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A STUDY OF MASS SCREENING FOR ACID SPHINGOMYELINASE DEFICIENCY ; THE RELATIONSHIP BETWEEN ENZYME ACTIVITY AND BLOOD CELL COUNTS

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Abstract
Lysosomal disease is a group of rare inherited disorders caused by malfunctions of lysosomal proteins, including more than 50 acid hydrolases. Progress in the molecular understanding of lysosomal disease has advanced the treatment, leading to the importance of mass screening for an individual disease to facilitate the earlier induction of treatment. Acid sphingomyelinase deficiency (ASMD) is a lysosomal disease that is ordinarily diagnosed by enzyme assays using biospecimens, lymphocytes, or fibroblasts. Here, we tested a study of a mass-screening method for ASMD using dried blood spot (DBS) samples. The subjects were 62 non-ASMD children admitted to our hospital and an ASMD-diagnosed patient. Acid sphingomyelinase (ASM) activity was determined by an enzyme assay using flow injection tandem mass spectrometry on DBS. In DBS samples from 62 subjects, the mean ASM activity was 5.7 ± 2.4 μmol/h/L (min 2.3, max 16.4, median 5.1). No sample showed a value below the reference cutoff of 1.5 μmol/h/L. The ASM activity of the patient with ASMD was 1.0 μmol/h/L, which was below the cut-off. These results demonstrate the feasibility of the DBS-based ASM enzyme assay for mass screening of ASMD. Furthermore, we discuss the correlation between ASM activity and blood laboratory tests in the subjects.

Key words : acid sphingomyelinase deficiency, dried blood spots, tandem mass spectrometry

Introduction

Lysosomal disease is a group of rare, intractable, inherited disorders caused by malfunctions of lysosomal proteins and includes more than 50 acid hydrolases[1]. Recent progress in the molecular understanding of lysosomal diseases has advanced the development of treatments ; this has led to the clinical application of enzyme replacement therapy, substrate reduction therapy, and chaperone therapy for individual lysosomal diseases[2]. This advance in treatment also emphasized the importance of a diagnostic method for the newborn screening program, a high-risk patient screening program, and a definitive diagnosis for this rare condition[3].

Acid sphingomyelinase deficiency (ASMD), classically termed Niemann-Pick disease types A and B, is an autosomal recessive disorder of lysosomal disease that is caused by genetic mutations (MIM#607608) in both al-

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leles of the *sphingomyelin phosphodiesterase 1* (*SMPD1)* gene, which encodes acid sphingomyelinase (ASM), resulting in reduced ASM activity. In ASMD, sphingomyelin, the substrate of ASM, accumulates intracellularly and causes various symptoms, including hepatosplenomegaly, respiratory disorders, and neurological symptoms[4]. There are three types of ASMD : type A, a severe form with rapidly progressive neurological symptoms and death in infancy ; type B, a chronic form with chronic visceral symptoms ; and type A/B, an intermediate form with varying degrees of severity and course of neurological symptom[5]. In ASMD, the first enzyme replacement therapy product, Olipudase alfa[6], was approved in 2022. This has led to an increased interest in ASMD as a treatable rare disease and the recognition of the importance of early diagnosis and the early introduction of treatment.

ASM activity can be measured in lymphocytes, cultured skin fibroblasts, or other biospecimens from patients. Dried blood spot (DBS) is another form of biospecimen in which blood samples are blotted and dried on a filter pad. DBS is suitable for mass screening because it can be easily shipped to an analytical laboratory and analyzed using various methods, including DNA amplification and enzyme assays. Recently, pilot studies of DBS-based newborn mass screening[3,7] and DBS-based high-risk patient screening[8] have been conducted, and they described the details of the studies, including ASM activities, incidence of ASMD, and genotypes of the ASMD subjects.

Since DBS involves whole blood collection, it contains both blood cells and plasma components. ASM activity is thought to originate from blood cells, but extracellular fluids such as plasma, urine, synovial fluid, and tear fluid have been reported to contain ASM that can be detected by enzyme assay[9]. The *SMPD1* gene is known to produce two ASMs : lysosomal ASM (L-ASM), which resides in lysosomes, and secretory ASM (S-ASM), which is released extracellularly[10,11]. L-ASM and S-ASM have been proposed to be produced by the differential transport of a common protein precursor[12]. It was recently hypothesized that S-ASM may be generated by exocytosis of lysosomes[13]. Therefore, the ASM activity of DBS could originate from both the blood cell and plasma components. However, blood cell counts depend on individual subjects. White blood cell (WBC) counts vary between 5,000/μL and 10,000/μL, suggesting that the blood cell count could influence the ASM activity of DBS.

This study aimed to conduct a mass screening for ASMD in our hospital and evaluate how blood laboratory data influence the ASM activity of DBS.

Material and Methods

This study was conducted in accordance with the Declaration of Helsinki and the protocols were approved by the Akita University Ethics Committee (No. 2091). All patient guardians provided written informed consent prior to participation.

Samples

Sixty-two non-ASMD infants and children admitted to the Department of Pediatrics of Akita University Hospital between February 2019 and June 2020 were enrolled in this study. The participants included 29 males and 33 females from 28 weeks of gestation to 15 years of age. To examine the differences in ASM activity according to age, the participants were alternately classified into the following three groups : (1) preterm infants (n=28), (2) full-term infants (n=24), and (3) others (n=10). Twenty-eight subjects were admitted because of low birth weight and premature birth. The other 34 subjects were admitted for the diagnosis of other diseases, including gastrointestinal disorders, thyroid dysfunction, and congenital anomalies. There were no cases of acute respiratory syncytial virus bronchiolitis[14], Kawasaki disease[15], sepsis[16], hemophagocytic syndrome[17], type 2 diabetes[18], or chronic heart failure[19] that have been reported to increase S-ASM activity. Table 1 summarizes the demographic characteristics of the subjects. DBSs were prepared along with laboratory blood tests. However, in neonatal care, routine blood tests are often performed by capillary blood collection ; therefore, blood counts and biochemical blood tests are not performed every time. The blood test data presented in Table 1 shows the results of 37 subjects who underwent DBS collection at the time of the blood count test. These 37 subjects were divided into three groups : preterm, full-

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Table 1. Demographic characteristics of the 62 subjects.

		Range
Age (years)	0.5 ± 2.1	(0-14)
Sex (number) (%)		
Male	29 (46.8%)	
Famale	33 (53.2%)	
ASM activity (μmol/h/L ; n=62)	5.7 ± 2.4	(2.3-16.4)
Blood laboratory data		
White blood cells (/μL ; n=37)	10,676 ± 3,380	(5,000-21,600)
Lymphocyte (/μL ; n=36)	5,459 ± 2,366	(1,430-11,880)
Neutrophil (/μL ; n=36)	3,808 ± 1,550	(1,230-8,040)
Eosinophil (/μL ; n=36)	548 ± 385	(0-1,340)
Basophile (/μL ; n=36)	50 ± 73	(0-250)
Monocyte (/μL ; n=36)	886 ± 428	(240-2,140)
Hematocrit (% ; n=37)	43.4 ± 7.4	(31.0-69.0)
Reticulocyte (×103/μL ; n=32)	77.6 ± 53.9	(24.1-288.8)
Platelet (×104/μL ; n=37)	39.0 ± 14.5	(10.6-79.1)
C-reactive protein (mg/L ; n=35)	0.1 ± 0.1	(0.0-0.4)

ASM, Acid Sphingomyelinase

term, and other. Their demographic characteristics are summarized in Table 2.

For sample collection, blood was dropped onto a filter paper. After drying at room temperature (approximately 26°C) for at least 5 h, 2 g of silica gel was added and stored at −80°C. Medical records were reviewed for patient age, sex, and blood laboratory data including WBC counts, lymphocytes, neutrophils, eosinophils, basophils, monocytes, hematocrit, reticulocytes, platelet counts, and C-reactive protein levels. DBS was also obtained from one patient with ASMD (type A/B) whose diagnosis had already been confirmed using the same method. The *SMPD-1* variants of the patient were confirmed as p.G494C/p.G494C. ASM activity in cultured skin fibroblasts was markedly decreased to 4.8 nmol/mg/h (residual ASM activity 7.6%). ASM activity in the skin fibroblasts was measured as previously described[20].

Determination of enzyme activity of ASM by flow injection-tandem mass spectrometry

ASM activity was determined using a Neo LSD kit (PerkinElmer, Turku, Finland). The kit contained buffer, extraction solution (ES, containing ethyl acetate), mobile phase, substrate, internal standard, and quality control DBS, which are necessary for measuring DBS activity. Methanol (MeOH) was purchased from Wako Pure Chemical Industries (Osaka, Japan).

The method for measuring ASM activity by tandem mass spectrometry (MS/MS) using DBS is shown in Figure 1. Briefly, on day 1, 30 μL of incubation cocktail containing the buffer, substrate, and internal standard of the enzyme was added to a 3.2 mm DBS and incubated at 37°C for 19 h with orbital shaking at 400 rpm. On day 2, the reaction was stopped with 100 μL of MeOH : ES and the mixed solution was transferred to a deep-well plate. Water (200 μL) and ES (400 μL) were then added and mixed. Centrifugation (700×g for 5 min) was performed, 50 μL of the upper layer was transferred to a U-bottom 96-well microplate, dried with nitrogen, and 100 μL of the mobile phase was added. Flow injection tandem mass spectrometry (MS/MS) was performed using a TSQ Vantage-AM triple quadrupole mass spectrometer (Thermo Fisher Scientific). Xcalibur 4.2 software (Thermo Fisher Scientific) was used for data acquisition and processing. The details of these methods are presented in Tables 3 and 4. ASM activity was expressed in μmol/h/L.

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Table 2. Demographic characteristics of 37 subjects with the same blood collection date for DBS and blood counts, by preterm group, full-term group, and other group.

	Preterm infants	Full-term infants	Others
Age (Med)	28-36 W (35)	37-41 W (37)	2 M-14 Y (2)
Number	16	12	9
Sex (number) (%)			
Male	10 (62.5)	2 (16.7)	6 (66.7)
Female	6 (37.5)	10 (83.3)	3 (33.3)
Weight (Med) (kg)	1.2-2.9 (2.0)	1.6-3.0 (2.4)	2.8-80.0 (9.0)
ASM activity (μmol/h/L)	6.1 ± 2.3	5.4 ± 1.2	7.8 ± 4.1
WBC (/μL)	10,606 ± 552	10,975 ± 826	10,863 ± 2030
Lymphocyte (/μL)	5,626 ± 514	5,151 ± 510	5,588 ± 1,312
Neutrophil (/μL)	3,451 ± 372	4,272 ± 460	3,825 ± 572
Diagnosis			
Low Birth Weight	13*	8	0
Congenital anomaly	2	0	4
Endocrine disorder	3	1	2
Digestive disorder	2	2	0
Respiratory failure	1	0	2
Heart disease	0	1	0
Malignant tumor	0	0	1

W, Weeks ; M, Months ; Y, Years ; Med, Median ; ASM, Acid Sphingomyelinase ; WBC, White Blood Cells.
* Some preterm infants have low birth weight and other overlapping diagnoses

Validation of the assay using quality control DBSs included this kit

The Neo LSD kit contains three quality control DBSs : low, medium, and high enzyme activities. The inter-day coefficient of variation (%) obtained using these DBSs examined four times was high control=9.7%, middle control=6.6%, and low control=3.9%, confirming the validity of the kit.

Assay cut-off values

The cutoff value was set at the 0.1th percentile. Owing to the limited number of samples in this study, a cut-off value of 1.5 μmol/h/L as described in a previous report measuring ASM activity using similar methods was used as the reference cut-off value[7]. This was 25% of the mean ASM activity of 6.0 μmol/h/L in 44,432 neonates.

Statistical analysis

Data were analyzed using the IBM SPSS Statistics software package (version 28.0) and are presented as mean±standard deviation (SD). The Pearson's correlation coefficient test was performed to examine the correlation between ASM activity and laboratory data. Independent sample t-tests were performed to compare mean differences between the two groups. Statistical significance was set at $P<0.05$.

RESULTS

ASM activity in DBS

The mean ASM activity of the DBSs from the 62 subjects was 5.7±2.4 μmol/h/L (min 2.3, max 16.4, median 5.1). No sample was below the reference cutoff value of 1.5 μmol/h/L. One sample was 2.0 μmol/h/L, below 2.1 μmol/h/L, and in the 0.1th percentile of 62 sub-

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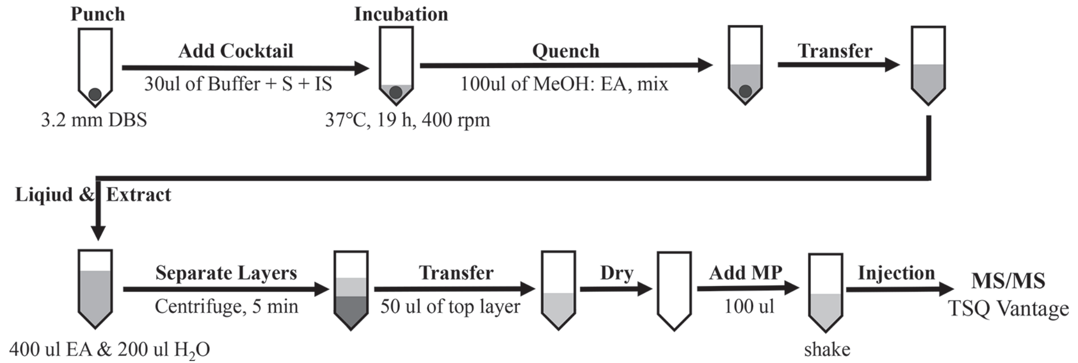

Figure 1.　Method for measuring ASM activity using flow injection-tandem mass spectrometry.
Using one punch from DBS, buffer containing substrate and internal standard were added, followed by incubation for 19 h to allow the enzyme reaction to proceed.　The product and internal standard were then extracted and analyzed using a mass spectrometer to calculate the enzyme activity.
DBS, dried blood spot ; S, substrates ; IS, internal standard ; MeOH, methanol ; EA, ethyl acetate ; MP, mobile phase ; MS/MS, tandem mass spectrometry.

Table 3.　Instrument parameters for the measurement of substrate, internal standard and product of ASM activity using MS/MS.

Interface	ESI (Electrospray ionization)
Polarity	Positive
Injection volume	2μL
Spray voltage	2.8 kV
Vaporizer temperature	0°C
Capillary temperature	270°C
Sheath gas pressure	25 L/h
Aux gas pressure	0 L/h
Analyzing mode	SRM (selected reaction monitoring)
Dwell time	0.1 s
Data format	Profile

jects.　The sample was reassayed and showed a value of 2.7 μmol/h/L.　This discrepancy in results could be due to the poor sample with low blood volume.　The ASM activity in DBS from a patient with ASMD was 1.0 μmol/h/L, which was below the cut-off values.　The ASM activities of DBSs are shown in Figure 2.

The ASM activity in DBS, WBC count, ASM activity, and lymphocyte count were positively correlated

The correlation between ASM activity and blood test data was examined in 37 subjects for whom DBS was collected at the time of a blood count and biochemical blood tests.　ASM activity and WBC count showed a significant positive correlation with Pearson's correlation test (r=0.44, p=0.006).　Furthermore, ASM activity and lymphocyte count showed a significant positive correlation with Pearson's correlation test (r=0.51, p=0.001).　Figure 3 shows the correlation diagram of the results.　No significant correlation was found between neutrophil count, basophil count, eosinophil count, monocyte count,

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Table 4. Product ion, precursor ion, Stacked-Lens, collision energy and retention times for the fragmentation of substrate, product and internal standard using MS/MS.

Species	Precursor ion (m/z)	Product ion (m/z)	S-Lends RF amplitude voltage (V)	Collision energy (V)
ASM-S	563.4	184.0	130	20
ASM-P	398.4	264.3	90	17
ASM-IS	405.4	264.3	90	16

ASM, acid sphingomyelinase

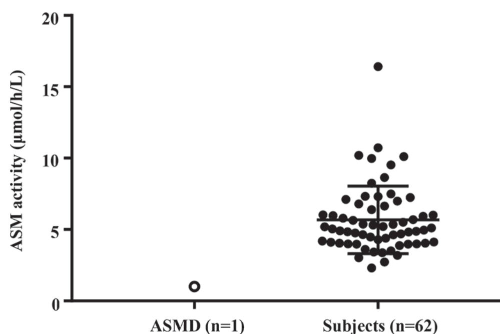

Figure 2. Acid sphingomyelinase activity in dried blood spots.
Acid sphingomyelinase (ASM) activity in 62 hospitalized patients and one patient with acid sphingomyelinase deficiency (ASMD) as per the activity in dried blood spots. All values represent the mean ± SD.

hematocrit value, reticulocyte count, platelet count, serum calcium, C-reactive protein, or serum ferritin (data not shown).

No significant differences were observed in ASM activity, WBC count, or lymphocyte count between the preterm and full-term groups.

To understand the effect of preterm birth on the possibility of retesting, we compared ASM activity, WBC count, and lymphocyte count between the preterm and full-term infant groups. Of the 37 subjects whose blood samples were collected on the same day, 16 preterm infants and 12 full-term infants were examined (Table 2). No significant differences were observed in the ASM activity (6.1±2.3 μmol/h/L vs. 5.4±1.2 μmol/h/L [p =0.30]), WBC counts (10,606±552 vs. 10,975±826 [p=0.70]), or lymphocyte counts (5,626±514 vs.

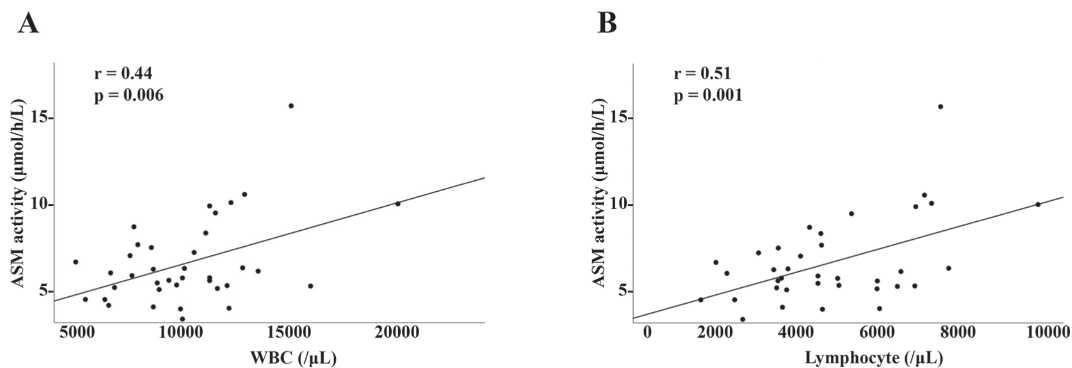

Figure 3. Correlation with acid sphingomyelinase activity.
(A) In 37 eligible patients, acid sphingomyelinase (ASM) activity positively correlated with white blood cell (WBC) count (r = 0.44, p = 0.006). (B) In 37 eligible patients, acid sphingomyelinase activity positively correlated with lymphocyte count (r = 0.51, p = 0.001).

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5,151±510 [p=0.53]) between the preterm and full-term infant groups.

Discussion

A study of mass screening for ASMD was conducted using DBSs in Japanese infants and children admitted to our hospital. This study revealed the proper distribution values of DBS-derived ASM activity in Japanese infants and children without ASMD. In this study, the patient with ASMD clearly tested positive on the DBS samples. Hence, this method provides a simplified and reliable mass-screening assay for ASMD. Our data may facilitate mass screening for ASMD in Japan.

When we make a diagnosis of ASMD, ASM activity has traditionally been measured using cultured skin fibroblasts or lymphocytes[21]. Subsequently, a DBS assay was developed, and pilot studies of expanded newborn mass screening and high-risk screening using DBS have recently been reported[3,7,8]. Investigation of the factors affecting ASM activity in DBS is important because they can lead to false-negative and false-positive results. The kit used in this study was evaluated for interference in accordance with CLSI (Clinical and Laboratory Standards Institute) document EP07-A2. Bilirubin and albumin levels were shown to have no effect on ASM activity in the DBS. However, other laboratory blood data were unavailable.

In this study, ASM activity during DBS was positively correlated with WBC counts and lymphocyte counts. Blood cells containing lysosomes as microgranules include white blood cells (WBCs), reticulocytes, and platelets. Mature erythrocytes are denucleated and do not contain lysosomes in their blood. In healthy adults, 17% of human blood ASM is present as L-ASM in peripheral blood mononuclear cells and 83% as S-ASM in the plasma[9]. We speculate that the positive correlation between WBC and lymphocyte counts and ASM activity in DBS is due to the influence of L-ASM on blood cells. Since residual ASM activity has previously been measured using lymphocytes, it is possible that L-ASM activity in lymphocytes is higher than that in other blood cells.

Alternatively, as shown in Tables 1 and 2, the subjects enrolled in this study were mostly infants, which may be due to the fact that the lymphocyte count is higher than the neutrophil count in terms of age. The WBC fraction is neutrophil-predominant after birth due to leukocyte growth factors, such as granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF), secreted from the placenta[22]. Thereafter, neutrophils decrease, becoming lymphocyte-dominant at 1-2 weeks of age and changing to neutrophil-dominant at around 6 years of age[23]. Neutrophil counts changed significantly, whereas lymphocyte counts did not change significantly from birth to early childhood. In this study, no significant difference was observed in the lymphocyte counts between the preterm and full-term groups. In addition, there was no significant difference in ASM activity during DBS between the preterm and full-term groups. This suggests that preterm infants may not be required to be retested when they grow up. However, more data are needed because this study did not compare the same cases.

In general, the residual enzyme activity of ASMD is less than 5% for type A and 15% for type B[9,24]. Since ASMD of type A has very low ASM activity, the enzyme activity in DBS is also apparently low, regardless of WBC and lymphocyte counts. However, in the case of type B, mild cases with a residual enzyme activity of nearly 20% have been reported[25,26], and WBC and lymphocyte counts may be problematic. In other words, false-negative results may occur. The ASM activity of carriers has been reported to be 20-50%[27,28], indicating that false positives may occur depending on WBC and lymphocyte counts. Further data accumulation is needed to establish a cut-off value that does not result in false negatives for patients with ASMD and false positives for carriers.

The limitations of this study include the small number of cases and the fact that early-preterm infants were not included in the study. In addition, the primary disease varied, and the possibility of bias cannot be ruled out. However, the background of the population varied in both newborns and high-risk screenings.

To the best of our knowledge, this is the first report to show that WBC and lymphocyte counts are involved in ASM activity during DBS. To prevent missed disease, further investigation and sharing of data is needed.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgements

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