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Rapid quantitative analysis of human serum sphingomyelin species using MALDI-TOF mass spectrometry with lipid hydrolase treatment

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ABSTRACT

Background: Sphingomyelin (SM) is a key component of extracellular membranes and lipoproteins, and plays roles in cell signaling and as a component of lipoproteins. SM species differ in terms of fatty acid (FA) composition. However, no simple, rapid, quantitative assay for identifying different SM species has yet been reported. In this study, lipid hydrolase treatment and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were used to identify serum SM species.
Methods: Sera were collected from healthy young individuals. To identify SM species, sera were treated with phospholipase A_2 and lipoprotein lipase, and lipids were extracted using the standard chloroform/methanol (2/1 v/v) method.
Results: We detected 15 peaks from serum using MALDI-TOF MS, which were assigned to SM species bound with FA components ranging from C15:0 to C24:2. The most prominent serum SM species was SM [C16:0], which accounted for approximately 26% of serum SM. Some SM species contained an odd-carbon FA (C15, C21, and C23), and these accounted for approximately 4% of serum SM. The reproducibility of major SM species within and between application positions on MS-sample plate was CV = 3.0%–7.9% and CV = 3.1%–6.8%, respectively. The concentration and dilution ratio were linearly related. The SM species composition of 10 healthy young subjects showed a similar profile.
Conclusions: We developed a rapid, and quantitative method for identifying serum SM species using lipid hydrolase treatment and MALDI-TOF MS. This method will be suitable for clinical laboratory studies to examine the associations between SM species and disease states.

1. Introduction

Sphingomyelin (SM) is a major component of extracellular membranes and lipoproteins, and as such is widely distributed in organ tissue, peripheral blood cells, and plasma [1]. SM plays a key role in maintaining the structural integrity of biological membranes, is a constituent of lipid microdomains (lipid rafts), and is involved in signal transduction [2–4]. Ceramide is the core molecule of most sphingolipids and can act as an intracellular messenger. Ceramides are generated following SM hydrolysis by neutral sphingomyelinase (SMase), and conversely, SM is synthesized from ceramide by SM synthase; this metabolic process is known as the SM cycle [5]. Molecules involved in the SM cycle are associated with a range of disease states.

SM is found in low-density lipoproteins (LDL) derived from human arteriosclerotic lesions, and is associated with the onset of atherosclerosis. Plasma SM concentrations and the SM/PC ratio are each considered independent risk factors for the development of arteriosclerosis [6]. With regard to the lipoprotein distributions, 7%, 50%, and 43% of plasma SM levels are found in very low-density lipoproteins (VLDL), LDL, and high-density lipoproteins (HDL), respectively [7]. Lipoprotein SM is derived from liver biosynthesis, from the diet, and from transfer between cells and lipoproteins [8]. High SM-deacylase activity is associated with atopic dermatitis, while SMase deficiency is a feature of Niemann–Pick disease [9,10]. Although perturbations in SM metabolism are associated with various types of cell dysfunction and pathogenesis, the associations between diseases and plasma SM levels are unknown.

SM is composed of ceramide and phosphocholine, while ceramide is composed of sphingoid base and fatty acid. The fatty acid moiety is usually a saturated fatty acid (SFA) or a monounsaturated fatty acid (MUFA) containing a long carbon chain. SM fatty acid composition differs according to tissue and animal species [11]. SM [C18:0] is abundant in human nerve tissue, and SM [C16:0] is abundant in human peripheral cells. The chain length and degree of fatty acid unsaturation influence membrane thickness and fluidity [12].

There are currently no simple methods for identifying SM fatty acid species. Thin layer chromatography (TLC) and reverse high-

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performance liquid chromatography (HPLC) are unable to clearly identify the various fatty acid moieties [13,14]. Although identification can be achieved using a combination of phospholipase C treatment and gas liquid chromatography, this is both time-consuming and laborious [15]. Although a recent study indicated that electrospray ionization mass spectrometry (ESI-MS) was a useful approach, the machine for analysis had to be reserved exclusively for that technique.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can be used to identify lipid species, and its advantages include ease of sample preparation, small sample mass required, short assay time, and overall simplicity of the procedure [16]. MALDI-TOF MS assays can provide quantifiable data with the use of internal control standards.

2. Materials and methods

2.1. Chemicals

Methanol and chloroform (HPLC grade), phospholipase A_2 (PLA_2: type I from *Crotalus adamanteus*; activity 320 U/mg), lipoprotein lipase (LPL: from *Pseudomonas* sp.; activity 1140 U/mg), sphingomyelinase (SMase: from *Staphylococcus aureus*; 100 U/mg protein), calcium chloride ($CaCl_2$), hydrochloric acid (HCl) and 2,5-dihydroxybenzonic acid (2,5-DHB) were from Wako Pure Chemical Industries, Ltd. N-lauroyl-D-*erythro*-sphingosylphosphorylcholine; lauroyl sphingomyelin (SM [C12:0]) as an internal standard material and N-palmitoyl-D-*erythro*-sphingosylphosphorylcholine; hexadecanoyl sphingomyelin (SM [C16:0]) was purchased from Avanti Polar Lipids, Inc. Trizma Base reagent was from Sigma-Aldrich Co. LLC.

2.2. Subjects

Healthy young volunteers (21 y; 3 males and 7 females) were recruited from among the Shinshu University student population. Each subject gave written informed consent, and the study protocol was approved by the Ethical Review Board of the Shinshu University School of Medicine. All procedures were performed according to the Helsinki Declaration of 1975 as revised in 1996.

2.3. Preparation of serum

Blood was drawn and allowed to clot at room temperature. After centrifugation at 1000 ×*g* for 10 min, serum samples were collected in plastic tubes and stored at −20 °C. In the analysis of SM molecular species, serum was stable for at least 1 year at −20 °C.

2.4. Lipid hydrolase treatment

Serum (100 μl) was added to a tube containing 50 μl of Tris–HCl buffer (45 mmol/l, containing 10 mmol/l $CaCl_2$, pH 8.0), 10 μl of PLA_2 (0.32 U/μl), and 20 μl of LPL (11.4 U/μl), followed by incubation for 20 min at 37 °C. To identify which MALDI-TOF MS peaks represented SM species, 30 μl of SMase (10.1 mU/μl) was then added and the mixture was incubated overnight at 37 °C.

2.5. Extraction of lipids

Lipid extraction from treated and untreated serum samples (100 μl) with 500 μl of the internal standard material (SM[C12:0], 1.6 μg/ml in C/M 2/1 v/v) was performed using a chloroform and methanol solution (C/M, 2:1 v/v). Samples were added to the C/M solution at a ratio of 1:5, and the mixture was shaken vigorously for 1 min. Distilled water was then added (1/5 of the C/M volume), the mixture gently rotated, and then centrifuged for 10 min at 500 ×*g* to separate the mixture into a lower phase (chloroform) containing lipids and an upper phase (water and methanol). The lower phase was collected and dried in a centrifugal concentrator. The resultant lipid was dissolved in a volume of C/M equal to that of the original serum sample.

2.6. Mass spectrometry

Mass spectrometry was performed using a MALDI-TOF MS instrument (Absciex TOF/TOF TM5800 system) in reflector mode to enhance spectral resolution. Lipid was measured in positive ion mode. Briefly, 5.0 μl of matrix solution (5 mg 2.5-DHB in 1 ml of chloroform/methanol, 2/1 v/v) was added to a micro-tube containing 1.0 μl of lipid sample, and then 0.2 μl of the mixture was applied to the metal sample plate, allowed to dry at room temperature, and the metal plate was then inserted into the MALDI-TOF MS analyzer. SM product ions were measured using a MALDI-TOF mass spectrometer in mass spectrometry/mass spectrometry (MS/MS) mode.

3. Results

3.1. Analysis of serum SM species

Untreated healthy young volunteers' sera were subjected to phospholipid analysis by MALDI-TOF MS. A typical SM species profile is shown in Fig. 1. Based on findings reported previously [21], peaks of *m/z* 758.4–834.4 represented phosphatidylcholine (PC) species, and those of *m/z* 853.5–905.5 represented triglyceride (TG) species. The peaks at *m/z* 703.4 and 725.4 were assigned to represent SM $[C16:0 + H]^+$ and SM $[C16:0 + Na]^+$, respectively. SM species peaks except SM [C16:0] were distributed in the mass/charge number ratio range overlapping with PC and TG peak positions.

3.2. Lipid hydrolase treatment

To identify SM species, lipid hydrolases were added to 100 μl of serum samples to hydrolyze PC and TG, as described in the Materials and methods section. The fatty acid in the *sn*-2 position of glycerophospholipids was hydrolyzed with PLA_2, and fatty acids in the α and β positions of TG were hydrolyzed with LPL. A 500 μl solution of the internal standard material (SM [C12:0], 1.6 μg/ml in C/M 2/1 v/v) was added to the treatment sample, the lipid was extracted, and the extracted mixture was analyzed by MALDI-TOF MS. In the resulting mass spectrum, PC and TG peaks could no longer be seen following enzyme treatment, as expected (Fig. 2). Thin layer chromatography (TLC) confirmed complete degradation of TGs and glycerophospholipids in serum treated with lipidolytic enzymes (data not shown). Sixteen peaks were detected at *m/z* 703.4–837.5 (Fig. 2-A). All of these peaks were further analyzed by MALDI-TOF/TOF-MS (MS/MS). The 16 peaks were found to represent SM species according to SM fragment detection by product ion analysis and confirmation of the presence of choline and phosphocholine residues. Furthermore, SM was hydrolyzed to ceramide and phosphocholine by SMase. The residual peaks in Fig. 2-B were confirmed not to be SM by MS/MS analysis. SMase treatment was found to result in almost complete reduction of all of these 16 peaks (Fig. 2-B). Each peak was assigned an SM species based on the long chain base (LCB; d18:1) and fatty acid chain length: C15:0, C16:1, C16:0, C18:1, C18:0, C20:1, C20:0, C21:0, C22:1, C22:0, C23:1, C23:0, C24:2, C24:1, and C24:0 (Table 1). The peaks at *m/z* 703.4 and 725.4 were assigned as hydrogen and sodium adduct ions of SM [C16:0], respectively.

3.3. Linearity and reproducibility

We examined the linearity of the MALDI-TOF MS SM assay. The hexadecanoyl sphingomyelin (SM [C16:0], 2 μg/μl) was serially (1, 1/2 and 1/4) diluted with C/M (2/1 v/v) and mixed with the internal standard material; lauroyl sphingomyelin (SM [C12:0], 1 μg/μl). The mixed sample was analyzed by MALDI-TOF MS. The equation describing