Effects of *Asterias amurensis*-derived Sphingoid Bases on the *de novo* Ceramide Synthesis in Cultured Normal Human Epidermal Keratinocytes

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Abstract: *Asterias amurensis* starfish provide several bioactive species in addition to being fishery waste. Glucosyl ceramides (GlcCers) were extracted from the viscera of these starfish and were isolated by silica gel column chromatography. Degraded GlcCers generated *A. amurensis* sphingoid bases (ASBs) that mainly consisted of the triene-type bases d18:3 and 9-methyl-d18:3. The effect of these bases on ceramide synthesis and content were analyzed using normal human epidermal keratinocytes (NHEKs). The bases significantly enhanced the *de novo* ceramide synthesis and gene expression in NHEKs for proteins, such as serine-palmitoyltransferase and ceramide synthase. Total ceramide, GlcCer, and sphingomyelin contents increased dramatically upon ASB treatment. In particular, GlcCer bearing very-long-chain fatty acids ($\geq$ C28) exhibited a significant content increase. These ASB-induced enhancements on *de novo* ceramide synthesis were only observed in undifferentiated NHEKs. This stimulation of the *de novo* sphingolipid synthesis may improve skin barrier functions.

Key words: *Asterias amurensis*, glucosylceramide, sphingoid base, keratinocyte, ceramide

1 INTRODUCTION

Mainly present on mammal skin surfaces, ceramides, which consist of an amide-linked fatty acid and a sphingoid base, act as a protective permeability barrier against transepidermal water loss and exogenous material invasion¹⁾. Their deficiency has been linked to atopic diseases (ADs)²⁾. Mono-hexosyl derivatives, such as glucosyl ceramides (GlcCers), exhibit various biological functions, such as anti-tumor, anti-inflammatory, plant growth regulating, and anti-microbial activities³⁻⁵⁾. Dietary GlcCers obtained from different sources can also improve skin condition⁶⁻⁸⁾, making them attractive therapeutic agents against AD. Several mechanisms suggest that absorbed sphingoid bases produced from GlcCer degradation by intestinal bacterial flora and enzymes activate the *de novo* ceramide synthesis on keratinocytes. This activation results from the enhancement of synthesis-associated gene expression upon exposure to plant-derived sphingoid bases⁹⁾.

Widely distributed in Hokkaido, the *Asterias amurensis* starfish damages fishing equipment as well as Japanese scallop and oyster aquafarming facilities. In addition,

15,000 tons of starfish are landed with other valuable species every year and treated as costly industrial waste in Hokkaido. On the other hand, this starfish contains bioactive compounds, such as astaxanthin¹⁰⁾, saponin¹¹⁾, and eicosapentaenoic acid-rich phosphatidylcholine¹²⁾. In particular, it comprises higher GlcCer amounts (0.7% of wet internal organ weight)¹³⁾ than plant sources^{14,15)}. Moreover, starfish-derived GlcCers consisted of structurally different sphingoid bases from other sources. Specifically, plants and fungi, including edible mushrooms, contain sphingadienine (d18:2) and 9-methyl-sphingadienine (9-Me-d18:2), respectively. Humans produce sphingosine (d18:1), sphinganine (d18:0), and phytosphingosine (t18:0)¹⁶⁾. *Asterias amurensis* GlcCers comprise relatively long-carbon-chain sphingoid bases, such as C22 sphingosine (d22:1), C22 sphingadienine (d22:2), and C18 sphingatrienine (d18:3), in addition to 9-methyl-sphingatrienine (9-Me-d18:3)^{13,17)}. In this study, sphingoid bases were prepared from *A. amurensis* GlcCers, and their impact on the ceramide *de novo* synthesis was examined using normal human epidermal keratinocytes (NHEKs) by high performance liquid chroma-

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tography tandem mass spectrometry (LC-MS/MS).

2 EXPERIMENTAL

2.1 General

HuMedia-KG2 medium, keratinocyte growth enhancers, and NHEKs from neonatal foreskin were purchased from Kurabo (Osaka, Japan). Sodium 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium (WST-1) and 1-methoxy-5-methylphenazinium methyl sulfate (1-methoxy PMS) were obtained from Dojindo Laboratories (Kumamoto, Japan). The PrimeScript RT Reagent Kit (Perfect Real Time) was purchased from Takara Bio Inc. (Shiga, Japan). TRIzol reagent was acquired from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA). N-heptadecanoyl-D-erythro-sphingosine (C17 ceramide; d18:1-17:0), N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine (C17 sphingomyelin; d18:1-17:0), D-glucosyl-1, 1'-N-lauroyl-D-erythro-sphingosine (C12 glucosyl ceramide; d18:1-12:0), and D-erythro-sphingosine (C17 sphingosine; d17:1) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA).

2.2 Isolation of starfish GlcCers and sphingoid bases

Asterias amurensis was collected along the coast of Mori, Hokkaido, Japan in October, 2011. Its total lipid was extracted using ethanol, filtrated with filter paper (Advantec No.1), and concentrated *in vacuo*. The crude extract was transferred to screw capped test tubes and diluted using five volumes of cold acetone before centrifugation ($1500 \times g$, 5 min, 0°C). The supernatant was discarded while polar lipids were isolated. Glycerolipids were hydrolyzed by incubation at 40°C for 2 h in the presence of 0.4 N KOH in methanol. The solvent was evaporated, and the residue was purified by silica gel column chromatography using a chloroform/methanol solvent gradient (10:0→9:1→8:2 (v/v)) as an eluent. The fraction eluted with 8:2 chloroform/methanol (v/v) was further purified by silica gel column chromatography using 9:1:0.05 chloroform/methanol/acetic acid (v/v/v) to yield the starfish GlcCers.

To analyze *A. amurensis* sphingoid bases (ASBs) at the molecular level, GlcCers were hydrolyzed according to slightly modified known procedures^{18, 19}. Specifically, purified GlcCers (250 mg) were hydrolyzed in 1:1 dioxane/10% aqueous $\text{Ba}(\text{OH})_2$ (v/v) at 110°C for 24 h. Heating in 1 M HCl-methanol solution generated numerous artifacts, thus ASBs preparation was done under alkali condition. The sphingoid bases were extracted with ethyl acetate and subsequently purified by preparative thin layer chromatography (silica gel 60 F254, Merck, Darmstadt, Germany) using 40:10:1 chloroform/methanol/2 N ammonium hydroxide (v/v/v). The obtained ASBs (80.9 mg) were analyzed by electrospray ionization-mass spectrometry (ESI-MS) and o-

phthalaldehyde (OPA)-derivatized high performance liquid chromatography (HPLC)²⁰.

2.3 Cell viability assay

Normal human epidermal keratinocytes were seeded in a 96-well plate at a density of 1.5×10^4 cells/ cm^2 and cultured to 90% confluence in HuMedia-KG2 medium. The medium was changed daily. After 4 days of cultivation, test samples in 0.1% dimethyl sulfoxide (DMSO) were added to the wells and incubated for 24 h. Subsequently, WST-1 and 1-methoxy PMS (10 μL) were added and incubated for 1.5 h. The absorbance at 450 nm was measured by quantifying the produced formazan dye. The cell viability was determined to compare the absorbance of control samples (0.1% DMSO).

2.4 *Asterias amurensis* sphingoid base treatment

The effect of prepared ASBs on the ceramide production was assessed in undifferentiated and differentiated NHEKs. For RNA and ceramide extractions, NHEKs were seeded in 24-well plates at a density of 1.5×10^4 cells/ cm^2 and in 55 cm^2 culture dishes at a density of 7.0×10^3 cells/ cm^2 , respectively. To generate undifferentiated NHEKs, cells were grown in the HuMedia-KG2 medium containing 0.07 mM Ca^{2+} and harvested at 90% confluence. At 90% confluence, the medium was changed to 2:1 Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (v/v) containing 1.2 mM Ca^{2+} and supplemented with 10% fetal bovine serum, 10 $\mu\text{g}/\text{mL}$ insulin, 0.4 $\mu\text{g}/\text{mL}$ hydrocortisone, and 50 $\mu\text{g}/\text{mL}$ ascorbic acid and cultured for 5 days to produce differentiated NHEK²¹. For gene expression and sphingolipid analyses, NHEKs were incubated in the presence of ASBs for 24 h and 5 days, respectively. Sphingoid bases were added as DMSO solutions at a final concentration of 0.1%, and the medium was changed daily.

2.5 RNA extraction and real time polymerase chain reaction (PCR)

The total RNA was isolated using the TRIzol reagent according to the manufacturer's protocol and subjected to cDNA synthesis using the PrimeScript RT reagent kit. Real time PCRs were performed using the KAPA SYBR Fast qPCR kit. Primers used in this study are listed in Table 1. Individual mRNA levels were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.6 Sphingolipid extraction and quantification by LC-MS/MS

Keratinocytes were scraped from culture dishes and subjected to lipid extraction using 1:2 chloroform/methanol (v/v) (3 mL) three times. Extracts were evaporated to dryness under a nitrogen stream. Glycerol lipids were saponified by adding 0.4 N NaOH in methanol (1 mL) to the residue and incubating the mixture at 40°C for 2 h. Chloroform (1 mL) and water (1 mL) were added to the solution to

Table 1 PCR primers used in this study.

Gene	Forward primer	Reverse primer
Ceramide synthase 1 (CerS1)	CAGTGACGTGCAGCTTGAGT	CTGGTGGCATAACAGGACCTT
Ceramide synthase 2 (CerS2)	TCTACGCCAAAGCCTCAGAT	TGCCACTGGTCAGGTAGAAA
Ceramide synthase 3 (CerS3)	TGTACACGATGTGGCTGACA	AAGGTGAAGGACCTGCAAGA
Ceramide synthase 4 (CerS4)	CTCGGTCCTGTACCACGAGT	CTGCTCCTTGAAATCCTTGC
Ceramide synthase 5 (CerS5)	TGCATCACTTGGTCACCAT	AGGGTCGTGTTTCTGAGATCCA
Ceramide synthase 6 (CerS6)	TCATCAACTCTTTGCCACACA	CAGAGATGCTCAAGCACTGAA
Serine palmitoyltransferase 2 (SPTLC2)	CCTGTCAGCAGCTCATACCA	GCCACAGAAGTGTGGTTCCT
Sphingolipid $\Delta 4$ -desaturase 1 (DES1)	CATGATTTCCCAACATTCC	TGGTGCCTCTTCATTCTTGA
Glucosyl ceramide synthase (GCS) ^a	GTTCGTCTCTTCTTGGTGC	AGAAGAGAGACACCTGGGAGC
β -glucocerebrosidase (GCase)	ACTGGGCAAAGGTGGTACTG	GCTGTACTGCATCCCTCGAT
Sphingomyelin synthase 1 (SMS1)	GCCAGGACTTGATCAACCTAACC	CCATTGGCATGGCCGTTCTTG
Sphingomyelin synthase 2 (SMS2)	CACCCAGTGGCTGTTTCTGA	TGCATTCCAGGCACAGGTAGA
Acid sphingomyelinase (aSMase)	TGGCTCTATGAAGCGATGGC	TTGAGAGAGATGAGGCGGAGAC
Elongase of very-long-chain fatty acids 1 (ELOVL1) ^b	AACTCTCCGTGCATGTCATAAT	TGCTTTTCCACCAAAGGTAGG
Elongase of very-long-chain fatty acids 3 (ELOVL3) ^b	GTATTGGGCAACCTCATTCCT	CCTTGGCTTCCTTCATGTAGT
Elongase of very-long-chain fatty acids 4 (ELOVL4) ^c	TTTGGTGGAAACGATACCTGA	AGTGCATCCATTGGGGAAG
Elongase of very long-chain fatty acids 5 (ELOVL5)	GTCTACCACCATGCCTCGAT	GGTCTGGATGATTGTTCAGCA
Elongase of very-long-chain fatty acids 6 (ELOVL6)	AGAGGTGAGCCGAAGATCAA	AGCAAATGCAGATGATGCAG
Elongase of very-long-chain fatty acids 7 (ELOVL7)	ACCTGGTGGTTTGGAGTCAA	TGGATGGCGACAATAACAAA
Involucrin (INV)	AGCCTTACTGTGAGTCTGGT	CCTCTTGCTTTGATGGGACC
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	GAGTCAACGGATTGGTTCGT	TTGATTTGGAGGGATCTCG

^a See Ref. 9.^b See the Primer Bank website (<http://pga.mgh.harvard.edu/primerbank/>).^c See Ref. 40. Primers specificities were checked by the its melting curve.

generate two phases. The hydrophobic phase was collected and analyzed by LC-MS/MS using a TripleTOF 5600 System (AB SCIEX, Foster City, CA, USA) equipped with an electrospray ionization probe and interfaced with a Prominence UFLC system (Shimadzu, Kyoto, Japan) according to a slightly modified reported method²². Extracted lipids (10 μ L) were injected into a column (InertSustain NH2, 5 μ m, 2.1 i.d. $\times$ 150 mm, GL Science, Tokyo, Japan). Solvents A and B consisted of 95:5:0.2 acetonitrile/methanol/formic acid (v/v) containing 5 mM ammonium formate and 100:0.2 methanol/formic acid (v/v) containing 5 mM ammonium formate, respectively. A sample was eluted at 0.2 mL/min through a 45 min solvent gradient: 0–5 min 0% B, 5–10 min from 0 to 20% B, 10–12 min hold 20% B, 12–15 min from 20 to 50% B, 15–22 min hold 50% B, 22–27 min from 50 to 80% B, 27–30 min hold, and 30–45 min from 80 to 0% B. The LC-MS/MS analysis was conducted in positive ion mode. Monitoring ions for individual ceramide (Cer), GluCer, and sphingomyelin (SM) molecular species were selected for their specific product ions. C17-Cer, C12-GluCer, and C17-SM were chosen as internal standards to quantify the corresponding molecular species. Data acquisition and analysis were performed using AnalystTF software version

1.7 (AB SCIEX).

2.7 Statistical analysis

Significant differences were determined by Student's *t*-test. In this test, $p < 0.05$ was considered as statistical significance. Data are presented as mean $\pm$ SD values.

3 RESULTS

3.1 *Asterias amurensis* sphingoid base composition

Molecular compositions of ASBs were determined by HPLC after fluorescent derivatization (Fig. 1). Each peak was identified by ESI-MS. The sphingoid bases mainly consisted of d18:3 (27.3 mol%), 9-Me-d18:3 (41.4 mol%), and d22:2 (9.2 mol% and 6.4 mol%), which displayed relatively shorter chains than in previous studies¹³.

3.2 Real time PCR mRNA analysis relative to *de novo* ceramide synthesis

Similarly to the major animal sphingoid base sphingosine, ASBs did not show cytotoxicity at a concentration of 30 μ M by WST-1 assay. Therefore, ceramide production assays

Fig. 1 HPLC chromatogram of OPA-derivatized ASBs. 1: d18:3 (27.3 mol%), 2: 9-Me-d18:3 (41.4 mol%), 3: d22:2 (9.2 mol%), 4: d22:2 (6.4 mol%), and others (15.7 mol%).

Fig. 2 mRNA changes during the *de novo* ceramide synthesis in ASB-treated NHEKs. (A) undifferentiated NHEK, (B) differentiated NHEK, (C) differentiation marker INV in undifferentiated NHEKs. Values appear as mean $\pm$ SD (n = 4). * $0.01 < p < 0.05$, ** $p < 0.01$.

Fig. 3 Selected ion chromatograms of extracted sphingolipids from NHEK (A) and MS/MS spectra of Cer (d18:1-C24:1, B), GlcCer (d18:1-C24:1, C), and SM (d18:1-C24:1, D).

Table 2 Ceramide quantification by LC-MS/MS.

Molecular species	Ceramide (nmol/55 cm ² culture dish)		
	ASBs		
	Control	10 μ M	30 μ M
d18:0-C16:0	1.696 $\pm$ 0.286	1.268 $\pm$ 0.274	2.095 $\pm$ 0.277
d18:0-C16:0	0.037 $\pm$ 0.003	0.053 $\pm$ 0.003	0.057 $\pm$ 0.008
d18:1-C18:1	0.017 $\pm$ 0.003	0.028 $\pm$ 0.004	0.034 $\pm$ 0.010
d18:1-C18:0	0.167 $\pm$ 0.026	0.288 $\pm$ 0.046 ^a	0.498 $\pm$ 0.079 ^{ab}
d18:0-C18:1	0.001 $\pm$ 0.000	0.001 $\pm$ 0.000	0.001 $\pm$ 0.000
d18:0-C18:0	0.005 $\pm$ 0.001	0.008 $\pm$ 0.002 ^a	0.012 $\pm$ 0.002 ^{ab}
d18:1-C20:1	0.004 $\pm$ 0.000	0.007 $\pm$ 0.002 ^a	0.009 $\pm$ 0.003 ^{ab}
d18:1-C20:0	0.041 $\pm$ 0.011	0.059 $\pm$ 0.014 ^a	0.081 $\pm$ 0.013 ^{ab}
d18:0-C20:0	0.002 $\pm$ 0.000	0.002 $\pm$ 0.000	0.003 $\pm$ 0.001
d18:1-C22:1	0.018 $\pm$ 0.007	0.039 $\pm$ 0.013	0.031 $\pm$ 0.007
d18:1-C22:0	0.184 $\pm$ 0.077	0.227 $\pm$ 0.078	0.390 $\pm$ 0.093 ^a
d18:0-C22:1	0.001 $\pm$ 0.000	0.002 $\pm$ 0.000	0.002 $\pm$ 0.000
d18:0-C22:0	0.006 $\pm$ 0.002	0.007 $\pm$ 0.002	0.012 $\pm$ 0.003
d18:1-C24:1	0.528 $\pm$ 0.120	0.859 $\pm$ 0.315	0.858 $\pm$ 0.115
d18:1-C24:0	0.433 $\pm$ 0.117	1.002 $\pm$ 0.231	1.051 $\pm$ 0.066
d18:0-C24:1	0.024 $\pm$ 0.010	0.037 $\pm$ 0.013 ^a	0.043 $\pm$ 0.009 ^a
d18:0-C24:0	0.035 $\pm$ 0.017	0.025 $\pm$ 0.008 ^a	0.034 $\pm$ 0.009 ^a
d18:1-C26:1	0.242 $\pm$ 0.114	0.543 $\pm$ 0.174 ^a	0.571 $\pm$ 0.158 ^a
d18:1-C26:0	0.199 $\pm$ 0.101	0.380 $\pm$ 0.103	0.352 $\pm$ 0.150
d18:1-C28:1	0.144 $\pm$ 0.125	0.162 $\pm$ 0.050	0.432 $\pm$ 0.233
d18:1-C28:0	0.021 $\pm$ 0.019	0.032 $\pm$ 0.006	0.073 $\pm$ 0.052
d18:1-C30:1	0.042 $\pm$ 0.041	0.041 $\pm$ 0.010	0.132 $\pm$ 0.085
d18:1-C30:0	0.005 $\pm$ 0.004	0.009 $\pm$ 0.002	0.013 $\pm$ 0.008
d18:1-C32:1	0.010 $\pm$ 0.010	0.009 $\pm$ 0.002	0.025 $\pm$ 0.014
d18:1-C32:0	0.007 $\pm$ 0.003	0.008 $\pm$ 0.002	0.013 $\pm$ 0.006

^a Significant difference from the control group at $p < 0.05$.^b Significant difference from the 10 μ M group at $p < 0.05$.

were conducted at 30 μ M in this study. Sphingoid base-treated NHEKs significantly enhanced the *de novo* ceramide biosynthesis of mRNA in undifferentiated, but not in differentiated cells after 24 h (Figs. 2A and 2B). The mRNA levels of *CerS2*, *CerS3*, *CerS4*, *SPTLC2*, *GCase*, *SMS2*, *aSMase*, *ELOVL4*, and *ELOVL7* increased 1.27-, 1.23-, 1.50-, 1.39-, 1.36-, 1.21-, 1.64-, 1.58-, and 1.40-fold, respectively, in the presence of 30 μ M ASBs. Involucrin (INV), a valuable keratinocyte differentiation maker characteristic of cornified envelope synthesis, increased dramatically (1.74-fold change) upon ASB treatment in undifferentiated NHEKs (Fig. 2C).

3.3 Ceramide, GlcCer, and sphingomyelin quantification by LC-MS/MS

Sphingolipid content rose with increasing cell concentration in undifferentiated NHEKs after five day exposure to 30 μ M ASBs. As shown in Fig. 3A, ceramide (d18:1-C24:1), GlcCer (d18:1-C24:1), and SM (d18:1-C24:1) eluted at 2.71, 7.59, and 20.23 min, respectively. In our HILIC-HPLC condition, ceramide, GlcCer, and SM showed similar retention time with on relation to amide-linked fatty acid chain. Quantification of each sphingolipid molecular species were done with multi reaction monitoring method using the key MS/MS product ion signals of ceramide, GlcCer, and sphingomyelin which linked to d18:1 are m/z 264, 264, and 184, to d18:0 are m/z 266, 266, and 186²²⁾. Detected molecular species are listed in Tables 2, 3 and 4. Total ceramide,

Table 3 Glucosyl ceramide quantification by LC-MS/MS.

Molecular species	Glucosyl ceramide (nmol/55 cm ² culture dish)		
	ASBs		
	Control	10 μ M	30 μ M
d18:0-C16:0	2.008 $\pm$ 0.378	1.831 $\pm$ 0.385	2.136 $\pm$ 0.154
d18:1-C18:1	0.005 $\pm$ 0.003	0.009 $\pm$ 0.003	0.021 $\pm$ 0.003 ^a
d18:1-C18:0	0.026 $\pm$ 0.014	0.087 $\pm$ 0.021 ^a	0.144 $\pm$ 0.031 ^{ab}
d18:1-C20:1	0.003 $\pm$ 0.001	0.004 $\pm$ 0.001	0.012 $\pm$ 0.002 ^a
d18:1-C20:0	0.016 $\pm$ 0.014	0.027 $\pm$ 0.007	0.054 $\pm$ 0.013 ^{ab}
d18:1-C22:1	0.024 $\pm$ 0.010	0.033 $\pm$ 0.007	0.057 $\pm$ 0.012 ^{ab}
d18:1-C22:0	0.111 $\pm$ 0.038	0.144 $\pm$ 0.025	0.364 $\pm$ 0.040 ^{ab}
d18:1-C24:1	0.601 $\pm$ 0.197	0.721 $\pm$ 0.124	1.721 $\pm$ 0.074 ^{ab}
d18:1-C24:0	0.462 $\pm$ 0.165	0.535 $\pm$ 0.089	1.038 $\pm$ 0.140 ^{ab}
d18:1-C26:1	0.382 $\pm$ 0.109	0.362 $\pm$ 0.083	0.665 $\pm$ 0.093 ^{ab}
d18:1-C26:0	0.207 $\pm$ 0.087	0.230 $\pm$ 0.050	0.297 $\pm$ 0.058
d18:1-C28:1	0.070 $\pm$ 0.025	0.051 $\pm$ 0.008	0.122 $\pm$ 0.024 ^{ab}
d18:1-C28:0	0.012 $\pm$ 0.004	0.012 $\pm$ 0.004	0.021 $\pm$ 0.006 ^{ab}
d18:1-C30:1	0.012 $\pm$ 0.004	0.013 $\pm$ 0.004	0.030 $\pm$ 0.009 ^{ab}
d18:1-C30:0	0.002 $\pm$ 0.001	0.002 $\pm$ 0.001	0.004 $\pm$ 0.001 ^{ab}

^a Significant difference from the control group at $p < 0.05$.^b Significant difference from the 10 μ M group at $p < 0.05$.**Table 4** Sphingomyelin quantification by LC-MS/MS.

Molecular species	Sphingomyelin (nmol/55 cm ² culture dish)		
	ASBs		
	Control	10 μ M	30 μ M
d18:1-C14:0	1.764 $\pm$ 0.216	1.857 $\pm$ 0.083	1.912 $\pm$ 0.459
d18:1-C16:1	1.086 $\pm$ 0.107	1.376 $\pm$ 0.042 ^a	1.691 $\pm$ 0.218 ^a
d18:1-C16:0	3.177 $\pm$ 0.398	3.466 $\pm$ 0.210	3.325 $\pm$ 0.716
d18:0-C16:0	2.533 $\pm$ 0.321	2.525 $\pm$ 0.161	2.807 $\pm$ 0.551
d18:1-C18:1	0.179 $\pm$ 0.043	0.242 $\pm$ 0.009	0.482 $\pm$ 0.030 ^{ab}
d18:1-C18:0	0.684 $\pm$ 0.063	0.640 $\pm$ 0.026	1.238 $\pm$ 0.123 ^{ab}
d18:0-C18:0	0.145 $\pm$ 0.033	0.125 $\pm$ 0.006	0.354 $\pm$ 0.011 ^{ab}
d18:1-C20:1	0.138 $\pm$ 0.019	0.300 $\pm$ 0.015 ^a	0.580 $\pm$ 0.053 ^{ab}
d18:1-C20:0	0.442 $\pm$ 0.045	0.378 $\pm$ 0.023	0.584 $\pm$ 0.003 ^{ab}
d18:0-C20:0	0.102 $\pm$ 0.021	0.103 $\pm$ 0.003	0.206 $\pm$ 0.008 ^{ab}
d18:1-C22:1	1.102 $\pm$ 0.166	1.153 $\pm$ 0.065	1.471 $\pm$ 0.279
d18:1-C22:0	1.271 $\pm$ 0.124	1.281 $\pm$ 0.084	1.826 $\pm$ 0.224 ^{ab}
d18:0-C22:0	0.261 $\pm$ 0.053	0.283 $\pm$ 0.015	0.474 $\pm$ 0.053 ^{ab}
d18:1-C23:1	0.244 $\pm$ 0.115	0.184 $\pm$ 0.009	0.415 $\pm$ 0.053 ^b
d18:1-C23:0	0.421 $\pm$ 0.313	0.168 $\pm$ 0.010	0.349 $\pm$ 0.022 ^b
d18:0-C23:0	0.103 $\pm$ 0.065	0.064 $\pm$ 0.005	0.158 $\pm$ 0.009 ^b
d18:1-C24:1	1.961 $\pm$ 0.229	2.224 $\pm$ 0.145	2.839 $\pm$ 0.584 ^a
d18:1-C24:0	0.444 $\pm$ 0.131	0.534 $\pm$ 0.033	0.710 $\pm$ 0.044 ^{ab}

^a Significant difference from the control group at $p < 0.05$.^b Significant difference from the 10 μ M group at $p < 0.05$.

Fig. 4 Total ceramides, GluCers, and sphingomyelins in undifferentiated NHEKs quantified by LC-MS/MS. Values appear as mean $\pm$ SD (For 10 μ M, $n = 3$; for 30 μ M, $n = 4$). *Significant difference from the control for $0.01 < p < 0.05$. **Significant difference from the control for $p < 0.01$.

GlcCer, and sphingomyelin contents increased 1.77-, 1.70-, and 1.33-fold, respectively, compared with control samples (Fig. 4). Ceramides and GluCers displayed similar amide-linked fatty-acid-chain-length profiles (Tables 2 and 3). Ultralong-chain fatty acids ($\geq C28$) linked to GluCers increased markedly upon exposure to 30 μ M ASBs. In the late NHEK differentiation stage, no significant differences were observed in ceramide content (data not shown).

4 DISCUSSION

The effects of ASBs on the sphingolipid biosynthesis activation in NHEKs were evaluated by extracting GluCers from *A. amurensis* and preparing their sphingoid bases. 4,8,10-Tri-unsaturated bases have been identified in ascidians, starfish, squid^{17, 23–25} and, rarely, in terrestrial plants^{26, 27}. They accounted for about 70% of isolated ASBs (d18:3 and 9-Me-d18:3), which also contained C22 bases (d22:2, 9.2 and 6.4 mol%). Shah *et al.* have reported that sphingoid bases comprised over 80% C22 sphingoid bases (d22:1 and d22:2) but only 11% C18 base (d18:3) in the cerebroside obtained from *A. amurensis* internal organs¹³. In contrast, Wu *et al.* have shown that d18:3 and d18:2 were major *A. amurensis* cerebroside constituents²⁸. Sphingoid bases present variable compositions under cold stress²⁹. The membrane fluidity is controlled with unsaturated carbon-carbon bonds and a methyl group at the C9 position³⁰. Therefore, the detected ASB molecular species may reflect the seawater conditions.

The expressions of *CerS3*³¹ and *INV*³² increase significantly upon keratinocyte differentiation, suggesting that ASBs facilitate NHEK differentiation. A previous study has demonstrated that some administrated sphingoid bases are transformed into ceramides by cultured keratinocytes³³.

To analyze the *de novo* sphingolipid production, ceramides, GluCers, and sphingomyelins, which originate from sphingosine and nonhydroxy fatty acids, were quantified. Although GluCers are the main precursors of stratum corneum ceramides in mice³⁴, the mRNA level of GCS did not increase in this study. On the other hand, the GCS activity was markedly enhanced when the keratinocyte differentiation medium was switched to a 1.4 mM Ca^{2+} -containing medium after four days²¹. mRNA analyses were examined only 24 h after ASB exposure, which may be insufficient incubation time to observe significant improvements in GCS mRNA level. Previous report showed that the mRNA levels of *CerS3*, *SPTLC2*, *GCase*, and *ELOVL4* increased 1.23-, 1.39-, 1.36-, 1.58-, and 1.58-fold, respectively, in the presence of 25 μ M sphingosine³³ which is mammalian predominant sphingoid base. On the other hand, other report showed that plant origin sphingoid bases increased sphingolipids *de novo* synthesis related mRNA levels but not 16.8 μ M sphingosine⁹. The concentration of ultralong-chain fatty acid-linked GluCers ($\geq C28$) rose dramatically, especially in the presence of 30 μ M ASBs in NHEKs. These ceramides are typically found in epidermis¹. *ELOVL4* null mice present deficiencies in ultralong-chain ceramides and the unique ω -O-acylcera-mides in the skin, which causes a loss in water retention ability and, consequently, neonatal death³⁵. *CerS3* deficient mice also lack ultralong-chain ceramides, resulting in neonatal death³⁶. Our results suggest that ASBs may improve skin barrier function. This is the first report on the stimulation of sphingolipid synthesis by GluCers derived from *A. amurensis*.

Previous reports have shown that peroxisome proliferator-activated receptor (PPAR) agonists promote keratinocyte differentiation³². These PPARs can be activated by sphingoid bases^{9, 37, 38}. According to Shirakura *et al.*⁹, the activation of PPAR γ requires a double bond at the C8 position of sphingoid bases. Bases such as d18:3 and 9-Me-d18:3 also bear a C8 double bond, indicating that ASBs may activate PPARs and induce ceramide production through a similar process. PPAR γ agonists strongly stimulate filaggrin and loricrin gene expressions but only enhance their corresponding protein levels during the NHEK differentiation stage³⁹. Therefore, sphingolipid production obeys different mechanisms depending on the NHEK differentiation. An attempt at separating ASBs by octadecylsilyl column chromatography failed to isolate the components with adequate purity because of their unusual elution behavior. Further investigations are necessary to identify the molecular mechanisms governing the influence of sphingoid bases on keratinocyte differentiation. Moreover, isolation methods need to be developed to obtain pure compounds from complex sphingoid base mixtures.

5 CONCLUSION

Sphingoid bases, such as the triene-type d18:3 and 9-Me-d18:3, were obtained by hydrolysis of *A. amurensis* GluCers. The impact of these bases on the *de novo* ceramide synthesis was assessed using cultured NHEKs. mRNA related to sphingolipid metabolism only increased at the cell proliferation stage, but not during differentiation. The bases also enhanced the ceramide content in differentiated NHEKs.

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