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Histological analyses by matrix-assisted laser desorption/ionization-imaging mass spectrometry reveal differential localization of sphingomyelin molecular species regulated by particular ceramide synthase in mouse brains

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ABSTRACT

Sphingomyelin (SM) is synthesized by SM synthase (SMS) from ceramide (Cer). SM regulates signaling pathways and maintains organ structure. SM comprises a sphingoid base and differing lengths of acyl-chains, but the importance of its various forms and regulatory synthases is not known. It has been reported that Cer synthase (CerS) has restricted substrate specificity, whereas SMS has no specificity for different lengths of acyl-chains. We hypothesized that the distribution of each SM molecular species was regulated by expression of the CerS family. Thus, we compared the distribution of SM species and CerS mRNA expression using molecular imaging. Spatial distribution of each SM molecular species was investigated using ultra-high-resolution imaging mass spectrometry (IMS). IMS revealed that distribution of SM molecular species varied according to the lengths of acyl-chains found in each brain section. Furthermore, a combination study using in situ hybridization and IMS revealed the spatial expression of CerS1 to be associated with the localization of SM (d18:1/18:0) in cell body-rich gray matter, and CerS2 to be associated with SM (d18:1/24:1) in myelin-rich white matter. Our study is the first comparison of spatial distribution between SM molecular species and CerS isoforms, and revealed their distinct association in the brain. These observations were demonstrated by suppression of CerS2 using siRNA in HepG2 cells; that is, siRNA for CerS2 specifically decreased C22 very long-chain fatty acid (VLCFA)- and C24 VLCFA-containing SMs. Thus, histological analyses of SM species by IMS could be a useful approach to consider their molecular function and regulative mechanism.

1. Introduction

Sphingolipids have important roles in the stabilization of membrane structures, and are signaling molecules [1–4]. Sphingomyelin (SM), which is the final product of the synthesis of sphingolipids, is a signaling regulator [5–7]. SM has also been reported to be involved in certain diseases. Niemann–Pick disease is a genetically inherited disease caused by a deficiency in sphingomyelinase, which causes accumulation of SM in some organs (e.g., liver, lungs, brain) and results in irreversible neurologic damage [6]. Additionally, a deficiency of sphingomyelin synthase 2 (SMS2) ameliorates diet-induced obesity, fatty liver disease, and insulin resistance [8–10].

Sphingolipids contain ceramide (Cer) as a backbone, in which a fatty acid (FA) is amide-linked to the sphingoid base (Sph). Most saturated and monounsaturated very long-chain fatty acids (VLCFAs) are found as acyl groups in sphingolipids. The *de novo* synthesis of sphingolipids begins in the endoplasmic reticulum (ER) with the conversion of L-serine and palmitoyl-CoA into Cer. These reactions are mediated by serine palmitoyltransferase, 3-ketosphinganine reductase, Cer synthase (CerS), and dihydroceramide desaturase 1 [11–13]. As a precursor of SM, part of Cer molecules is transferred from the ER to the Golgi by

Abbreviations: SM, sphingomyelin; SMS, sphingomyelin synthase; Cer, ceramide; CerS, ceramide synthase; LC/ESI-MS/MS, liquid chromatography/electrospray ionization-tandem mass spectrometry; IMS, imaging mass spectrometry; ISH, in situ hybridization; MALDI, matrix-assisted laser desorption/ionization; FTICR, Fourier transform ion cyclotron resonance; VLCFA, very long-chain fatty acid; HexCer, hexosylceramide; GlcCer, glucosylceramide; GalCer, galactosylceramide; LacCer, lactosylceramide

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Cer transport protein [14,15]. In the Golgi, Cer is converted to SM or glucosylceramide (GlcCer) and subsequently to lactosylceramide (LacCer) by SMS or UDP-glucose ceramide glucosyltransferase (Ugcg), respectively [16–19]. SM is synthesized from Cer by the transfer of phosphocholine (PCho) from phosphatidylcholine through SMS1 in the Golgi or SMS2 in plasma membranes [16,17,20].

Even though reports showing which SMS has specificity for different lengths of the acyl-chains of Cer are lacking, it is known that formation of an amide bond between acyl-CoA and the Sph is catalyzed by CerS [21,22]. Six CerS isoforms (CerS1–CerS6) have been identified and each one exhibits restricted substrate specificity to FAs with specific chain lengths [21–23]. For example, CerS1 is primarily responsible for the synthesis of C18 long-chain fatty acid (LCFA)-containing sphingolipids [24], whereas CerS2 is responsible for the synthesis of C22–C24 VLCFA-containing sphingolipids [25–28]. It has also been shown that CerS exhibits ubiquitous or tissue-specific distribution patterns [23]. For example, CerS1 is specifically expressed in the brain and skeletal muscle, whereas CerS2 is ubiquitously expressed in most tissues, in particular in the liver and kidneys [23]. Thus, the tissue-distribution patterns of Cer molecular species could be identified according to their substrate specificity.

FA compositions of SM range mainly from C16 to C24; C16:0, C24:0 and C24:1 FA-containing SMs are major components in most mammalian tissues [23]. However, the proportion of each FA among tissues varies considerably. For example, C24 VLCFAs are major components of SM in the liver and kidneys, whereas C18 LCFAs are major components of SM in the brain and skeletal muscle [23]. It has been reported that the FA composition of sphingolipids is markedly dependent on the cell types in each organ [24,28]. Neurons synthesize mainly C18 LCFA-containing sphingolipids [24], whereas oligodendrocytes and Schwann cells (which wind tightly around axons to form myelin sheaths) mostly synthesize C24 VLCFA-containing sphingolipids [28]. Thus, clarifying the spatial information for SM is important to understand the properties of SM and the functions or distributions of cells within tissue because each type of cell has different SM species. However, few reports have identified the distribution of each molecular species of SM, or compared SM and its distribution pattern of regulatory synthases, by histologic means.

Liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) is the “gold standard” method for the measurement of lipids within tissues. However, histologic information is lost by the extraction procedure. Matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-IMS) is expected to be a powerful tool to visualize the distribution of various molecules (including lipids) in tissue sections [29–33]. Several reports have shown that MALDI-IMS can be used for imaging of the sites of endogenous metabolites, especially glycerophospholipids and sphingolipids [34–38]. Furthermore, use of MS/MS allows the structures of imaged molecules to be identified [39,40]. Comparison of the distribution of lipid molecules and expression of their synthases would likely clarify their relationship, but no studies have compared them directly. However, it has been stated that conventional MALDI-time of flight-mass spectrometry (TOF-MS) cannot be employed for the identification of endogenous metabolites (including lipids) because very close mass peaks are often observed as a single overlapping peak owing to low mass resolution [41]. These limitations make identification of each peak in IMS by MS/MS very difficult. Conversely, Fourier transform ion cyclotron resonance-mass spectrometry (FTICR-MS) can be used to specifically identify the exact mass and isotopic fine structure of each molecule by its ultra-high-resolution [34,42,43]. Thus, MALDI-FTICR-IMS enables scholars to distinguish between structurally heterogeneous molecules according to their exact mass and to visualize them simultaneously.

Thus, the aims of this study were first to determine whether SM was differently distributed by its acyl-chain lengths, and second, to compare the distributions of SM with the expression patterns of CerS in the brain by employing MALDI-IMS of lipids and in situ hybridization (ISH) of metabolizing genes.

2. Materials and methods

2.1. Materials

High-performance liquid chromatography-grade methanol, acetonitrile, chloroform, n-hexane and 2-propanol were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Trifluoroacetic acid, ammonium acetate, potassium acetate, potassium hydroxide and hydrochloric acid were obtained from Wako Pure Chemical Co., Ltd. (Osaka, Japan). 2,5-dihydroxybenzoic acid (DHB) and formic acid were purchased from Sigma–Aldrich (Saint Louis, MO). Indium tin oxide (ITO) glass slides were obtained from Bruker Daltonics (Bremen, Germany). Complete Protease Inhibitor Cocktail was purchased from Roche Diagnostics (Basel, Switzerland).

SM (d18:1/16:0), SM (d18:1/18:0), SM (d18:1/18:1), SM (d18:1/24:0), SM (d18:1/24:1), SM (d18:1/16:0-d31), Cer (d18:1/16:0), Cer (d18:1/18:0), Cer (d18:1/18:1), Cer (d18:1/22:0), Cer (d18:1/24:0), Cer (d18:1/24:1), Cer (d18:1/16:0-d31), GalCer (d18:1/24:1) and LacCer (d18:1/18:0) standards were purchased from Avanti-Polar Lipids Inc. (Alabaster, AL). TRIzol RNA Separation Reagent and the SuperScript III First-Strand Synthesis system for reverse transcription-polymerase chain reaction (RT-PCR) were obtained from Life Technologies (Carlsbad, CA). An RNeasy Mini Kit was purchased from Qiagen (Venlo, The Netherlands). SYBR Premix Ex Taq II and Perfect Real Time Primer were obtained from Takara Bio Inc. (Otsu, Japan).

Silencer Select Negative Control siRNA, Pre-designed siRNA, Opti-MEM and Lipofectamine RNA iMAX Transfection Reagent were purchased from Life Technologies. Dulbecco's Modified Eagle's Medium (DMEM) containing glucose (1.0 mg/mL) was obtained from Nacalai Tesque (Suita, Japan). Fetal bovine serum was purchased from GE Healthcare Life Sciences (Logan, Utah). Lipoprotein-depleted serum (LPDS) was obtained from Sigma–Aldrich. Micro BCA Assay Reagent was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Cell Lysis Buffer was obtained from Cell Signaling Technology, Inc. (Danvers, MA).

2.2. Animals

The animal research protocol was approved by the Animal Care and Use Committee of the Graduate School of Medicine, Hokkaido University (Sapporo, Japan). The protocol conformed to the *Guide for the Care and Use of Laboratory Animals* (US National Institutes of Health, Bethesda, MD).

Male C57BL/6 N mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Mice (age, 4 weeks) were housed in separate cages under controlled temperature and humidity with a 12 h light–dark cycle for 1 week. Tissues were removed from 5 weeks of age under isoflurane anesthesia after 4 h of fasting and weighed. They were snap-frozen on dry ice, and stored at −80 °C until analyses.

2.3. Culture and transfection of cells

HepG2 cells were maintained in culture medium (DMEM supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic (100×) liquid), and cultured at 37 °C in an atmosphere of 5% CO_2. Lipofectamine RNA iMAX Transfection Reagent and Silencer Select siRNA (Negative Control Number 1, part number: 4390843; Negative Control Number 2, part number: 4390846; CerS2 Number 1, part number: 4427037, ID: s26788; CerS2 Number 2, part number: 4427037, ID: s26789; SMS2 Number 1, part number: 4392420, ID: s46645; SMS2 Number 2, part number: 4392420, ID: s46646) were diluted in Opti-MEM. Diluted siRNA were added to diluted Lipofectamine RNA iMAX