.

***Key words:*** ceramide, sphingomyelin-based liposomes, three-dimensional cultured skin epidermis model

## Introduction

The skin comprises the epidermal and dermal layers, which together acts as a barrier, protecting the body from physical, chemical and microbial damage. The stratum corneum is the outer layer of the epidermis and comprises keratinocytes and intercellular lipids. The intracellular lipids of the stratum corneum include ceramide, cholesterol, fatty acids and cholesterol esters. These lipids adopt a lamellar structure to perform the barrier function. Intracellular lipids —ceramide in particular play the most important role in the barrier function of the stratum corneum [1-4].

Ceramide species are generated through either the glucosylceramide (GlcCer) pathway or the sphingomyelin (SM) pathway. Generally, ceramides NS and AS are synthesized from sphingomyelin, while all ceramide species are synthesized from glucosylceramide [5,6]. A lack of various sphingolipids, such as ceramide, or a deficiency of sphingolipid metabolic enzymes can cause diseases including Niemann-Pick disease and Gaucher's disease [7, 8].

Here we focus on the precursors of ceramide—GlcCer and SM—to elucidate the mechanism controlling increased ceramide levels in the skin. We have previously reported that the application of GlcCer-based liposomes (GlcCer-L) to a three-dimensional cultured human epidermis model (Labcyte Epi-Model) increased ceramide AS content [9]. Moreover, the application of sphingomyelin-based liposomes (SM-L) to a three-dimensional cultured human epidermis model increased levels of ceramides NS, NP, AS and AP [10]. However, why the application of SM-L induced an increase in not only ceramides NS and AS but also ceramides NP and AP in the Labcyte Epi-Model is unknown.

In this study, we prepared SM-L and applied them to a three-dimensional cultured human epidermis model to clarify the influence of SM-L on ceramide metabolic enzymes on three-

dimensional cultured human epidermis.

## Materials and Methods

### *Cells*

LabCyte Epi-Model 6D and culture medium were obtained from Japan Tissue Engineering Co. Ltd. (Gamagori, Aichi, Japan).

### *Reagents*

Sphingomyelin was purchased from NOF Co. Ltd. (Tokyo, Japan). Sphingomyelinase from *Bacillus cereus* was obtained from Sigma Aldrich (St. Louis, MO, USA). Hydroxy ceramide and non-hydroxy ceramide were purchased from Matreya (Pleasant Gap, PA, USA). Ceramides NP and AP were obtained from Evonik (Essen, Germany). L-ascorbic acid phosphate magnesium salt n-hydrate, chloroform, isopropyl alcohol, ethanol, methanol and acetic acid were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Phosphate buffered saline (PBS), RNA iso Plus, PrimeScript® RT reagent Kit and SYBR® *Premix Ex Taq*™ were purchased from Takara Bio Inc. (Kusatsu, Shiga, Japan). Conduritol B epoxide (CBE) was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA).

*Preparation of sphingomyelin-based liposomes (SM-L)*

SM-L were prepared using the thin-layer film method and the freezing and thawing method. Briefly, sphingomyelin was dissolved in chloroform, dried under reduced pressure and the thin-layer film hydrated with PBS. The multi-lamella vesicles were freeze-thawed using liquid nitrogen and then extruded through polycarbonate membrane filters (pore size 100 nm;

Nuclepore, Whatman, GE Healthcare Life Sciences, Buckinghamshire, UK). Finally, the SM-L were ultracentrifuged at 4 °C, 230,000 × g for 15 min (himac CS120GX II, Hitachi Ltd., Tokyo, Japan). The liposomal pellets were resuspended in PBS.

*Determination of the ceramide class produced by sphingomyelin-based liposomes*

Sphingomyelinase (SMase) from *Bacillus cereus* (1 U) was added to 10 mg/mL of SM-L and incubated at 37 °C for 24 h. Ceramides were developed using high-performance thin layer chromatography (HPTLC). Samples were developed twice with chloroform:methanol:acetic acid (190:9:1 v/v/v). The various ceramides were visualized by treatment with a 10% $CuSO_4$, 8% $H_3PO_4$ aqueous solution, and heated to 180 °C for 10 min. The amount of ceramide present was quantitatively determined using a densitometer.

*Application of SM-L to LabCyte Epi-Model*

The three-dimensional cultured human epidermis model was cultured at 37 °C in a 5% $CO_2$ atmosphere. SM-L (10 or 30 mg/mL) was applied to the stratum corneum side of the three-dimensional cultured human epidermis model followed by incubation at 37 °C for 7 days. Medium was changed every day (1 mM CBE as an inhibitor of β-glucocerebrosidase (β-GCase) was dissolved in the medium where indicated). After 7 days, the medium containing ascorbic acid was exchanged for enhanced keratinization medium for 24 h.

*Lipid extraction*

Lipids in the three-dimensional cultured human epidermis model were extracted according to

the methods of Bligh and Dyer [11]. Briefly, the skin was dissolved in a chloroform:methanol (2:1 v/v) solution and sonicated using a probe-type sonicator (Advanced Sonifier Model 250A, Branson, Danbury, CT, USA; 50 W, 10 min). The extracted lipid solution was dried under $N_2$ gas, and dissolved in a chloroform:methanol (2:1 v/v) solution. The presence of the various ceramides was determined by HPTLC as described above.

*Real-time PCR*

SM-L was applied to the stratum corneum side of the three-dimensional cultured human epidermis model followed by a 24 h incubation at 37 °C. Total RNA was then extracted using RNAiso Plus. cDNA was synthesized from total RNA by reverse-transcription using a Prime Script™ reagent kit. Real-time PCR was performed using SYBR *Premix Ex Taq*™ and the Step One Plus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

**Results**

*Observation of the various ceramide classes produced by sphingomyelin-based liposomes*

Figure 1 shows the various ceramides isolated from SM-L by HPTLC. Generally, ceramides NS and AS are synthesized from sphingomyelin, while all ceramide species are synthesized from glucosylceramide [5-7]. After the reaction of SM-L with SMase, ceramide NS was detected, however, the other ceramide classes were not observed.

*Figure 1*

*Expression of β-GCase and SMase mRNA*

SM and GlcCer are metabolized to ceramide by SMase and β-GCase respectively [12-14]. SM-L (10 or 30 mg/mL) was applied to the stratum corneum side of the three-dimensional cultured human epidermis model for 24 h. The mRNA expression levels of both β-GCase and SMase increased after application of 10 and 30 mg/mL SM-L ($p = 0.06$ and $p < 0.05$, respectively)

*Figure 2*

*The ceramide content of the three-dimensional cultured human epidermis model changes following application of SM-L and treatment with β-GCase inhibitor*

One mM of the β-GCase inhibitor CBE [15], with or without 10 mg/mL SM-L, was applied to the stratum corneum side of the three-dimensional cultured human epidermis model for 7 days. The contents of ceramides NS, NP, AS and AP were all decreased following treatment with CBE (without SM-L; Fig. 3). However, ceramide NS was significantly increased ($p < 0.05$) after treatment with CBE and application of SM-L. The levels of other ceramides were also slightly increased compared with the CBE treatment group, but these increases were not significant).

*Figure 3*

**Discussion**

We have previously reported that the application of various sizes of SM-L to a three-

dimensional cultured skin model can increase the content of ceramides NS, NP, AS and AP [10]. However, only ceramides NS and AS are synthesized from sphingomyelin [5]. In this study, we prepared SM-L and applied them to the Labcyte Epi-Model to characterize the influence of SM-L on ceramide metabolic enzymes in the three-dimensional cultured human epidermis. First, mixtures of SM-L and *Bacillus cereus* derived SMase were developed by HPTLC. Only ceramide NS was generated from SM-L (Fig. 1). Generally, ceramides NS and AS are synthesized from sphingomyelin, while other ceramide species are synthesized from GlcCer [5,6]. However we have previously reported that application of SM-L to the Labcyte Epi-Model increased levels of ceramides NS, NP, AS and AP. These finding suggest that SM affects not only SMase but also another factor. We next applied SM-L to the Labcyte Epi-Model and measured levels of β-GCase and SMase mRNA expression. Both were increased 24 h after application of 30 mg/mL SM-L (Fig. 2). This indicates that application of SM-L induced expression of both SMase (a SM metabolic enzyme) and β-GCase (a GlcCer metabolic enzyme).

Finally, we added the β-GCase inhibitor CBE to the medium [16] and applied SM-L to the Labcyte Epi-Model. Ceramides NS, NP and AS contents in the Labcyte Epi-Model were significantly decreased compared with control group following treatment with CBE. Additionally, ceramide AP content was decreased compared with the control group by treatment with CBE (Fig. 3). Contrastingly, ceramide NS content was significantly increased following treatment with CBE and SM-L compared with the CBE-alone treatment group. Additionally, other ceramide species were increased compared with the CBE treatment group though these changes were not significant. These findings suggest that SM was metabolized to ceramide NS. Furthermore, SM may regulate the expression levels of SMase and β-GCase. This suggests that other ceramide species were produced following SM application. Our findings suggest that

SMase and β-GCase expression levels were upregulated and ceramide content was subsequently increased following application of SM-L to the three-dimensional cultured skin epidermis model. Therefore, the application of SM-L to skin influences the ceramide metabolism pathway.

**Conflict of interest**

The authors have no financial conflicts of interest to disclose concerning this manuscript.

**Reference**


1) P.M. Elias, Epidermal lipids, barrier function, and desquamation. *J. Invest. Dermatol.*, **80**, (1983) 44-49.
2) G. Imokawa, M. Hattori, A possible function of structural lipid in the water-holding properties of the stratum corneum. *J. Invest. Dermatol.*, **84**, (1985) 282-284.
3) G. Imokawa, H. Kuno, M. Kawai, Stratum corneum lipids serve as a bound-water modulator. *J. Invest. Dermatol.*, **96**, (1991) 845-851.
4) G. Imokawa, A. Abe, K. Jin, Y. Higaki, M. Kawashima, A. Hidano, Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *J. Invest. Dermatol.*, **96**, (1991) 523-526.
5) Y. Uchida, M. Hara, H. Nishio, E. Sidransky, S. Inoue, F. Otsuka, A. Suzuki, P. M. Elias, W.M. Holleran, S. Hamanaka, Epidermal sphingomyelins are precursors for selected stratum corneum ceramides. *J. Lipid Res.*, **41**, (2000) 2071-2082.
6) S. Hamanaka, M. Hara, H. Nishio, F. Otsuka, A. Suzuki, Y. Uchida, Human epidermal glucosylceramides are major precursors of stratum corneum ceramides. *J. Invest. Dermatol.*, **119**, (2002) 416-423.

7) M. Schmuth, M.Q. Man, F. Weber, W. Gao, K.R. Feingold, P. Fritsch, P.M. Elias, W.M. Holleran, Permeability barrier disorder in Niemann-Pick disease: sphingomyelin-ceramide processing required for normal barrier homeostasis. *J. Invest. Dermatol.*, **115**, (2000) 459-466.

8) W.M. Holleran, E.I. Ginns, G.K. Grundmann, M. Fartasch, C.E. McKinney, P.M. Elias, E. Sidransky, Consequences of beta-glucocerebrosidase deficiency in epidermis. Ultrastructure and permeability barrier alterations in Gaucher disease. *J. Clin. Invest.*, **93**, (1994) 1756-1764.

9) Y. Tokudome, M. Endo, F. Hashimoto, Application of glucosylceramide-based liposomes increased the ceramide content in a three-dimensional cultured skin epidermis. *Skin Pharmacol. Physiol.*, **27**, (2014) 18-24.

10) Y. Tokudome, M. Jinno, H. Todo, T. Kon, K. Sugibayashi, F. Hashimoto, Increase in Ceramide Level after Application of Various Sizes of Sphingomyelin Liposomes to a Cultured Human Skin Model. *Skin Pharmacol. Physiol.*, **24**, (2011) 218-223.

11) E. G. Bligh, W. J. Dyer, A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, **37**, (1959) 911-917.

12) W. M. Holleran, Y. Takagi, G. K. Menon, G. Legler, K. R. Feingold, P. M. Elias, Processing of epidermal glucosylceramides is required for optimal mammalian cutaneous permeability barrier function. *J. Clin. Invest*., **91**, (1993) 1656-1664.

13) J. M. Jensen, S. Schütze, M. Förl, M. Krönke, E. Proksch, Roles for tumor necrosis factor receptor p55 and sphingomyelinase in repairing the cutaneous permeability barrier. *J. Clin. Invest.*, **104**, (1999) 1761-1770.

14) W. M. Holleran, Y. Takagi, G. K. Menon, S. M. Jackson, J. M. Lee, K. R. Feingold, P. M. Elias, Permeability barrier requirements regulate epidermal beta-glucocerebrosidase. *J.*

*Lipid Res*. **35**, (1994) 905-912.

15) S. J. Yang, S. G. Ge, Y. C. Zeng, S. Z. Zhang, Inactivation of alpha-glucosidase by the active-site-directed inhibitor, conduritol B epoxide. *Biochim. Biophys. Acta*, **29**, (1985) 236-240.

16) N. Yoshida, E. Sawada, G. Imokawa, A reconstructed human epidermal keratinization culture model to characterize ceramide metabolism in the stratum corneum. *Arch. Dermatol. Res*., **304**, (2012) 563-577.

Figure captions

Fig. 1 The HPTLC pattern of SM-L and SM-L + SMase mixtures.

Fig. 2 mRNA expression levels of SMase (a) and β-GCase (b) after 24 h application of 10 or 30 mg/mL SM-L to three dimensional cultured epidermis. Data represent means ± SD (n = 3).

*$p < 0.05$ versus control, Dunnet test

Fig. 3 Ceramide NS (a), NP (b), AS (c), AP (d) contents in the three dimensional cultured epidermis after application of 10 mg/mL SM-L and treatment of 1 mM β-GCase inhibitor.

Ten mg/mL SM-based liposomes were applied to the stratum corneum (SC) side for 7 days. Data represent means ± SD of three experiments. *$p < 0.05$, ***$p < 0.001$, Tukey's test

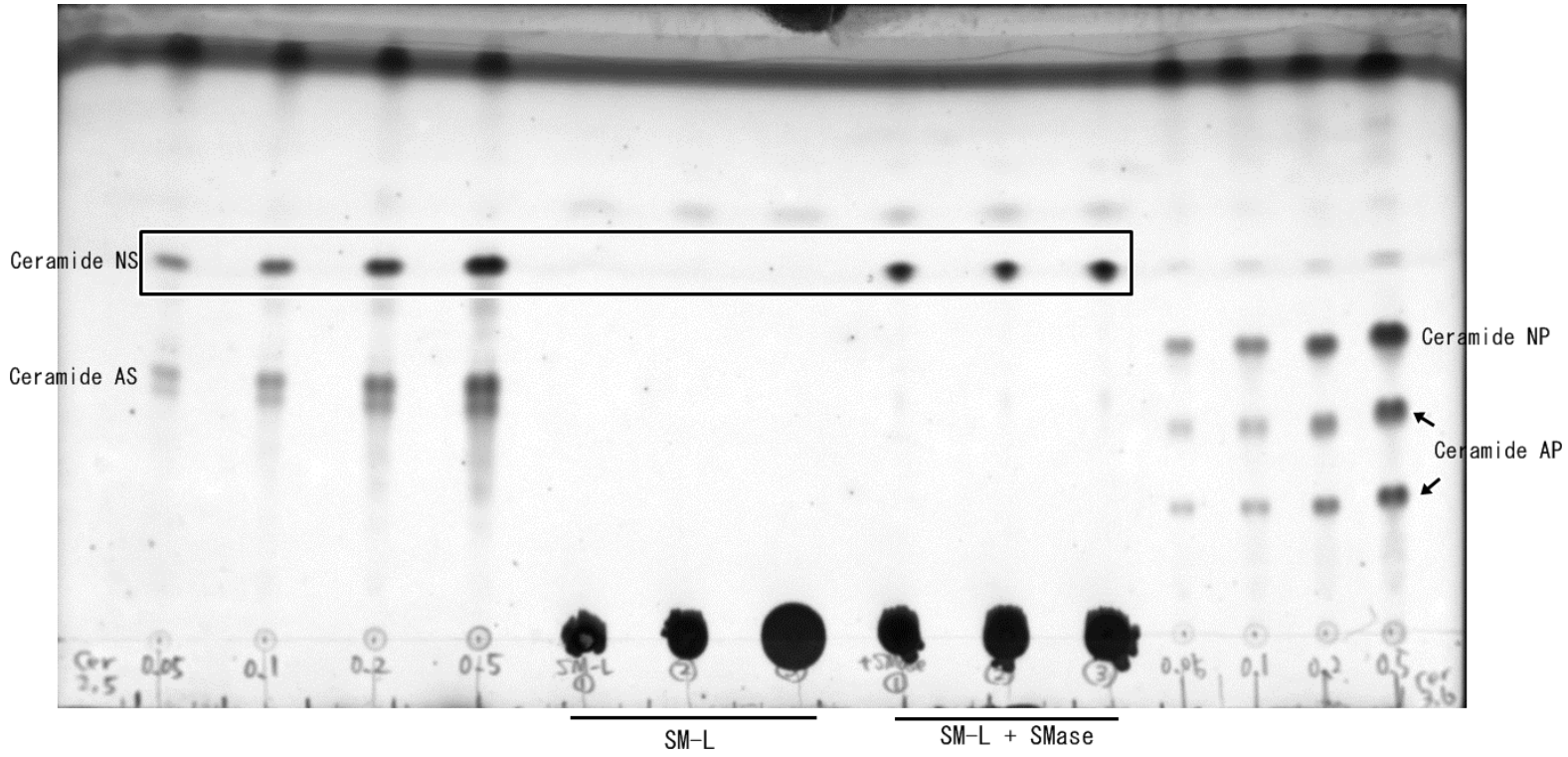
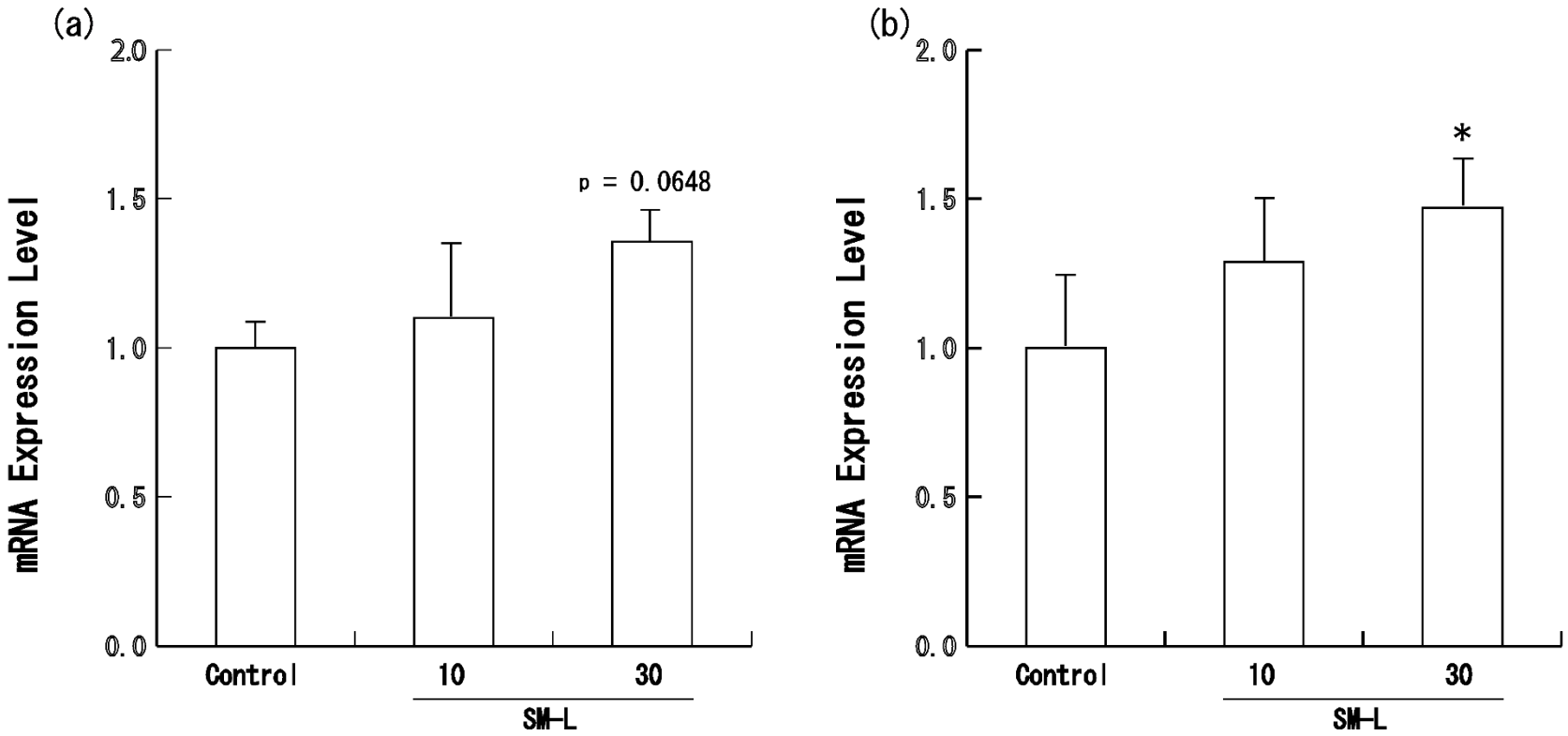

Ceramide NS
Ceramide AS
Ceramide NP
Ceramide AP
SM-L
SM-L + SMase

Fig. 1


(a)
mRNA Expression Level
2.0
1.5
1.0
0.5
0.0
p = 0.0648
Control
10
30
SM-L
(b)
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1.5
1.0
0.5
0.0
*
Control
10
30
SM-L

Fig. 2

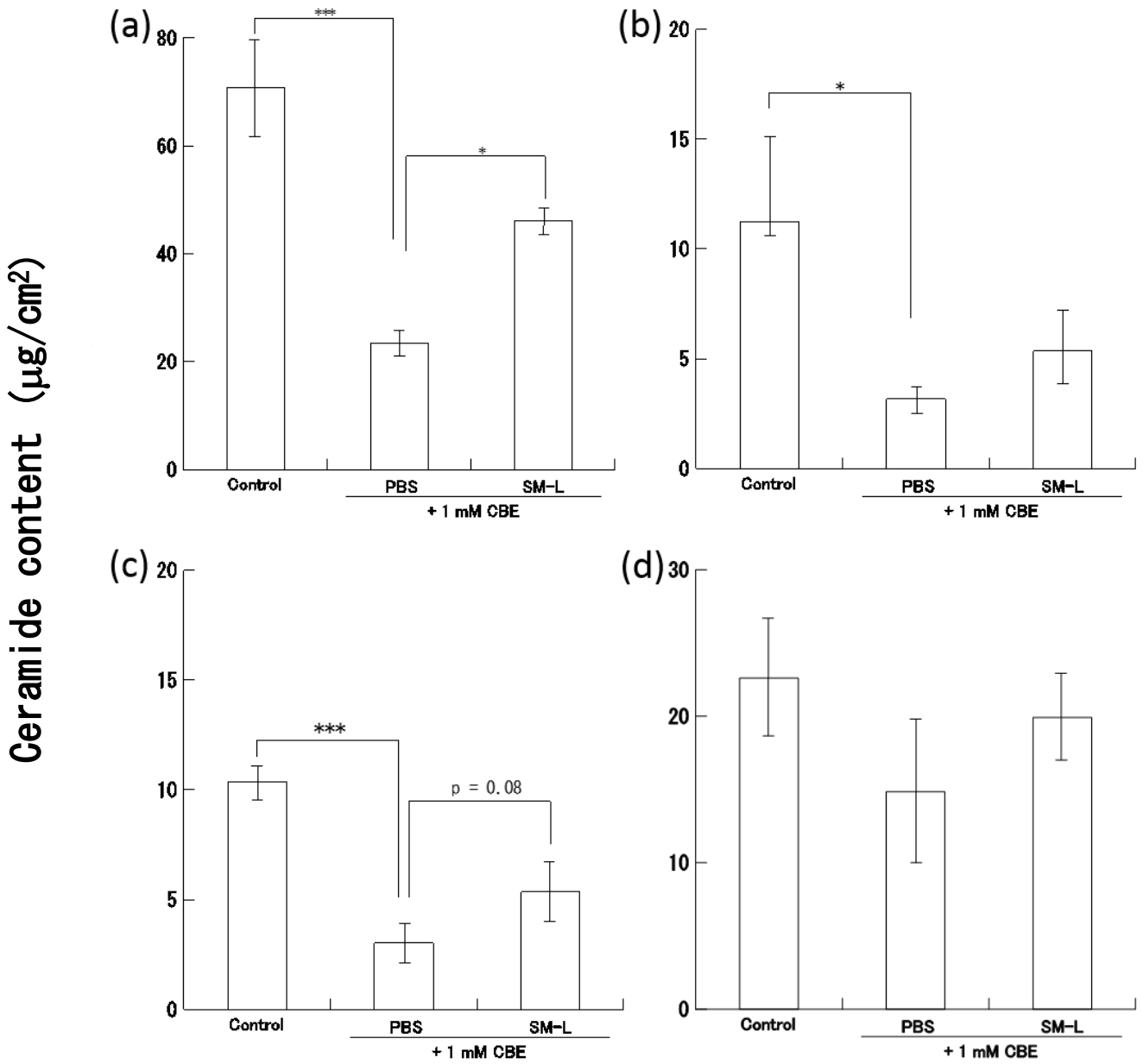


Fig. 3