Acetic Acid Bacterial Lipids Improve Cognitive Function in Dementia Model Rats

HIROYUKI FUKAMI,*,[†] HIDEKI TACHIMOTO,[†] MIKIYA KISHI,[†] TAKAYUKI KAGA,[†] AND
YASUKAZU TANAKA[‡]

[†]Central Research Institute, Mizkan Group Corporation, Handa-shi, Aichi 475-8585, Japan, and
[‡]Department of Functional Biogerontology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku,
Tokyo 173-0015, Japan

Acetic acid bacteria, fermentative microorganisms of traditional foods, have unique alkali-stable lipids (ASL), such as dihydroceramide which is a precursor of sphingolipids. Sphingolipids are important components of the brain tissue. We examined the effect of oral administration of ASL in a rat model of dementia (7-week-old, male) with a basal forebrain lesion. In a water maze test, the dementia model rats demonstrated poor spatial orientation. The administration of ASL (165 or 1650 mg/kg of body weight per day, for 14 days) produced a significant improvement in learning ability in the dementia model rats. In vitro experiments showed ASL had the ability to promote neurite outgrowth in pheochromocytoma (PC12) cells. Among the ASL components, dihydroceramide has the most potent effect on the differentiation of PC12 cells. It is highly possible that oral administration of dihydroceramide-containing ASL reverses the decline in cognitive function in dementia.

KEYWORDS: Acetic acid bacteria; *Acetobacter*; alkali-stable lipids; sphingolipid; dihydroceramide; cognitive function; dementia

INTRODUCTION

Acetic acid bacteria are fermentative microorganisms found in traditional foods, such as vinegar, fermented milk (e.g., kefir, Caspian Sea yogurt), and nata de coco (*1−3*). They have characteristic lipid components which are limited to some Gram-negative bacteria: phospholipids (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol), coenzymes Q, and alkali-stable lipids (ASL). Comprehensive analysis and identification of ASL components showed that ASL consist of hopanoids (terpenoid compounds), sphingolipids (dihydroceramide and sphinganine), amino lipids, and free fatty acids (*cis*-vaccenate) (*4−7*), which are characterized in only a few bacteria among the fermentative microorganisms. All the sphingolipids in acetic acid bacteria have sphinganine as the sphingoid base and (2-hydroxypalmitoyl)sphinganine (dihydroceramide) as the main compound (*7*). Alternately, higher organisms contain a large variety of sphingolipids, such as glycosphingolipids and sphingomyelin; dihydroceramide-containing sphingolipids are only minor components (*8*). Therefore, it is very unique that dihydroceramide accumulates in acetic acid bacteria with such a high purity.

In animals, dihydroceramide is converted to ceramide, which is then transferred to various sphingolipids. Sphingolipids are important lipid components in the brain. For example, ceramide is reported to affect the development and survival of nerve cells (*9−12*). Gangliosides, composed of sialic acid and oligosaccharides conjugated to ceramide, are also shown to enhance the activity of

neurotrophic factors in events such as neuronal dendritic elongation, to participate in the formation of synapses, and to accelerate the release of acetylcholine from synapses (*13−15*). Subcutaneous injection with gangliosides was demonstrated to improve symptoms in Alzheimer's disease (AD) patients (*16*). These reports suggest that intake of ceramide and/or its derivatives might have favorable effects on various brain functions such as cognition, learning, and memory.

Cognitive functions such as working memory and space orientation are widely accepted to deteriorate with advancing age and dementia in experimental animals and humans. Such deterioration is caused by disorders in synaptic communication (*15*). In the case of AD patients, the function of cholinergic neurons was observed to be disordered in the basal nucleus of Meynert (*17*). Acetylcholinesterase inhibitors have been approved as treatment reagents for depressed cholinergic neurons (*18*). Various nutrients, such as docosahexaenoic acid (*19, 20*) and phosphatidylserine (*21*), have also been reported to reverse declines in cognitive function in experimental animals and humans. However, the effects of oral intake of ceramide and/or its derivatives on cognitive function have not yet been reported.

We focused on the possibility that acetic acid bacterial lipids, containing pure dihydroceramide, a precursor of the various sphingolipids in the brain, might improve cognitive function in dementia and/or aging. In this study, we extracted ASL from *Acetobacter* derived from vinegar and examined the effects of oral administration of ASL on learning and memory ability in Meynert nucleus-lesioned rats. These rats are reported to be used as a model of dementia in the evaluation of therapeutic agents (*22, 23*). We also examined the neuronal effect of ASL using pheochromocytoma (PC12) cells to

*To whom correspondence should be addressed: Central Research Institute, Mizkan Group Corp., 2-6, Nakamura-cho, Handa-shi, Aichi 475-8585, Japan. Telephone: +81-569-24-5128. Fax: +81-569-24-5029. E-mail: h-fukami@mizkan.co.jp.

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determine which component of ASL has the most potent effect on the differentiation of these cells.

MATERIALS AND METHODS

Preparation of ASL in Acetic Acid Bacteria. The extraction of total lipids from acetic acid bacteria and weakly alkaline treatment were performed according to a previous report (*24*). Briefly, solvent [1:1:0.8 (v/v) chloroform/methanol/water mixture] was added to the dry powder of *Acetobacter aceti* subsp. *xylinum* NBI 1002, derived from vinegar fermentation, and the mixture was incubated at room temperature with occasional shaking. Partitioning of the mixture was performed via addition of 1.25 volumes of chloroform and water. The lower layer was collected as total lipids. The total lipids were treated with 0.4 M KOH in methanol to decompose glyceryl phospholipids. The alkaline-treated lipids (ASL) were obtained from the reaction mixture by Folch's method (*25*).

Compositional Analyses of ASL. ASL was applied on a silica gel thin layer plate (Silica Gel 60, 20 cm × 20 cm, Merck Ltd. Japan, Tokyo, Japan), and the plate was developed with a chloroform/methanol mixture (96:4, v/v) or a chloroform/methanol/acetic acid mixture (65:25:10, v/v). Lipid spots were visualized with 50% H_2SO_4 and/or ninhydrin. To determine the yields of the major ASL components, ASL was fractionated by silica gel column chromatography (Wako gel C-100, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and each purified fraction was weighed and confirmed to be a single spot in TLC. ASL was further analyzed by HPLC to confirm the composition using benzoylated ASL (*26*). The dried ASL was reacted with 0.5 mL of a benzoyl chloride/anhydrous pyridine mixture (1:9, v/v) for 15 min at 70 °C, followed by addition of 0.5 mL of a methanol/water mixture (8:2, v/v). The benzoylated ASL was cleaned by being passed through a Sep-PakC18 column (Nihon Waters K. K., Tokyo, Japan) equilibrated with a methanol/water mixture (8:2, v/v), and the sample was eluted with methanol. After the sample had been redissolved in a small amount of a hexane/2-propanol mixture (100:0.8, v/v), benzoylated ASL was introduced into the HPLC system equipped with a Lichrospher 100 CN column (5 μm, 250 mm × 4 mm, Merck Ltd. Japan). Separation of each ASL component was performed with a hexane/2-propanol mobile phase (100:0.8, v/v) at a flow rate of 1.0 mL/min. Each HPLC peak was observed using a UV detector at 230 nm. Purified (2-hydroxypalmitoyl)sphinganine (dihydroceramide, purity of >98% by TLC), C_{35}-pentacyclic terpene alcohol (tetrahydroxybacteriohopane, purity of >98% by TLC), and D-*erythro*-dihydrosphingosine (sphinganine, purity of >98%, Sigma-Aldrich Japan Co., Tokyo, Japan) were used to make the standard curves for quantitative analysis.

Preparation of the Dementia Model Rat. Seven-week-old male Crlj: Wistar rats (SPF, Japanese Charles River, Ltd., Yokohama, Japan) were used. They were anesthetized with pentobarbital sodium (40 mg/kg of body weight) and fixed in a stereotaxic retainer. Ibotenic acid (5 μg) was injected into the Meynert nucleus of rats with a syringe pump through a guide cannula. Ibotenic acid is a neurotoxin, used to make the focal brain dysfunctional (*22, 23*). The rats were maintained with free access to a solid commercial diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water at a controlled temperature (21−25 °C) and humidity (42−63%) with a 12 h light−dark cycle. The CRF-1 diet contained the following nutrients: 78 g of water/kg, 224 g of protein/kg, 57 g of lipid/kg, 66 g of mineral/kg, 31 g of fiber/kg, 447 g of nitrogen-free extract/kg, and 15027 kJ of energy/kg. Handling of the experimental animals was performed according to the guidelines for "Guiding Principals for the Care and Use of Laboratory Animals" and the ethical treatment of laboratory animals at the Central Research Institute of the Mizkan Group Corp. (Aichi, Japan).

Animal Treatment. Ibotenic acid-treated rats were divided into four groups: high-ASL dosage (H-ASL) group, low-ASL dosage (L-ASL) group, tacrine-administered group, and vehicle-administered (nondemented) group. A fifth group was comprised of rats not treated with ibotenic acid. Each group consisted of 10 rats. ASL and tacrine were dispersed in the vehicle consisting of 3% (w/v) glyceryl monomyristate esters and administered through a stomach tube. The H-ASL group was given 1650 mg of ASL/kg of body weight per day (corresponding to 90 mg of dihydroceramide/kg of body weight per day), and the L-ASL group was given 165 mg of ASL/kg of body weight per day (corresponding to 9 mg of dihydroceramide/kg of body weight per day). These dosages were selected according to a previous report, in which monosialotetrahexosylganglioside (GM1) improved learning ability in rats with lesioned forebrain nuclei by intraperitoneal injection (30 mg/kg of body

weight per day, for 22 days) (*27*). As a positive control, tacrine (tetrahydroaminoacridine, Sigma-Aldrich Japan Co.), an acetylcholinesterase inhibitor, was administered (1 mg/kg of body weight per day) to the tacrine group (*24*). Administrations in all five groups were performed for 14 days after the ibotenic acid injection.

Water Maze Test.   *Water Maze Apparatus.* A round pool (148 cm in diameter and 44 cm in height) was used for the water maze test. Water (at 17−18 °C) had been put into the pool up to the height of 32 cm to hide the platform made of transparent acrylic fiber that could not be identified by sight (12 cm in diameter and 30 cm in height). The surface of the pool was divided into four areas. The platform was set up at the center of one area (36 cm from the center of the pool), and a lamp was set up behind the platform outside the pool as a spatial cue. The first water maze test was performed 11 days after administrations were completed, and the test was performed for a total of 5 days in all five groups.

Learning Trial. With the head facing the wall of the pool, the rat was placed in the water at a different point among five points in each trial. The time it took the animal to reach the platform (goal latency, seconds) was measured. Success in reaching the platform was recorded if the rat arrived on the platform within 90 s and stayed for 30 s. From the first to the fourth day, goal latency was measured twice daily (AM and PM). When a rat could not reach the platform on the first day, it was put on the platform for 30 s and then returned to the breeding cage.

Probe Trial. On the fifth day of the water maze test, the platform was removed in this probe trial. Rats were placed in a position diagonal to the location where the platform had been placed originally and were allowed to swim. The frequency of crossing the area of the original placement of the platform was recorded.

Cell Culture and Treatment. PC12 cells were obtained from the RIKEN (Ibaraki, Japan) cell bank. The culture medium consisted of DMEM containing 5% fetal bovine serum (FBS), 10% horse serum, 100 μg/mL streptomycin, and 100 units/mL penicillin. Cells were cultured on 35 mm collagen-coated dishes at 37 °C under 5% CO_2. After the cells had become confluent, they were treated with 0.25% trypsin, and 2 mL of a solution with a density of 0.5×10^6 cells/mL was disseminated to a new dish. Cells were cultured for 3−5 h, and after adhesion to the dish, ASL or purified ASL component was added to the culture medium. Free fatty acid and the methyl ester inhibit cell adhesion so they were removed from ASL by silica gel column chromatography. Each lipid fraction was properly diluted with dimethyl sulfoxide (DMSO), and 20 μL of the diluted solution was added. Nerve growth factor 2.5S (Sigma-Aldrich Japan Co.) was added to a final density of 0.05 μg/mL for the positive control (*12*). Pure DMSO was given for the negative control.
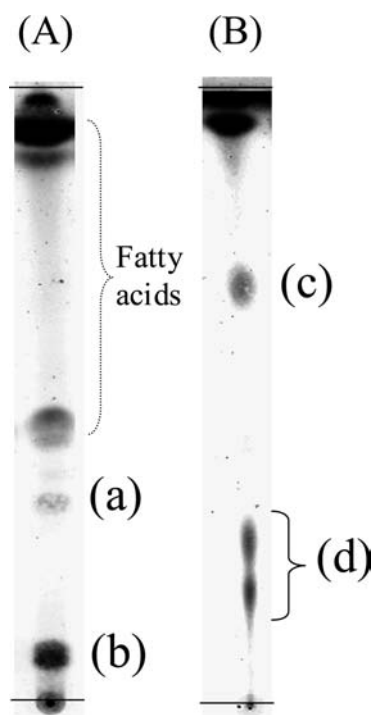
Evaluation of the Ratio of Differentiated Cells. Forty-eight hours after addition of ASL or each ASL component, morphological changes in PC12 cells were observed by fluorescent phase-contrast microscopy. Ten photographs (∼100−200 cells/piece) were taken for each dish (AxioVision 3.0, Carl Zeiss Japan Co., Ltd., Tokyo, Japan). All cells and cells with neurites in each photograph were counted manually for the calculation of the ratio of developed cells. Cells were considered to be developed when the length of the neurite extensions was greater than the diameter of the cell body.

Statistical Analysis. For the water maze test, to confirm the effect of ibotenic acid treatment, the comparison between the nondemented group and the vehicle group was tested by the Wilcoxon rank sum test. The comparison between vehicle group and each sample-administered group was tested by the Kluscal−Wallis test with the Shirley−Williams test. For analysis of the learning trial in the water maze test, the learning rate (decreasing rates in goal latency) of each rat was calculated by regression analysis; data were excluded if the R^2 value was less than 0.1. A Student's *t* test was used to compare differences between vehicle and each added component during analysis of the rates of developed PC12 cells. All data were analyzed by a statistical program (Excel Statistics 2006, Social Survey Research Information Co., Ltd., Tokyo, Japan). Data are expressed as means $\pm$ the standard error of the mean (SEM), and $p < 0.05$ was considered significant.
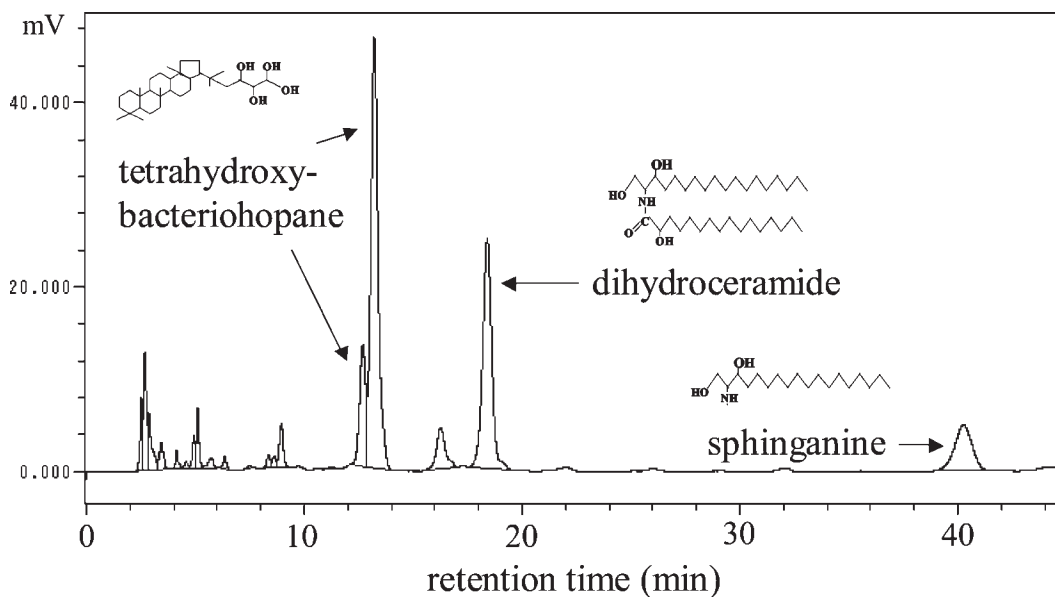
RESULTS

Composition of ASL. The yields of total lipids and ASL from a dry powder of *Acetobacter* were 16.1 and 5.0%, respectively. **Figure 1** shows thin layer chromatograms of ASL. Four major

lipid spots on the TLC (spots a−d) were detected. After fractionation of the four major ASL lipids by silica gel column chromatography, they were identified by IR, GC−MS, and an amino acid analyzer (4−7) as (a) (2-hydroxypalmitoyl)sphinganine (dihydroceramide), (b) C_{35}-pentacyclic terpene alcohol (tetrahydroxybacteriohopane), (c) sphinganine, and (d) amino lipids (tauro-ornithine lipid, ornithine lipid, and lyso-ornithine lipid). **Figure 2** shows a HPLC chromatogram of the benzoylated ASL lipids. The contents of the major lipids in ASL, tetrahydroxybacteriohopane, (2-hydroxypalmitoyl)sphinganine, and sphinganine, were determined using the purified standard compounds (**Table 1**).

Water Maze Test. None of the rats in the five groups exhibited abnormal observations or variations in body weight and dietary intake throughout the experiment. In the nondemented group, goal latencies in the learning trial were linearly shortened throughout the trials (**Figure 3**). In the vehicle-administered dementia model rats, however, goal latencies were not shortened in each learning trial. The mean goal latencies were shortened more promptly in every trial in the rats in the H-ASL and L-ASL groups when compared to the vehicle group ($p < 0.05$). This result indicates that ASL administration improved the learning

Figure 1. Thin layer chromatogram of the lipids in *A. aceti* subsp. *xylinum* NBI 1002 after treatment with a weak alkaline solution (ASL). Lipids were detected by 50% H_2SO_4. (**A**) Developed with a chloroform/methanol mixture (96:4) and (**B**) developed with a chloroform/methanol/acetic acid mixture (65:25:10). (a) Dihydroceramide, (b) tetrahydroxybacteriohopane, (c) sphinganine, and (d) amino lipids.

Table 1. Contents of the Major Components of ASL in *A. aceti* subsp. *xylinum* NBI 1002

| compound	content (%, w/w)
tetrahydroxybacteriohopane	11.5[a]
dihydroceramide	5.5[a]
sphinganine	3.2[a]
amino lipids	48.3[b]

[a] Values were determined by HPLC analysis. Mean values of the sum of three independent determinations. [b] Values were determined by the weights of ASL and purified fraction.
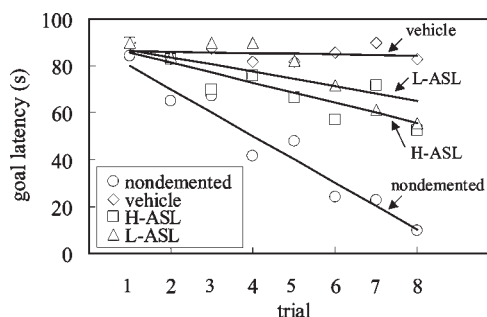
Figure 3. Measurements of goal latency (seconds) in the learning trial of the water maze test in nondemented rats and three groups (vehicle, H-ASL, and L-ASL) of dementia model rats ($n = 10$). Scatter plots of mean goal latencies with the best fit line in each group by regression analysis are shown.

Figure 2. HPLC chromatogram of the benzoylated derivatives of ASL lipids in *A. aceti* subsp. *xylinum* NBI 1002.

ability in the dementia model rats. The tacrine group, used as a positive control, exhibited a learning pattern similar to that of the L-ASL group, and there was a significant difference ($p < 0.05$) in the learning rate compared with that of the vehicle group (data not shown).

The probe trial in which the platform was removed was conducted after the learning trial. In the vehicle group, a significantly lower frequency of crossing the area where the platform had been located was observed when compared with that of the nondemented animals (**Figure 4**). The frequency of passing over the previous location of the platform was significantly greater in the H-ASL, L-ASL, and tacrine-treated dementia groups in comparison with the vehicle group; the highest value was obtained in the H-ASL dementia group. These results suggest
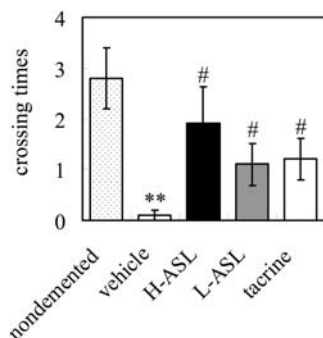
Figure 4. Probe trial of the water maze test in nondemented rats and four groups of dementia model rats ($n = 10$). Data are expressed as the mean number of times within 90 s that rats crossed the place where the platform had been located in the water maze test, and results are means ± SEM. Asterisks denote a significance level of $p < 0.01$ in the vehicle group compared with the nondemented group. Number signs denote a significance level of $p < 0.05$ in each administered group compared with the vehicle group.

that ASL, like tacrine, improves learning ability in dementia model rats.

Effect of ASL on Cell Differentiation. PC12 cells were cultivated in the presence of total ASL and fractionated lipid classes of ASL for 48 h before evaluation of induced neural differentiation. The proportion of neurite-bearing cells to total cells increased significantly after addition of total ASL (**Figure 5A,B**). The addition of dihydroceramide significantly increased the proportion of differentiated cells in a concentration-dependent manner (**Figure 5C**); however, no significant increase in the proportion of differentiated cells was observed after addition of the other lipid components of ASL. At the higher concentration ($> 50 \mu g/$ mL) of the lipid components of ASL, the adhesion of PC12 cells was inhibited and almost no effect on differentiation was observed. It is thought that the high concentration of lipid causes nonspecific inhibition of cell growth. *cis*-Vaccenate, a major fatty acid in crude ASL (*7*), did not affect cell differentiation (data not shown).

DISCUSSION

ASL from acetic acid bacteria consist of four major lipids: amino lipids, hopanoid compounds (e.g., tetrahydroxybacterio-hopane), dihydroxyceramide, and sphinganine (**Table 1**). The characteristic feature of ASL is that it contains a highly pure free dihydroceramide with sphinganine as the sphingoid base. Dihydroceramide is known to be a precursor of the various sphingolipids, such as gangliosides, that are located especially in the brain and are expected to improve cognitive function in dementia (*16*, *27*).

To elucidate the effect of oral administration of ASL extracted from *Acetobacter* on dementia model rats, a behavioral study using a water maze test was conducted. High and low dosages of ASL or tacrine were administered to dementia model rats, and their measured abilities (space orientation, learning, and memory) were significantly improved when compared to those of the
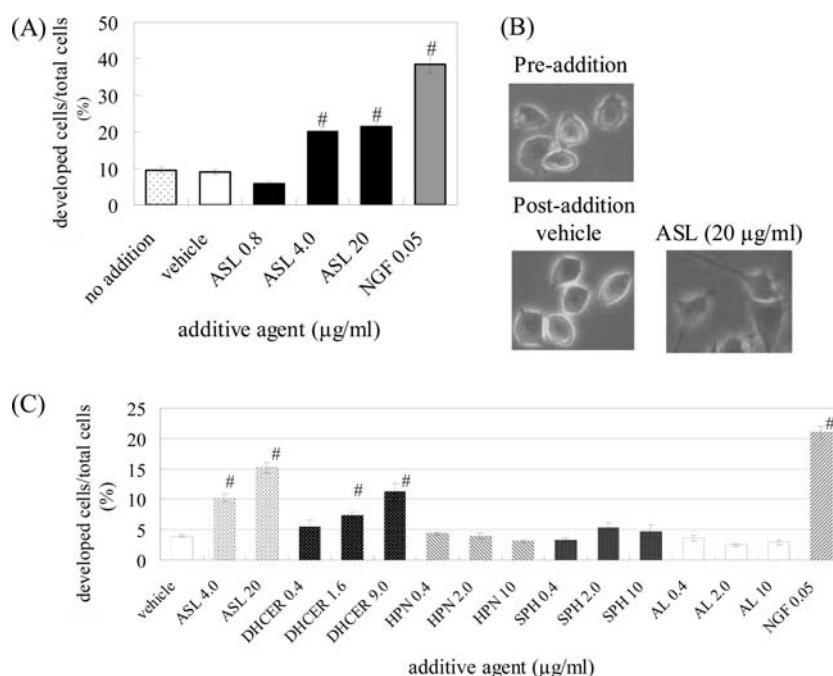
Figure 5. Effects of ASL on the differentiation of PC12 cells. (**A**) Proportions of differentiated PC12 cells to total cells 48 h after addition of ASL ($n = 10$). (**B**) Morphological differences in PC12 cells before and after addition of DMSO and ASL. (**C**) Proportions of differentiated PC12 cells to total cells 48 h after addition of each lipid class ($n = 10$). All results are means ± SEM. Number signs denote a significance level of $p < 0.01$ compared with the cells to which vehicle had been added. Abbreviations: NGF, nerve growth factor; DHCER, dihydroceramide; HPN, hopanoids; SPH, sphinganine; AL, amino lipids.

vehicle only group of rats. These results suggest that ASL could improve cognitive function in dementia model rats.

In vitro experiments showed that ASL promoted neurite outgrowth in PC12 cells. Among the ASL components, dihydroceramide was shown to have a differentiation effect on PC12 cells. Therefore, dihydroceramide, or a derivative metabolized in the cells, is considered to be a potent component affecting the function of nerve cells.

Dihydroceramide is synthesized from serine and palmitoyl-CoA and has sphinganine as its sphingoid base, which does not contain a double bond in the molecule. In animal tissues, the main sphingoid base is sphingosine, which is an unsaturated molecule. In the experiments in which radiolabeled sphinganine or sphingosine was orally administered to rats, the amounts of sphinganine detected in the intestinal tract lymph were remarkably higher than those of sphingosine (*28*), suggesting that ingested sphinganine-based lipids are more available for absorption and utilization in animal tissues than other sphingoid bases.

The effect of dihydroceramide on PC12 cells in this study displayed nerve growth factor-like effects, perhaps through common receptors such as the tyrosine kinase type receptor (*14*). Nerve growth factors are reported to be potential therapeutic agents (*15*) but cannot cross the blood−brain barrier. Ceramide and its glycosylated derivatives were shown to have important effects on neuronal function in the central nervous system. For example, in vitro studies showed that ceramide can promote dendrite and axon elongation and survival of neuronal cells (*9−12*) and inhibit cytotoxicity from reactive oxygen or amyloid-β peptide (*29*). The levels of gangliosides, which are representative derivatives of ceramide in the brain, decrease with age from the juvenile stage and reach a level of approximately one-third at 85 years of age (*30*). Gangliosides are reported to improve cognitive function in experimental animals and AD patients (*27*, *16*). These reports suggest that orally administered dihydroceramide from acetic acid bacteria might be converted to its metabolites, such as gangliosides, and exert its beneficial effects on cognitive function in the damaged brain.

In conclusion, the results of this study suggest that oral administration of dihydroceramide-containing ASL can reverse the decline in cognitive function in dementia and/or in elderly persons, and we suggest dihydroceramides are nutritionally important for brain function. We are currently studying the absorption kinetics and neurochemical effects of orally administered ASL and/or dihydroceramide in the central nervous system. We are also seeking further evidence of their effects on cognitive function in aged experimental animals and elderly persons.

ABBREVIATIONS

AD, Alzheimer's disease; AL, amino lipids (tauro-ornithine lipid, ornithine lipid, and lyso-ornithine lipid); ASL, alkali-stable lipids; DHCER, dihydroceramide [(2-hydroxypalmitoyl)sphinganine]; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GM1, monosialotetrahexosylganglioside; H-ASL, high ASL dosage; HPN, hopanoids; L-ASL, low ASL dosage; NGF, nerve growth factor; PC12, pheochromocytoma; SPH, sphinganine; TLC, thin layer chromatography.

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