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Distribution of Acid Sphingomyelinase in Human Various Body Fluids

Ikuko Takahashi, Tsutomu Takahashi, Tamaki Abe, Wataru Watanabe[1] and Goro Takada

Department of Pediatrics and [1]Orthopedics, Akita University School of Medicine, Akita 010-8543

Takahashi, I., Takahashi, T., Abe, T., Watanabe, W. and Takada, G. *Distribution of Acid Sphingomyelinase in Human Various Body Fluids.* Tohoku J. Exp. Med., 2000, **192** (1), 61-66 —— Enzyme activities of acid sphingomyelinase (ASM) were determined in various human cell-free body fluids, serum, cerebrospinal fluid, urine, salivary fluid, tear fluid, and synovial fluid, using assay buffers with or without Zn^{2+}-cation. Although ASM activity was not detected in the cerebrospinal fluid, the other fluids demonstrated significant enzyme activities of ASM. All ASMs detected in the fluids were stimulated by the addition of Zn^{2+}-cation, suggesting that those enzymes are secretory ASM derived from ASM gene. We suggest a possible enzymatic diagnosis of Niemann-Pick disease types A and B using those body fluids. Interestingly, salivary and tear fluids showed much higher activities of ASM than those of the other fluids. Because sphingolipids, especially sphingomyelin, are major constituents of a normal diet, especially, milk, eggs, and meat products, we suggest that ASM in the salivary gland may play an important role in the digestion of sphingomyelin in a normal diet. ———— acid sphingomyelinase; tear fluid; salivary fluid

Sphingomyelinase hydrolyzes sphingomyelin to form phosphocholine and ceramide. Among several mammalian enzymes catalyzing this reaction, a lysosomal acid sphingomyelinase (ASM, EC 3.1.4.12) has been well characterized even at the molecular level more than other sphingomyelinases (Brady et al. 1966; Schuchman et al. 1991). ASM is important for sphingolipid turnover in cell. ASM activation and ceramide generation have emerged as an important signaling pathway transducing diverse biological effects of cytokine receptors like the tumor necrosis factor receptor or Fas (Cifone et al. 1993; Wiegmann et al. 1994; Boucher et al. 1995). Recently, the ASM gene has been shown to give rise to two ASMs; one is lysosomal ASM, which is cation-independent, and the other is secretory ASM, which is fully or partially dependent on Zn^{2+} for its activity (Schissel et al.

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Address for reprints: Tsutomu Takahashi, M.D., Department of Pediatrics, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan.

e-mail: tomy@med.akita-u.ac.jp

1996). This dependency of the enzyme on Zn^{2+} cation is explained as follows (Schissel et al. 1998). The ASM gene gives rise to the common mannosylated precursor protein that gets into either the lysosomal trafficking pathway or the secretory pathway. Containing several highly conserved zinc-binding motifs, the enzyme is exposed to cellular pools of Zn^{2+}, saturated with Zn^{2+}, and activated in the lysosomal pathway. This activation does not occur in the secretory pathway. Secretory Zn^{2+}-dependent ASM may have significant physiologic roles, since extracellular sphingomyelin hydrolysis may be involved in some of the non-lysosomal processes. A recent report described the possession of two ASMs, termed ASM-1 and ASM-2 encoded by two distinct genes, in *C. elegans*. ASM-1 is preferentially expressed in the embryo, whereas ASM-2 is predominantly expressed in postembryonic stages (Lin et al. 1998). ASM-1 is found almost entirely in a secreted form whereas only 20% of ASM-2 is secreted. Despite the discovery of some secretory ASMs, little is known about the functions of these enzymes.

To find the extracellular distribution in humans, we determined the ASM enzyme activities in serum, cerebrospinal fluid, urine, salivary fluid, tear fluid, and synovial fluid from various subjects. In this report, we show a possible enzymatic diagnosis of Niemann-Pick disease using body fluids and discuss the functions of extracellular ASM.

Materials and Methods

Serum samples were collected from 10 normal adults and from one patient with Niemann-Pick disease as a disease control (Takahashi et al. 1992). Cerebrospinal fluids (CSF) were collected from two patients with undiagnosed neurological diseases demonstrating the normal ASM activities of white blood cells. Urine and salivary fluid samples were collected from normal adults. Tear fluids were sampled from normal adults by the stimulation of onion vapor. Synovial fluids were aspirated from 10 patients with rheumatoid arthritis (RA) and 10 patients with osteoarthritis (OA). All of these samples were frozen, so that all analyses could be performed at the same time. Using a modification of the method proposed by Chatterjee and Ghosh (1991), assays of cation-independent ASM and Zn^{2+}-dependent secretory ASM were carried out as follows: ^{14}C-sphingomyelin was purchased from NEN® Life Science Products (Boston, MA, USA). For the assays of cation-independent ASM and Zn^{2+}-dependent secretory ASM, the final 200 μl assay mixture consisted of 100 μl of sample, 50 μl of sodium acetate buffer (1.0 M, pH 5.0) containing 4% Triton X-100, and 50 μl of substrate (20 nmol, ^{14}C-sphingomyelin, 0.08 μCi/20 nmol in 0.2% taurodeoxycholic acid). When added, the final concentration of ethylenediamine tetraacetic acid (EDTA) and Zn^{2+} were 0.02 mM and 0.1 mM, respectively. The reaction was initiated by adding the substrate solution and carried out at 37°C for 6 hours. The assay was terminated with 100 μl of ice-cold 30% Trichloroacetic acid and 200 μl of 2.5%

bovine serum albumin. The contents of the tubes were vortexed, allowed to settle for 5 minutes at room tempereture, and then centrifuged for 5 minutes at 3000 rpm. Five hundred μl of the supernatant was carefully withdrawn and transferred into glass scintillation vials. The radioactivity was measured directly after mixing with 4.5 ml Clear-sol II (Nakalai Tesque Inc., Kyoto) in a liquid scintillation counter (LSC 950, Aloka, Tokyo). The effects of pHs on the hydrolysis of sphingomyelin by tear fluid and salivary fluid were examined with 0.1 M glycine-HCl buffer (pH 3.0), 0.1 M acetate buffer (pH 4.0 and pH 5.0), and 0.1 M phosphate buffer (pH 6.0 and pH 7.0).

Statistical analysis was performed by *t*-test using Stat View-J4.5 (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

ASM activities of various body fluids were shown in Table 1. Cation-independent ASM exists in the serum, however it was intensified about two-fold by the presence of Zn^{2+} cation. Both ASM activities were very low level in the serum from type B Niemann-Pick disease, demonstrating the authenticity of this assay method. In the cerebrospinal fluids (CSFs), ASM activity was not detected even by the stimulation of Zn^{2+} cation. Urinary ASM activities were detected and were less active than that of serum. Unexpectedly, tear and salivary fluids demonstrated much higher activities than those of serum. In synovial fluids from patients with RA and OA, the levels of ASM activities were approximately two times higher than those of serum from normal controls. There was no difference in the enzyme activities between the two disorders. Under the assay conditions, the ASM activity recorded in tear fluid had optimum activity at approximately pH 5 and that of salivary fluid was at pH 4 (Fig. 1).

Table 1. *Acid sphingomyelinase activities of human various body fluids*

	ASM activity (nmol/0.1 ml/6 hours)	
	Zn^{2+} (−) EDTA (+)	Zn^{2+} (+)
Serum ($n=10$)	0.79±0.23	1.55±0.75*
Serum (NPD)	0.03	0.04
Cerebrospinal fluid ($n=2$)	N.D.	N.D.
Urine ($n=4$)	0.59±0.29	0.76±0.28**
Salivary fluid ($n=4$)	4.29±1.82	6.47±1.95*
Tear fluid ($n=3$)	11.30±1.92	12.73±0.85
Synovial fluid (RA) ($n=10$)	1.24±0.81	2.42±0.54**
Synovial fluid (OA) ($n=10$)	0.94±0.82	2.40±1.38**

N.D., not detected; NPD, Niemann-Pick disease.
*$p<0.05$ vs. ASM activity with Zn^{2+}(−) EDTA(+) buffer.
**$p<0.01$ vs. ASM activity with Zn^{2+} (−) EDTA (+) buffer.

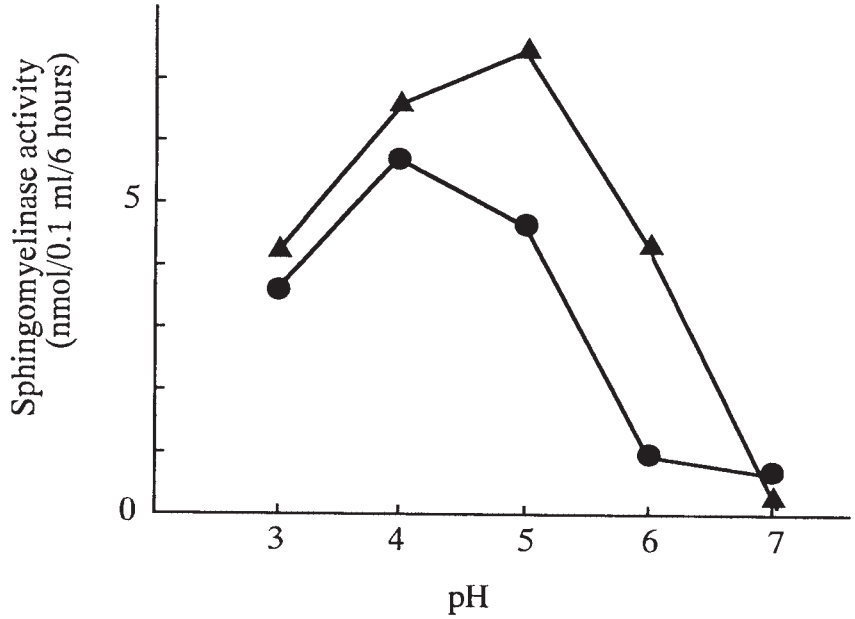

Fig. 1. Effect of pH on the hydrolysis of sphingomyelin by human saliva or tear fluid. One hundred μl of saliva or tear fluid was used in each incubation with EDTA buffer ($n = 1$, details in Methods). ▲, Tear fluid; ●, Salivary fluid.

Discussion

In human serum, we detected cation-independent ASM activity which was stimulated by the addition of Zn^{2+} cation. The ASM activity was not detected in the serum of the patient with type B Niemann-Pick disease, showing that the detected ASM activity originated from ASM gene. Because human vascular endothelial cells are known to secrete massive amounts of ASM in cell culture studies, the serum ASM enzyme may originate from vascular endothelial cells and macrophages of various tissues (Marathe et al. 1998). In this study, we, for the first time, report the presence of ASM activity in tear and salivary fluids. We do not directly know whether these enzymes in tear and salivary fluids are identical to products of the ASM gene. However, these enzymes showed optimum activities at acidic pH (pH 4–5) and were stimulated by additing Zn^{2+} cation, strongly suggesting that these enzymes are products of the ASM gene. A number of lysosomal acid hydrolases are well known to be present in tears in concentrations between 2 and 10 times those in serum (Van Haeringen 1981). The lacrimal gland is the main source of the lysosomal enzyme in tears. Lysosomal enzyme activities in tears are used for diagnosis and identification of the carrier of several inborn errors of metabolism. The function of tear ASM is unclear, because the patients with Niemann-Pick disease do not present ophthalmological disorder, except for cherry-red spots in the eye fundus. Recently, Nyberg et al. (1998) reported the presence of ASM activity in human milk. Human milk contains approximately 0.2 mmol/liter sphingomyelin, making sphingomyelin the dominant phospholipid, comprising 0.4% of total milk or 40% of total milk phospholipids. They demon-

strated that human milk possses principal enzyme activities required for the first two steps in the degradation of sphingomyelin, that is, an ASM that cleaves sphingomyelin to ceramide and phosphocholine and alkaline ceramidase that hydrolyzes ceramide further to sphingosine and fatty acid. In human milk, the optium activity of ASM was at approximately pH 5. They considered that hydrolysis of sphingomyelin could be carried out in the stomach. Other new enzyme, alkaline SMase of which optium activity is pH 9, has been demonstrated in human intestine and bile. No alkaline SMase was found in the stomach (Duan et al. 1996). This enzyme has been also considered having important role in the digestion of the dietary sphingomyelin. The salivary ASM that we reported here may have the same functions as milk ASM for nutritional purposes even in adult humans, because sphingolipids are constituents of a normal diet, especially, milk, eggs, and meat products. We suggest that salivary ASM contributes to the digestion of sphingomyelin in a normal diet. To confirm this role, activities of ASM in gastrointestinal tract should be assayed.

ASM activity was not detected in the cerebrospinal fluid. Most of the daily CSF production is from the choroid plexus which is formed by invaginating the ependyma into the ventricular system. Our result shows the inability of ependymal cells to produce ASM enzymes. We detected the ASM, which is stimulated by Zn^{2+} cation, in the urine as previously known. We also have shown that the synovial fluid of patients with RA or OA contains high levels of ASM enzymes. Lysosomal enzymes in synovial fluid are derived from two sources, including synovial tisssues and inflammatory cells such as polymorphonuclear leukocytes and macrophages. Some lysosomal enzymes, N-acetyl-β-D-glucosaminidase, lysozyme, and β-glucuronidase, are known to increase in the synovial fluids of patients with RA when compared to those of patients with OA (Yoshida et al. 1992). However, our results did not demonstrate any differences between ASM activities in RA and OA.

In summary, ASM enzymes are distributed in various extracellular fluids of human tissues. Activities of those enzymes are intensified by the addition of Zn^{2+}-cation in the assay buffer, suggesting that those enzymes are secretory ASM derived from ASM gene. Salivary and tear fluids demonstrate higher enzyme activities of ASM than those of other body fluids. We propose that ASM in the salivary gland may play an important role in the digestion of sphingomyelin in a normal diet.

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