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Dietary Ceramide Prepared from Soy Sauce Lees Improves Skin Barrier Function in Hairless Mice

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Abstract: Dietary sphingolipids such as glucosylceramide and sphingomyelin are known to improve the skin barrier function of damaged skin. In this study, we focused on free-ceramide prepared from soy sauce lees, which is a byproduct of soy sauce production. The effects of dietary soy sauce lees ceramide on the skin of normal mice were evaluated and compared with those of dietary maize glucosylceramide. We found that transepidermal water loss value was significantly suppressed by dietary supplementation with soy sauce lees ceramide as effectively as or more effectively than maize glucosylceramide. Although the content of total and each subclass of ceramide in the epidermis was not significantly altered by dietary sphingolipids, that of 12 types of ceramide molecules, which were not present in dietary sources, was significantly increased upon ingestion of maize glucosylceramide and showed a tendency to increase with soy sauce lees ceramide intake. In addition, the mRNA expression of ceramide synthase 4 and involucrin in the skin was downregulated by sphingolipids. This study, for the first time, demonstrated that dietary soy sauce lees ceramide enhances skin barrier function in normal hairless mice, although further studies are needed to clarify the molecular mechanism.

Key words: ceramide, glucosylceramide, soy sauce lees, maize, skin barrier function

1 Introduction

The skin provides a barrier to protect the body from dehydration and irritants. Maintaining normal skin barrier function is important for health. The stratum corneum (SC) of the epidermis, composed of terminally differentiated keratinocytes and intercellular lipids such as ceramides, cholesterol, and free fatty acids, acts as the primary skin barrier structure. In particular, ceramides are essential for barrier function, accounting for ~50% of SC lipids[1, 2]. There are various molecular species of ceramide in the SC, which are classified into subclasses, according to the structural components of the sphingoid base and fatty acyl chain, as shown in **Fig. 1**. Numerous studies have found abnormal ceramide composition in SC with compromised barrier function[3−9]. It has been revealed that the content of ceramides in the SC of patients with atopic dermatitis and psoriasis is lower than that in healthy subjects[3, 4]. In particular, the level of ceramide [NP] in the SC was reported to be negatively correlated while the level of ceramide [NS] containing a short acyl chain was positively correlated with transepidermal water loss (TEWL) values[6−8]. The TEWL values are an indicator of the skin barrier function. We have previously reported that the level of ceramide [P-OS] specifically found in the SC negatively correlated with TEWL values in a dry-skin hairless mouse model[10]. In addition, disruption of ceramide synthesis-related genes has been demonstrated to be involved in the pathogenesis of skin diseases characterized by barrier dysfunction[7, 11, 12]. Thus, the homeostasis of the content and composition of ceramides in SC is critical for normal skin barrier function.

Ceramide is the basic structure of sphingolipids, which are constituents of eukaryotic membranes and are frequently conjugated to polar headgroups, such as phosphocholine and sugars. Recently, dietary sphingolipids from various foods have attracted much attention owing to their protective effects on skin barrier function. We have shown that dietary glucosylceramide, sphingomyelin, and ceramide 2-aminoethylphosphonate improved the compro-

Sphingoid base \ Fatty acyl chain	Non-hydroxy [N]	α-Hydroxy [A]	ω-Hydroxy [O]	Esterified ω-hydroxy [EO]	Protein-bound ω-hydroxy [P-O]
Phytosphingosine [P]	Ceramide [NP]	Ceramide [AP]	Ceramide [OP]	Ceramide [EOP]	Ceramide [P-OP]
6-Hydroxy sphingosine [H]	Ceramide [NH]	Ceramide [AH]	Ceramide [OH]	Ceramide [EOH]	Ceramide [P-OH]
Dihydrosphingosine [DS]	Ceramide [NDS]	Ceramide [ADS]	Ceramide [ODS]	Ceramide [EODS]	Ceramide [P-ODS]
Sphingosine [S]	Ceramide [NS]	Ceramide [AS]	Ceramide [OS]	Ceramide [EOS]	Ceramide [P-OS]
4,14-Sphingadiene [SD]	Ceramide [NSD]	Ceramide [ASD]	Ceramide [OSD]	Ceramide [EOSD]	Ceramide [P-OSD]

Fig. 1 Nomenclature for ceramide subclasses in human stratum corneum.

mised barrier function of a dry-skin mice model via the activation of ceramide synthesis in the skin[10, 13, 14]. In addition, we have previously reported that dietary glucosylceramide and sphingomyelin lowered the TEWL values in the skin of normal mice, thus indicating strengthened normal barrier function of the skin[13].

In the present study, we aimed to evaluate the effect of dietary sphingolipids on the skin of normal mice. Ceramide prepared from soy sauce lees was used as a new dietary supplement of sphingolipids while maize glucosylceramide acted as a source of conventional dietary sphingolipids. Soy sauce lees is a byproduct of soy sauce made from soybean, wheat, koji, and yeast and the crude fat accounts for around 10% of total weight[15]. Although the utilization of soy sauce lees has been limited, it is expected to become a new source of sphingolipids. We have previously found that soy sauce lees ceramide, mainly composed of phytosphingosine and 4-hydroxy-8-sphingenine as sphingoid bases, is partly absorbed into the blood by oral administration without digestion[16]. Since ceramides are not abundant in common foodstuffs, the effect of dietary ceramide, including soy sauce lees ceramide, on skin has not yet been evaluated. Therefore, we investigated the barrier function, ceramide profile, and the mRNA expression in genes related to ceramide synthesis in the skin of normal mice fed soy sauce lees ceramide and compared it with those fed maize glucosylceramide.

2 Experimental Procedures

2.1 Materials

Ceramides prepared from soy sauce lees were kindly donated by Genuine R&D Co., Ltd.(Fukuoka, Japan). The composition of the molecules was analyzed using the LC-MS/MS method described below(Table 1). Glucosylce-ramide from maize was purchased from Nagara Science Co., Ltd.(Gifu, Japan). The composition of the molecules was also analyzed using LC-MS/MS(Table 2). Standard ceramides: N-palmitoyl-phytosphingosine(t18:0-C16:0, ceramide[NP]), N-lignoceroyl-phytosphingosine(t18:0-C24:0, ceramide[NP]), N-(2'-(R)-hydroxylignoceroyl)-phytosphingosine(t18:0-C24:0(OH), ceramide[AP]), N-palmitoyl-D-erythro-sphingosine(d18:1-C16:0, ceramide[NS]), N-lignoceroyl-D-erythro-sphingosine(d18:1-C24:0, ceramide[NS]), N-(2'-(S)-hydroxypalmitoyl)-D-erythro-sphingosine(d18:1-C16:0(OH), ceramide[AS])were purchased from Avanti Polar Lipids, Inc.(Alabaster, AL, USA). Standard glucosylceramides: β-glucosyl-N-(2'-(R)-hydroxystearoyl)-4E,8Z-sphingadienine(Glc-d18:2-C18:0(OH), glucosylceramide[ASD]), β-glucosyl-N-(2'-(R)-hydroxyarachidoyl)-4E,8Z-sphingadienine(Glc-d18:2-C20:0(OH), glucosylceramide[ASD]), β-glucosyl-N-(2'-(R)-hydroxybehenoyl)-4E,8Z-sphingadienine(Glc-d18:2-C22:0(OH), glucosylceramide[ASD]), β-glucosyl-N-(2'-(R)-hydroxylignoceroyl)-4E,8Z-sphingadienine(Glc-d18:2-C24:0(OH), glucosylceramide[ASD]), β-glucosyl-N-(2'-(R)-hydroxybehenoyl)-4-hydroxy-8-sphingenine(Glc-t18:1-C22:0(OH), glucosylceramide[AH]), β-glucosyl-N-(2'-(R)-hydroxylignoceroyl)-4-hydroxy-8-sphingenine(Glc-t18:1-C24:0(OH), glucosylceramide[AH])were purchased from Nagara Science Co., Ltd. Standard ω-hydroxyceramide(OS): N-omega-hydroxy-C30:0-D-erythro-ceramide were purchased from Matreya LLC, Inc.(Pleasant Gap, PA, USA). Other chemicals and solvents were of reagent grade, except for high-performance liquid chromatography(HPLC)-grade solvents used for HPLC analysis.

2.2 Animals and diets

All animal studies were conducted in accordance with protocols approved by the Animal Experimentation Committee of Kyoto University(Approval No. 29-35). Four-

Table 1   Composition of ceramide molecular species in soy sauce lees ceramide.

Ceramide molecular species	Precursor ion (Q1) *m/z* [M+H]$^+$	Product ion (Q3) *m/z*	Composition (%)
t18:0-C16:0	556.5	282.3	0.05
t18:0-C18:0(OH)	600.6	282.3	0.07
t18:0-C18:0	584.6	282.3	0.02
t18:1-C20:0(OH)	626.6	280.3	0.01
t18:0-C20:0(OH)	628.6	282.3	0.15
t18:1-C21:0(OH)	640.6	280.3	0.07
t18:1-C22:0(OH)	654.6	280.3	2.88
t18:0-C22:0(OH)	656.6	282.3	4.48
t18:1-C23:0(OH)	668.6	280.3	1.59
t18:0-C24:0 (OH)$_2$	700.6	282.3	8.85
t18:0-C22:0	640.6	282.3	0.29
t18:0-C23:0(OH)	670.6	282.3	2.62
t18:1-C24:0(OH)	682.6	280.3	11.37
t18:0-C25:0(OH)$_2$	714.7	282.3	1.07
t19:0-C24:0(OH)$_2$	714.7	296.3	0.46
t18:0-C24:0(OH)	684.6	282.3	38.29
t18:1-C25:0(OH)	696.6	280.3	1.89
t20:0-C24:0(OH)$_2$	728.7	310.3	1.86
t18:0-C24:0	668.7	282.3	1.41
t18:0-C25:0(OH)	698.7	282.3	5.21
t19:0-C24:0(OH)	698.7	296.3	1.27
t18:1-C26:0(OH)	710.7	280.3	1.31
t20:0-C24:0(OH)	712.7	310.3	13.14
t20:0-C25:0(OH)	726.7	310.3	1.14
t20:0-C26:0(OH)	740.7	310.3	0.52

week-old female albino hairless mice (Hos:HR-1), established by inbreeding the Skh:HR-1 mice, were obtained from Hoshino Laboratory Animals, Inc. (Ibaragi, Japan) and individually housed in plastic cages at 25℃ with a 12 h light/dark cycle[17]. The mice were allowed free access to standard AIN-93G purified diet and distilled water during a one-week acclimation period and then randomly divided into three groups: control group with AIN-93G diet (n = 5); 0.1% Cer group with AIN-93G diet containing 0.1% soy sauce lees ceramide (n = 6), or 0.1% GlcCer group with AIN-93G diet containing 0.1% maize glucosylceramide (n = 6) (Table S1). Body weight and diet intake of each mouse were measured daily during the two-week experimental period. At the end of 14 d, the mice were fasted for 12 h but were allowed free access to distilled water and were subsequently sacrificed under isoflurane anesthesia, and

the dorsal skin specimens were collected immediately. To analyze mRNA expression, part of the skin specimens was stored in RNAlater (Qiagen, Valencia, CA, USA) at −80℃ until use. The skin specimens for lipid analysis were frozen immediately at −80℃.

2.3 Measurement of skin barrier functions

TEWL values and the skin hydration index of the dorsal skin of the mice were measured at 20-22℃ and 40% relative humidity, on days 0, 7, 11, and 14 of the experimental period using a Tewameter TM 300 and Corneometer CM 825 (Courage Khazaka electronic GmbH, Cologne, Germany), respectively.

2.4 Evaluation of wrinkles on skin

On day 14 of the experimental period, the skin surface

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Table 2 Composition of glucosylceramide molecular species from maize.

Glucosylceramide molecular species	Precursor ion (Q1) *m/z* [M+H]⁺	Product ion (Q3) *m/z*	Composition (%)
d18:2-C16:0(OH)	714.6	262.3	4.59
d18:3-C18:0(OH)	740.6	260.3	0.77
d18:2-C18:0(OH)	742.6	262.3	13.54
d18:3-C20:0(OH)	768.6	260.3	2.00
d18:2-C19:0(OH)	756.6	262.3	0.48
d18:2-C20:0(OH)	770.6	262.3	36.07
t18:1-C22:0(OH)	816.7	280.3	3.56
d18:2-C22:0(OH)	798.6	262.3	9.67
d18:3-C24:0(OH)	824.7	260.3	0.93
t18:1-C24:0(OH)	844.7	280.3	12.61
d18:2-C24:0(OH)	826.6	262.3	14.52
d18:2-C25:0(OH)	840.7	262.3	1.27

replicas were collected from the dorsal skin using a silicone product(ASB-01, Asahi Biomed, Yokohama, Japan)under isoflurane anesthesia[14]. To assess the degree of wrinkling, parameters(number of wrinkles, average depth of wrinkles, wrinkle area ratio, and wrinkle volume ratio)of the skin replica were measured using a skin wrinkle analysis system (Asahi Biomed).

2.5 Extraction of ceramides and glucosylceramides from mouse epidermis

Epidermal lipid extraction was performed as previously described[14]. The skin epidermis(4 mm diameter pieces) was separated from the dermis using Dispase II(neutral protease, grade II, Roche Diagnostics GmbH Mannheim, Germany)in Hanks' balanced salt solution + (HBSS(+), Nacalai Tesque, Kyoto, Japan). The epidermis was immersed in chloroform/methanol(2:1, v/v)overnight at 4℃, and epidermal lipids were extracted. To remove glycerolipids, total lipids were saponified with 1 mL of 0.4 M KOH in methanol. Then, the alkali-stable lipids were loaded onto a solid-phase extraction column(Sep-Pak, Silica Cartridges, sorbent 120 mg, 55-105 μm particle size, Waters, Milford, MA, USA)equilibrated with chloroform. Sep-Pak was washed with 2 mL of chloroform and eluted using 6 mL of chloroform-methanol(9:1, v/v)to obtain the ceramide and glucosylceramide fractions. After extraction of epidermal lipids as described above, the epidermal residue was immersed in 1 M KOH in 95% methanol by overnight incubation at room temperature to release the ω-hydroxyceramides bound to the SC by ester bonds(ceramide[P-OS]). Subsequently, the ceramide[P-OS]fractions were extracted with chloroform/methanol(2:1, v/v)after neutralization with acetic acid. The protein level in the residue was quantified using a DC Protein Assay kit(Bio-Rad Laboratories, CA, USA)according to the manufacturer's instructions to normalize the ceramide levels in SC. The solution fraction was dried under a stream of N_2 gas and re-dissolved in methanol(1 mL). Finally, an aliquot(1-5 μL)of this solution was injected into the LC-MS/MS, as described in section 2.6.

2.6 LC-MS/MS analysis of ceramides and glucosylceramides

Analysis of ceramide molecules, other than ceramide [P-OS], was performed using a triple-quadrupole linear ion trap mass spectrometer(LC-QTRAP/MS)(QTRAP 5500; SCIEX, Tokyo, Japan)equipped with a heated electrospray ionization(ESI)interface, as described previously[16]. Ceramide molecules were monitored in the positive ion multiple reaction monitoring(MRM)mode(**Table S2**). In this analytical method, 4-hydroxy-8-sphingenine and 6-hydroxy sphingosine in the ceramide structure were not distinguished and were grouped as t18:1[H]. Furthermore, 4,14-sphingadiene and 4,8-sphingadienine in the ceramide structure were not distinguished and were grouped as d18:2 or[SD][18]. Ceramides were quantified using the standard curves of each molecular species of ceramides. Molecules without a corresponding standard were quantified using standard curves of structurally similar compounds: t18:0-C16:0 for ceramides composed of trihydroxy sphingoid base([P]and[H])and C16-19 non-hydroxy fatty acyl chain([N]), t18:0-C24:0 for ceramides composed of trihydroxy sphingoid base([P]and[H])and C20-26 non-hydroxy fatty acyl chain([N]), t18:0-C24:0(OH)for ceramides composed of trihydroxy sphingoid base([P]and[H]) and hydroxy fatty acyl chain([A]), d18:1-C16:0 for cerami-

des composed of dihydroxy sphingoid base and C16-19 non-hydroxy fatty acyl chain([N]), d18:1-C24:0 for ceramides composed of dihydroxy sphingoid base([SD], [S] and[DS])and C20-26 non-hydroxy fatty acyl chain([N]), d18:1-C16:0(OH)for ceramides composed of dihydroxy sphingoid base([SD], [S]and[DS])and hydroxy fatty acyl chain([A]). Analysis of ω-hydroxyceramide molecules(ceramide[P-OS])was performed using LC-QTRAP/MS equipped with an atmospheric pressure chemical ionization interface, according to a previous method with some modifications[14]. ω-Hydroxyceramide molecules were detected in the positive ion MRM mode. The precursor ion to product ion mass transition for each molecule was optimized, as shown in **Table S3**. The optimized ionization source conditions were as follows: nebulizer current, 2 μA; GS1, 30 psi; curtain gas, 20 psi; interface heater, 400℃; declustering potential, 85 V; entrance potential, 10 V; collision energy, 10 V; and collision cell exit potential, 12 V. Mobile phase A consisted of 2 mM ammonium acetate in water, and mobile phase B consisted of 1 mM ammonium acetate in methanol. A TSKgel ODS-100Z(2.0×50 mm inner diameter, 3 μm, Tosoh, Tokyo, Japan)was eluted at a flow rate of 0.2 mL/min using the following gradient: 0–5 min, 90%–99% B; 5–30 min, 99% B; 30–35 min, 99%–90% B; 35–40 min, 90% B. Column temperature was maintained at 40℃. ω-Hydroxyceramides were quantified using standard curves of the standard, d18:1-C30:0(OH). Analysis of glucosylceramide molecules was performed using LC-QTRAP/MS equipped with a heated ESI interface in the positive ion MRM mode, according to our previously published quantitative analysis method(**Table S4**)[16]. Glucosylceramides were quantified using standard curves for each molecular species of glucosylceramides. Glucosylceramides containing sphingoid bases d18:2 and d18:3 and without an actual standard were quantified using standard curves of structurally similar compounds: Glc-d18:2-C18:0(OH), Glc-d18:2-C20:0(OH), Glc-d18:2-C22:0(OH), and Glc-d18:2-C24:0(OH)were used to quantify molecules having hydroxy fatty acyl chain fatty acid with a carbon chain length C15-19, C20, C22 and C24-25, respectively. All data were collected using the Analyst 1.6.3 software(SCIEX).

2.7 RNA preparation and real-time qRT-PCR

Total RNA extraction, cDNA synthesis, and real-time quantitative qPCR were performed as described previously, with modifications[14]. Total RNA was extracted from the whole-skin samples in RNAlater using Sepasol reagent (Nacalai Tesque), following the manufacturer's instructions. cDNAs were synthesized using ReverTra Ace (Toyobo, Osaka, Japan)with random hexamers. For RT-PCR, 3 μL of diluted cDNA was mixed with 7 μL iQ SYBR Green Supermix(Bio-Rad Laboratories)containing 2 μL PCR primer(5 μM; primer sequences are shown in **Table S5**). Real-time qRT-PCR was performed using a DNA

Engine Option system(Bio-Rad Laboratories). The thermal cycling conditions were 3 min at 95℃ for 1 cycle, followed by amplification for 40 cycles with melting for 15 s at 95℃, and annealing and extension for 30 s at 60℃. The expression level of each mRNA was normalized to that of β-actin (*Actb*)mRNA as an internal control.

2.8 Statistical analyses

Data are presented as the mean±standard error of the mean(SEM). Statistical analyses were performed using one-way analysis of variance(ANOVA), followed by Tukey–Kramer test to identify significant differences between the groups($p < 0.05$).
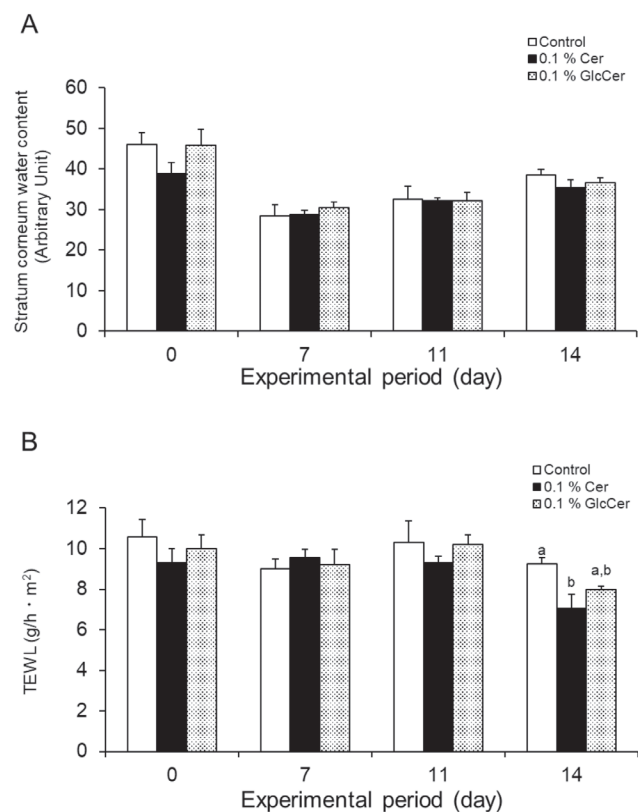
Fig. 2 Effect of dietary sphingolipids on stratum corneum water content(A)and TEWL values(B)in normal mice fed different diets(control group, n=5; 0.1% Cer and 0.1% GlcCer groups, n=6). The dorsal skin measurements performed on days 0, 7, 11, and 14 of the experimental period. Data presented as means±SE and were analyzed by one-way ANOVA, followed by Tukey–Kramer tests. Bars with different letters in each group show significant difference at $p < 0.05$.

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3 Results

### 3.1 Effect of dietary ceramide and glucosylceramide on skin conditions

Daily food intake and body weight were not significantly different among the groups during the experimental period（data not shown）. After the experimental period, the tissue weights（liver, spleen, kidney, and heart）were not significantly different among the groups. The TEWL value in the 0.1％ Cer group was significantly lower than that in the control group at day 14, whereas there was no significant difference in the water content of the stratum corneum（Fig. 2）. In the 0.1％ GlcCer group, the TEWL value tended to decrease, but was not significantly different compared to that of the 0.1％ Cer group（Fig. 2）. The results suggested that dietary sphingolipids could strengthen the skin barrier function of the hairless mice. The level of wrinkle formation did not differ among the groups（Fig. 3）.

3.2 Effect of dietary ceramide and glucosylceramide on ceramide in epidermis

The specific molecular species found only in soy sauce lees ceramide or maize glucosylceramide were not detected in the epidermis of the 0.1％ Cer and 0.1％ GlcCer group, respectively. The total amount of ceramide in the epidermis was not different among the groups（Fig. 4A）. The amount of each ceramide subclass was also not different among the groups（Fig. 4B）. Among the 218 molecular species of ceramide, the amounts of 12 molecules（t17:0-C17:0, t18:0-C15:0, t17:0-C26:0（OH）, t17:0-C24:0（OH）, d17:0-C17:0, d18:0-C15:0, d18:0-C18:1, d18:0-C26:1, d18:0-C22:0（OH）, d18:0-C25:0（OH）, d17:1-C18:2, and d18:2-C25:0）were significantly higher in the 0.1％ GlcCer group compared with those in the control group. These molecules tended to increase more in the 0.1％ Cer group than in the control group（Fig. 4C）. In addition, the amount of d18:2-C20:0（OH）in the 0.1％ GlcCer group was higher than in the control and 0.1％ Cer groups.
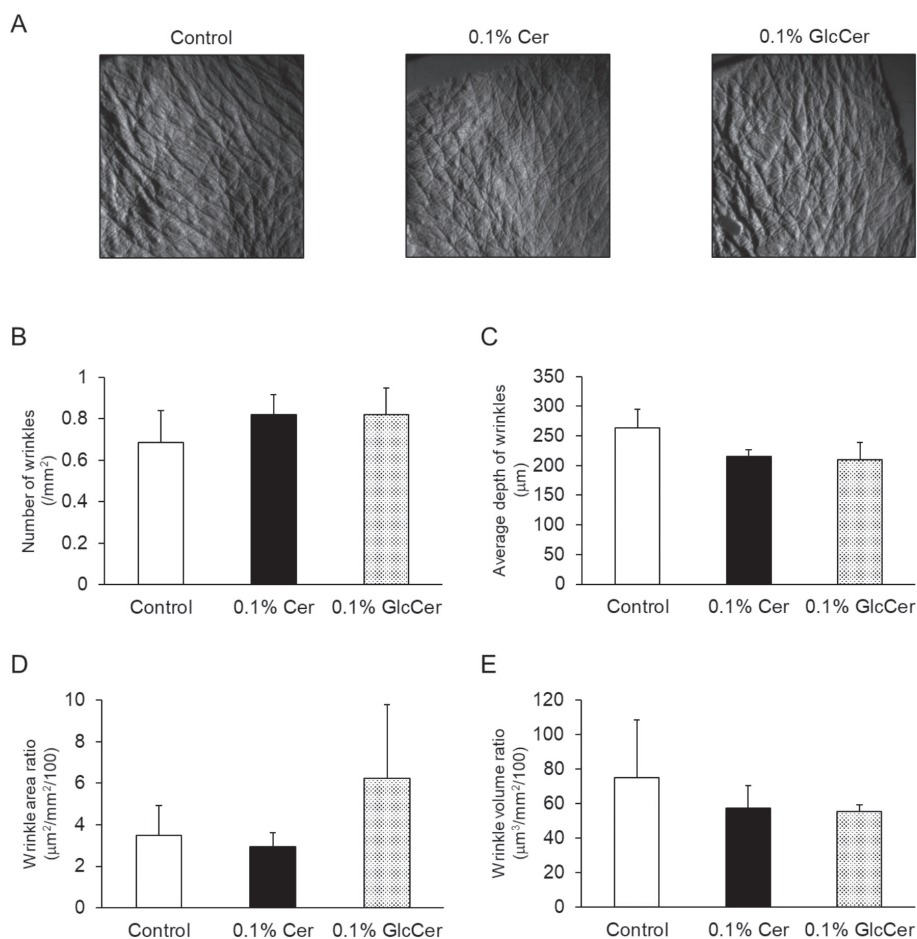
Fig. 3　Representative photographs of replicas taken from mice dorsal skin（A）. The images show skin wrinkles in each group. The number（B）, the average depth of wrinkles（C）, wrinkle area ratio（D）, and volume ratio（E）analyzed by an imaging analyzer. Data presented as means ± SE（control group, n = 5; 0.1％ Cer and 0.1％ GlcCer groups, n = 6）.
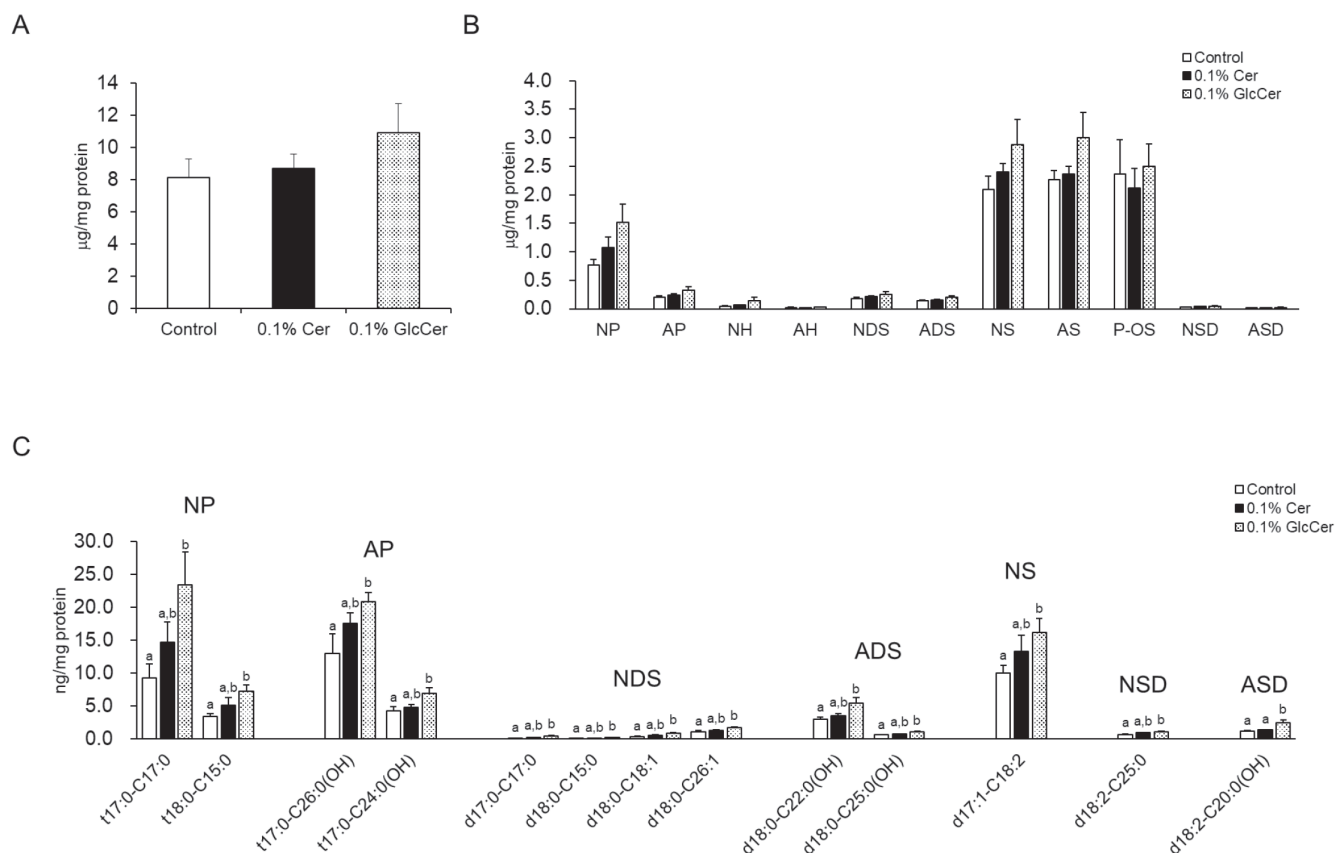
Fig. 4 Levels of total ceramide（A）, each ceramide class（B）, and significantly changed ceramide molecules（C）in mice epidermis fed different diets（control group, n＝5; 0.1％ Cer and 0.1％ GlcCer groups, n＝6）. Data presented as means±SE and were analyzed by one-way ANOVA, followed by Tukey–Kramer tests. Bars with different letters in each group showed significant difference at $p < 0.05$.

3.3 Effect of dietary soy sauce lees ceramide and maize glucosylceramide on mRNA expression in skin

We analyzed the levels of mRNA expression in ceramide synthases（*Cers2-6*）and protein-bound ω-hydroxyceramide synthesis-related genes（*Pnpla1*, *Cyp4f39* and *Tgm1*）in the dorsal skin（**Figs. 5A** and **5B**）. The mRNA expression level of *Cers4* in 0.1％ GlcCer group was significantly lower compared with that of the control group（**Fig. 5A**）. In the 0.1％ Cer group, the mRNA expression level of *Cers4* tended to decrease, as compared to the control group. Furthermore, the levels of mRNA expression in the corni-fied envelope（CE）formation-related genes（*Ivl*, *Lor*, *Tgm3*）, epidermal differentiation markers（*Krt5*, *Krt14*, *c-Fos*, *c-Jun*, *Notch1* and *Tgfb*）, natural moisturizing factor（NMF）production-related genes（*Flg*, *Flg2*, *Casp14* and *Blmh*）, and the inflammation marker *Tnfa* were inves-tigated（**Figs. 5C-5F**）. The mRNA expression level of *Ivl* in the 0.1％ Cer group was significantly lower compared with that of the control group（**Fig. 5C**）. In the 0.1％ GlcCer group, the mRNA expression level of *Ivl* tended to de-crease, as compared to the control group.

4 Discussion

This study, for the first time, demonstrated that dietary soy sauce lees ceramide enhances skin barrier function in normal hairless mice, as effectively or more effectively than maize glucosylceramide. Our finding is consistent with a previous report that dietary sphingolipids, such as gluco-sylceramide and sphingomyelin, could also improve skin barrier function under normal conditions[13]. On the other hand, dietary ceramide and glucosylceramide did not affect skin hydration or wrinkle formation. Our findings provide new insights into the effects of dietary ceramide and gluco-sylceramide on normal skin.

In this study, we evaluated the effect of dietary ceramide and glucosylceramide on ceramide composition in the epi-dermis, which is important for barrier function. We have previously reported that dietary sphingolipids improved skin barrier function by modulating sphingolipid metabo-lism in dry skin mice model fed on an HR-AD diet[13, 14]. Pre-vious results showed that the decreased level of ceramide ［P-OS］in this mice model was significantly increased by dietary sphingolipids, including glucosylceramide[14]. In this study, however, the total and subclass amounts of ceramide

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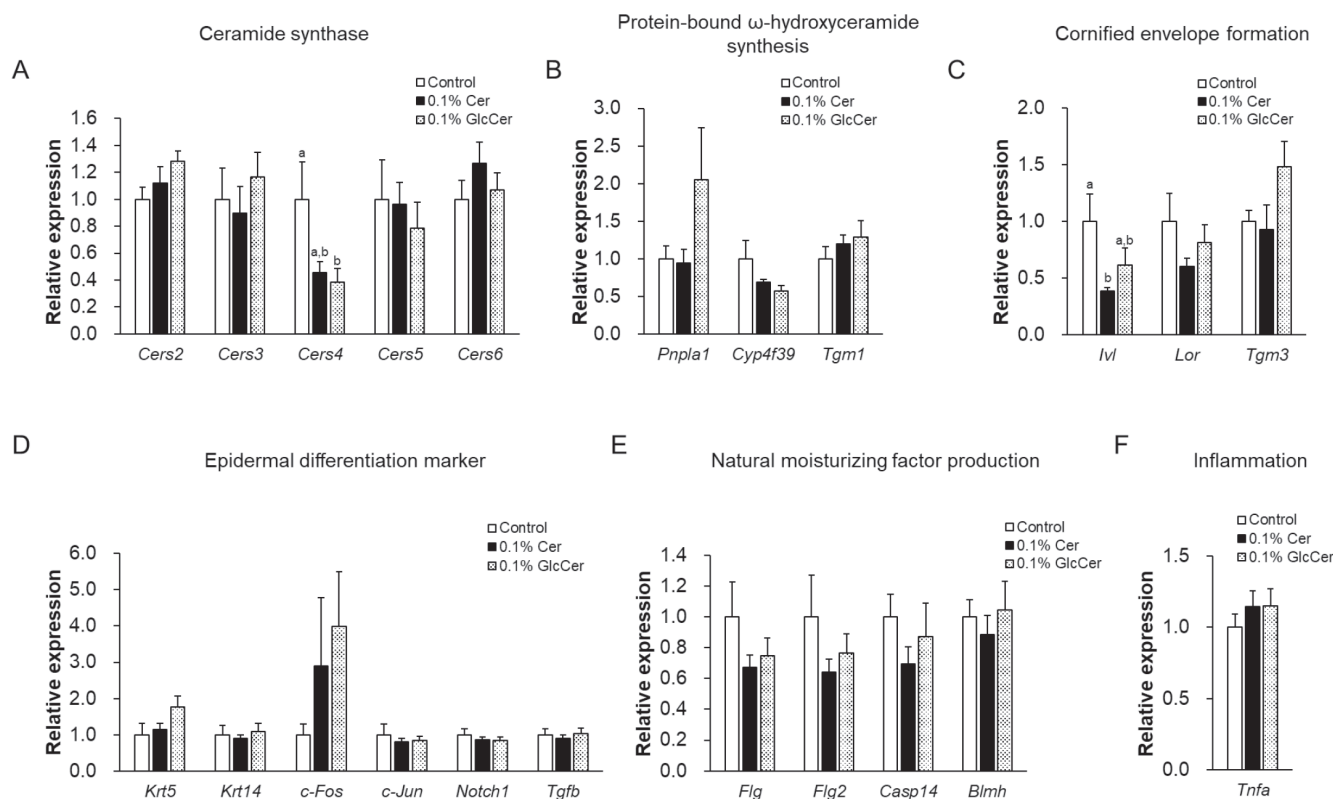
Fig. 5   Effect of dietary sphingolipids on the levels of mRNA expression in genes related to ceramide synthases (A), ω-hydroxyceramide synthesis-related genes (B), the cornified envelope formation-related genes (C), the epidermal differentiation marker genes (D), natural moisturizing factors (E) and the inflammation marker gene (F) in whole skin of normal mice fed different diets (control group, n = 5; 0.1% Cer and 0.1% GlcCer groups, n = 6). Relative amounts of mRNA levels were quantified by real-time RT-PCR. The expression of *Actb* mRNA was used as an internal control. Values presented as means ± SE and were analyzed by one-way ANOVA, followed by Tukey–Kramer tests. Bars with different letters in each group showed significant difference at $p < 0.05$.

in the epidermis of normal mice were not significantly altered by dietary ceramide and glucosylceramide. As we examined the ceramide molecular species of the epidermis in detail, 12 molecules, not contained in dietary sources, were significantly increased in the 0.1% GlcCer group and tended to increase in the 0.1% Cer group compared with the control group. Among them, the level of t17: 0-C17: 0, which is classified as ceramide [NP] known to correlate with TEWL, showed a greater increase by dietary sphingolipids than other ceramide molecules. In addition, dietary soy sauce lees ceramide and maize glucosylceramide were not detected as intact molecules in the epidermis of normal mice. Therefore, it can be inferred that the alteration of endogenous ceramides in the epidermis by dietary sphingolipids may contribute to skin barrier improvement under normal conditions. Especially, ceramide subclasses composed of phytosphingosine, [NP] and [AP], which showed a tendency to increase by dietary ceramide and glucosylceramide in this study, have been reported to lower the permeability and strengthened the lipid barrier of model SC membrane[19]. Although d18:2-C20:0 (OH) ceramide mole-

cule, which is a major ceramide backbone of maize glucosylceramide, significantly increased only in the 0.1% GlcCer group, the molecule could not be distinguished from the isomer consisting of endogenous 4,14-sphingadiene by our analytical method.

Several reports have shown that dietary sphingolipids upregulate the expression of genes related to skin barrier, such as those involved in ceramide synthesis and cornified envelope formation, in mouse models of skin disorders[13, 20−23]. However, our results indicated that *Cers4* and *Ivl* were downregulated in normal mice fed sphingolipids. *Cers4* is the predominant type of ceramide synthase in the epidermis and uses C18–C22 -CoA for the acyl chain moiety. It has been reported that balancing *Cers4* expression is necessary to maintain skin barrier function[7, 12, 24]. Involucrin is a major cornified envelope protein to which ceramide binds, and its lipid-protein complex is known to be essential for skin barrier function[25]. However, we could not prove whether the changes in mRNA expression levels in this study were reflected in the ceramide profile and skin barrier function. Further studies such as a metabolomic

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analysis including not only sphingolipids but also their metabolites are needed to clarify the molecular mechanism of the skin-improving effects of dietary ceramide and glucosylceramide under normal conditions.

Conflict of Interest

The authors declare no conflicting interest.

Contributions

Investigation, K.O.; Writing-original draft, K.O.; Resources, S.H., M.M., and T.U.; Supervision, Y.M. and T.S.; Conceptualization, T.S.; Funding acquisition, S.H., M.M., T.U., and T.S.; Project administration, T.S.; Writing-review and editing, T.S.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess21128

References

1) Weerheim, A.; Ponec, M. Determination of stratum corneum lipid profile by tape stripping in combination with high-performance thin-layer chromatography. *Arch. Dermatol. Res.* **293**, 191-199（2001）.
2) Yardley, H.J.; Summerly, R. Lipid composition and metabolism in normal and diseased epidermis. *Pharmacol. Ther.* **13**, 357-383（1981）.
3) Motta, S.; Monti, M.; Sesana, S.; Caputo, R.; Carelli, S.; Ghidoni, R. Ceramide composition of the psoriatic scale. *Biochim. Biophys. Acta* **1182**, 147-151（1993）.
4) Di Nardo, A.; Wertz, P.; Giannetti, A.; Seidenari, S. Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta Derm. Venereol.* **78**, 27-30（1998）.
5) Ishikawa, J.; Narita, H.; Kondo, N.; Hotta, M.; Takagi, Y.; Masukawa, Y.; Kitahara, T.; Takema, Y.; Koyano, S.; Yamazaki, S.; Hatamochi, A. Changes in the ceramide profile of atopic dermatitis patients. *J. Invest. Dermatol.* **130**, 2511-2514（2010）.
6) Ishikawa, J.; Shimotoyodome, Y.; Ito, S.; Miyauchi, Y.; Fujimura, T.; Kitahara, T.; Hase, T. Variations in the ceramide profile in different seasons and regions of the body contribute to stratum corneum functions. *Arch. Dermatol. Res.* **305**, 151-162（2013）.
7) Ito, S.; Ishikawa, J.; Naoe, A.; Yoshida, H.; Hachiya, A.; Fujimura, T.; Kitahara, T.; Takema, Y. Ceramide synthase 4 is highly expressed in involved skin of patients with atopic dermatitis. *J. Eur. Acad. Dermatol. Venereol.* **31**, 135-141（2017）.
8) Yokose, U.; Ishikawa, J.; Morokuma, Y.; Naoe, A.; Inoue, Y.; Yasuda, Y.; Tsujimura, H.; Fujimura, T.; Murase, T.; Hatamochi, A. The ceramide［NP］/［NS］ratio in the stratum corneum is a potential marker for skin properties and epidermal differentiation. *BMC Dermatol.* **20**, 6（2020）.
9) Yamamoto, M.; Sassa, T.; Kyono, Y.; Uemura, H.; Kugo, M.; Hayashi, H.; Imai, Y.; Yamanishi, K.; Kihara, A. Comprehensive stratum corneum ceramide profiling reveals reduced acylceramides in ichthyosis patient with CERS3 mutations. *J. Dermatol.* **48**, 447-456 （2021）.
10) Morifuji, M.; Oba, C.; Ichikawa, S.; Ito, K.; Kawahata, K.; Asami, Y.; Ikegami, S.; Itoh, H.; Sugawara, T. A novel mechanism for improvement of dry skin by dietary milk phospholipids: Effect on epidermal covalently bound ceramides and skin inflammation in hairless mice. *J. Dermatol. Sci.* **78**, 224-231（2015）.
11) Jennemann, R.; Rabionet, M.; Gorgas, K.; Epstein, S.; Dalpke, A.; Rothermel, U.; Bayerle, A.; van der Hoeven, F.; Imgrund, S.; Kirsch, J.; Nickel, W.; Willecke, K.; Riezman, H.; Gröne, H.-J.; Sandhoff, R. Loss of ceramide synthase 3 causes lethal skin barrier disruption. *Hum. Mol. Genet.* **21**, 586-608（2012）.
12) Peters, F.; Tellkamp, F.; Brodesser, S.; Wachsmuth, E.; Tosetti, B.; Karow, U.; Bloch, W.; Utermöhlen, O.; Krönke, M.; Niessen, C.M. Murine epidermal ceramide synthase 4 is a key regulator of skin barrier homeostasis. *J. Invest. Dermatol.* **140**, 1927-1937.e5（2020）.
13) Duan, J.; Sugawara, T.; Hirose, M.; Aida, K.; Sakai, S.; Fujii, A.; Hirata, T. Dietary sphingolipids improve skin barrier functions via the upregulation of ceramide synthases in the epidermis. *Exp. Dermatol.* **21**, 448-452 （2012）.
14) Tomonaga, N.; Manabe, Y.; Aida, K.; Sugawara, T. Dietary ceramide 2-aminoethylphosphonate, a marine sphingophosphonolipid, improves skin barrier function in hairless mice. *Sci. Rep.* **10**, 13891（2020）.
15) Okubo, K.; Tomita, M.; Tsujino, M.; Yamauchi, F.; Otomo, K.; Sato, S.; Utsumi, N. Chemical properties of proteins in pressed cake from soy sauce mash. *J. Brew. Soc. Japan* **84**, 869-875（1989）.
16) Ohta, K.; Hiraki, S.; Miyanabe, M.; Ueki, T.; Aida, K.;

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Manabe, Y.; Sugawara, T. Appearance of intact molecules of dietary ceramides prepared from soy sauce lees and rice glucosylceramides in mouse plasma. *J. Agric. Food Chem*. doi: 10.1021/acs.jafc.0c07259（2021）. *in press.*

17）Suzuki, O.; Koura, M.; Noguchi, Y.; Uchino-Yamada, K.; Matsuda, J. Zygosity determination in hairless mice by PCR based on Hr^hrg Gene analysis. *Exp. Anim.* **62**, 267-273（2013）.

18）Kawana, M.; Miyamoto, M.; Ohno, Y.; Kihara, A. Comparative profiling and comprehensive quantification of stratum corneum ceramides in humans and mice by LC/MS/MS. *J. Lipid Res.* **61**, 884-895（2020）.

19）Uche, L.E.; Gooris, G.S.; Beddoes, C.M.; Bouwstra, J.A. New insight into phase behavior and permeability of skin lipid models based on sphingosine and phytosphingosine ceramides. *Biochim. Biophys. Acta* **1861**, 1317-1328（2019）.

20）Ideta, R.; Sakuta, T.; Nakano, Y.; Uchiyama, T. Orally administered glucosylceramide improves the skin barrier function by upregulating genes associated with the tight junction and cornified envelope formation. *Biosci. Biotechnol. Biochem.* **75**, 1516-1523（2011）.

21）Hasegawa, T.; Shimada, H.; Uchiyama, T.; Ueda, O.; Nakashima, M.; Matsuoka, Y. Dietary glucosylceramide enhances cornified envelope formation via transgluta-minase expression and involucrin production. *Lipids* **46**, 529-535（2011）.

22）Takatori, R.; Le Vu, P.; Iwamoto, T.; Satsu, H.; Totsuka, M.; Chida, K.; Shimizu, M. Effects of oral administration of glucosylceramide on gene expression changes in hairless mouse skin: Comparison of whole skin, epidermis, and dermis. *Biosci. Biotechnol. Biochem.* **77**, 1882-1887（2013）.

23）Kawada, C.; Hasegawa, T.; Watanabe, M.; Nomura, Y. Dietary glucosylceramide enhances tight junction function in skin epidermis via induction of Claudin-1. *Biosci. Biotechnol. Biochem.* **77**, 867-869（2013）.

24）Levy, M.; Futerman, A.H. Mammalian ceramide synthases. *IUBMB Life* **62**, 347-356（2010）.

25）Marekov, L.N.; Steinert, P.M. Ceramides are bound to structural proteins of the human foreskin epidermal cornified cell envelope. *J. Biol. Chem.* **273**, 17763-17770（1998）.