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Research Report

Cerebrospinal fluid biomarkers showing neurodegeneration in dogs with GM1 gangliosidosis: Possible use for assessment of a therapeutic regimen

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Abbreviations:

ANOVA, analysis of variance

AST, aspartate aminotransferase

CNS, central nervous system

CSF, cerebrospinal fluid

HPTLC, high performance thin-layer chromatography

LDH, lactate dehydrogenase

MBP, myelin basic protein

NANA, *N*-acetylneuraminic acid

NSE, neuron-specific enolase

PBS, phosphate-buffered saline

ABSTRACT

The present study investigated cerebrospinal fluid (CSF) biomarkers for estimating degeneration of the central nervous system (CNS) in experimental dogs with GM1 gangliosidosis and preliminarily evaluated the efficacy of long-term glucocorticoid therapy for GM1 gangliosidosis using the biomarkers identified here. GM1 gangliosidosis, a lysosomal storage disease that affects the brain and multiple systemic organs, is due to an autosomal recessively inherited deficiency of acid β -galactosidase activity. Pathogenesis of GM1 gangliosidosis may include neuronal apoptosis and abnormal axoplasmic transport and inflammatory response, which are perhaps consequent to massive neuronal storage of GM1 ganglioside. In the present study, we assessed some possible CSF biomarkers, such as GM1 ganglioside, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), neuron-specific enolase (NSE) and myelin basic protein (MBP). Periodic studies demonstrated that GM1 ganglioside concentration, activities of AST and LDH, and concentrations of NSE and MBP in CSF were significantly higher in dogs with GM1 gangliosidosis than those in control dogs, and their changes were well related with the months of age and clinical course. In conclusion, GM1 ganglioside, AST, LDH, NSE and MBP could be utilized as CSF biomarkers showing CNS degeneration in dogs with GM1 gangliosidosis to evaluate the efficacy of novel therapies proposed for this disease. In addition, we preliminarily treated an affected dog with long-term oral administration of prednisolone and evaluated the efficacy of this therapeutic trial using CSF biomarkers determined in the present study. However, this treatment did not change either the clinical course or the CSF biomarkers of the affected dog, suggesting that glucocorticoid therapy would not be effective for treating GM1 gangliosidosis.

1. Introduction

GM1 gangliosidosis, a lysosomal storage disease that affects the brain and multiple systemic organs, is due to an autosomal recessively inherited deficiency of acid β -galactosidase activity (Suzuki et al., 2001). The substrates of the enzyme, such as GM1 ganglioside, and glycoconjugates with terminal β -galactose accumulate especially in the lysosomes of neurons, resulting in the manifestation of progressive neurodegeneration and brain dysfunction. The pathogenesis of GM1 gangliosidosis, perhaps consequent to massive neuronal storage of GM1 ganglioside and related glycoconjugates, is not completely understood. Neuronal apoptosis is found in GM1 gangliosidosis (Tessitore et al., 2004) and other neurodegenerative diseases: GM2 gangliosidosis (Huang et al., 1997) and Alzheimer's disease (Su et al., 1994; Lassmann et al., 1995). Abnormal axoplasmic transport, resulting in deficit of myelin, is also found in GM1 gangliosidosis (van der Voorn et al., 2004). Furthermore, inflammatory responses are considered to contribute to the pathogenesis or disease progression in several neurodegenerative diseases including GM1 gangliosidosis (Gehrmann et al., 1995; Bianca et al., 1999; Wada et al., 2000; Jeyakumar et al., 2003).

At present, only symptomatic therapy is available for patients with GM1 gangliosidosis in both human and veterinary medicine. Allogeneic bone marrow transplantation and enzyme replacement therapy are effective for some lysosomal diseases in which only visceral organs are affected (Schiffmann and Brady, 2002), but these therapeutic methods are generally not applicable for neurodegenerative diseases such as GM1 gangliosidosis. Gene therapy is another promising approach for the potential treatment of neurodegenerative diseases that are caused by a single-gene defect, but this method is still in the experimental stage. Recently, the effects of a molecular approach, i.e., chemical chaperone therapy, for restoration of mutant

α -galactosidase in Fabry disease (Fan et al., 1999; Asano et al., 2000) and mutant β -galactosidase in GM1 gangliosidosis (Matsuda et al., 2003) were reported. These novel therapies are expected to be effective and safe, but all of these therapies need further investigation using appropriate animal models prior to application to humans.

In animals, naturally occurring GM1 gangliosidosis has been recorded in cats, dogs, sheep and calves (Suzuki et al., 2001). In addition, a mouse model lacking a functional β -galactosidase gene has been generated by homologous recombination and embryonic stem cell technology (Hahn et al., 1997; Matsuda et al., 1997), and a recently advanced mouse model that lacks an original β -galactosidase gene and also expresses a certain human gene mutation has been developed as an appropriate model of human disease (Matsuda et al., 2003). In general, mouse models can be easily used for therapeutic trials because of the small body size, high fertility and short survival period. However, it is difficult to estimate neurological symptoms because these are not as definite as those in humans, although recently a new assessment system for motor and reflex functions in mice with neurogenetic diseases has been developed (Ichinimiya et al., 2006). In contrast, canine and feline models show definite neurological features similar to those of humans. Especially, canine disease is an excellent model of human disease because canine and human β -galactosidases are structurally similar (Suzuki et al., 2001). However, dogs are not as fertile, making it more difficult and expensive for many individuals to be used in therapeutic trials. If it is possible to evaluate the degree or extent of degeneration in the central nervous system (CNS) using samples obtained from living individuals, it will lessen the number of dogs necessary to determine the efficacy of potential therapeutic programs for GM1 gangliosidosis and simultaneously contribute to the welfare of experimental animals.

GM1 gangliosidosis in shiba dogs was initially identified in 2000 (Yamato et al., 2000). Since then, a closed breeding colony has been maintained at the Graduate School of Veterinary Medicine, Hokkaido University (Sapporo, Hokkaido, Japan) (Yamato et al., 2003). The homozygous recessive mutation causing GM1 gangliosidosis in shiba dogs has been identified as a deletion of C nucleotide 1647 in the putative coding region for canine β -galactosidase gene (Yamato et al., 2002a), which can be detected by the PCR-based DNA assay (Yamato et al., 2004a and 2004b). Affected shiba dogs show deficiency of β -galactosidase activity and storage of GM1 ganglioside in the CNS, and manifest neurological symptoms of progressive motor dysfunctions starting from 5-6 months of age and finally die by 15 months of age following a clearly defined clinical course (Yamato et al., 2000 and 2003), which may be associated with the progression and severity of pathological degeneration and the level of GM1 ganglioside accumulation in the CNS. Shiba dogs with GM1 gangliosidosis have clinical features similar to the human juvenile form of this disorder. This canine model is expected to contribute to developing novel therapeutic methods.

In the present study, we assessed some possible cerebrospinal fluid (CSF) biomarkers, such as GM1 ganglioside, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), neuron specific enolase (NSE) and myelin basic protein (MBP) using CSF samples that were periodically collected from both affected dogs and control dogs. We expected that the levels of these CSF biomarkers would show the degree or extent of CNS degeneration in dogs with GM1 gangliosidosis and could be utilized to evaluate the efficacy of novel therapeutic methods.

It has been reported that inflammation in the CNS is related to pathogenesis in mouse models of GM1 and GM2 gangliosidoses, so depression of the inflammatory reaction is

thought to ameliorate clinical features of GM1 gangliosidosis. Actually, depression of CNS degeneration and amelioration of clinical features were observed in GM2 gangliosidosis model mice by deletion of macrophage inflammatory protein-1 α , one of the important factors participating in the inflammatory reaction (Wu et al., 2004). Therefore, we preliminarily tried glucocorticoid therapy at an anti-inflammatory dose of prednisolone in a shiba dog with GM1 gangliosidosis to determine whether this treatment could ameliorate the clinical symptoms or delay death.

The present study investigated CSF biomarkers for estimating CNS degeneration, then evaluated the efficacy of long-term glucocorticoid therapy for GM1 gangliosidosis using the biomarkers determined here.

2. Results

2.1. GM1 ganglioside in CNS tissues and CSF

The changes of GM1 ganglioside content in the cerebrum, cerebellum and spinal cord in dogs with GM1 gangliosidosis are shown in Fig. 1. GM1 ganglioside concentration increased with age in months in all CNS tissues until the terminus of life, and the rate of increase was marked in cerebellum and cerebrum. In two control dogs, GM1 ganglioside concentration in cerebrum, cerebellum and spinal cord were 2.07, 0.60 and 0.77 nmol *N*-acetylneuraminic acid (NANA) per mg of protein (mean of two dogs), respectively. These levels in control dogs were lower than those in a newborn affected dog (Fig. 1).

Changes in CSF GM1 ganglioside concentration in affected dogs and control dogs are shown in Fig. 2. The concentration of CSF GM1 ganglioside in dogs with GM1 gangliosidosis was already significantly higher than that in control dogs at 2 months of age,

then increased further with age in months. The level increased markedly after 5 months of age until the terminus of life. In contrast, the concentration of CSF GM1 ganglioside in control dogs was maintained at a low level throughout the experimental period. The change of CSF GM1 ganglioside concentration in affected dogs was well consistent with the change in GM1 ganglioside concentration in affected CNS tissues (Figs. 1 and 2).

2.2. Candidate biochemical components for CSF biomarkers

Fourteen biochemical components in CSF were analyzed in affected shiba dogs and normal dogs as candidate CSF biomarkers showing CNS degeneration of GM1 gangliosidosis (Table 1). As a result, the AST and LDH activities in CSF were significantly higher in affected dogs than in normal dogs.

2.3. AST and LDH activities in CSF and tissues

In the present study, we periodically measured CSF activities of AST and LDH for possible biomarkers in dogs with GM1 gangliosidosis and carrier dogs as control (Figs. 3 and 4), because these enzyme activities were significantly higher in affected dogs than in control dogs in the preceding examination (Table 1). In this serial examination, the CSF AST activity was already significantly higher in affected dogs than in control dogs at 2 months of age (Fig. 3). The AST activity in affected dogs continued to increase until 7 months of age, then maintained at the highest level until 12 months of age. However, the activity at 13 months of age seemed to decrease to the level at 4-6 months of age. In contrast, AST activity in control dogs remained constant at a very low level throughout the experimental period. The change in CSF LDH activity in affected dogs was very similar to that in CSF AST activity (Fig. 4). The

LDH activity was significantly higher in affected dogs than in control dogs at 2 months of age, then increased until 7 months of age. Thereafter, it slightly decreased toward the terminus of disease, although the level remained high.

Tissue distributions of AST and LDH activities were investigated to determine the location of these enzymes in CNS tissues and the differences between affected and normal dogs. However, there was almost no difference in tissue distribution and enzyme activity in each organ between affected and control dogs (data not shown).

2.4. NSE and MBP in CSF

In addition, we periodically measured the CSF concentrations of NSE and MBP in affected dogs and control dogs (Figs. 5 and 6). The CSF NSE concentration in affected dogs increased gradually until the terminus of disease and showed a significant increase compared with that in control dogs after 4 months of age but not at 6 months of age (Fig. 5). The CSF MBP concentration in affected dogs rapidly increased after 9 months of age and was significantly higher than that in control dogs (Fig. 6). However, there was no significant difference between the two groups at 2 to 8 months of age.

2.5. Glucocorticoid therapy in a dog with GM1 gangliosidosis

The clinical features of the affected dog treated with prednisolone were not ameliorated. The dog finally reached the terminal period relatively early at 11 months of age compared with other untreated affected dogs, although there were no apparent side-effects in the treated affected dog on serial hematological examinations (data not shown). Furthermore, there was no difference in body weight between the treated dog and other untreated affected dogs (data

not shown).

The findings of CSF biomarkers obtained from the treated affected dog are shown in Figs. 2-6 using solid lines with open circles. The CSF biomarkers, i.e., GM1 ganglioside, AST, LDH, NSE and MBP, were not changed or improved as compared with those in untreated dogs with GM1 gangliosidosis. The AST activity and NSE concentration in CSF seemed to be higher in the treated dog than in untreated affected dogs.

3. Discussion

As a candidate CSF biomarker showing the degree or extent of CNS degeneration in GM1 gangliosidosis, we first targeted the concentration of GM1 ganglioside since it is a major pathogenic material stored in CNS tissues in this disease. To estimate the usefulness of CSF GM1 ganglioside concentration as a biomarker, changes of GM1 ganglioside in CNS tissues and CSF were investigated and compared (Figs. 1 and 2). As a result, both the GM1 ganglioside concentration in CNS tissues (Fig. 1) and the GM1 ganglioside concentration in CSF (Fig. 2) showed a positive correlation with the months of age and progression of clinical symptoms in dogs with GM1 gangliosidosis. As a result of these investigations, it was demonstrated that the increase in CSF GM1 ganglioside agrees with the accumulation of GM1 ganglioside and pathological deterioration in the brains of dogs with GM1 gangliosidosis. It was reported that the increased concentration of GM1 ganglioside in CSF reflects the accumulated GM1 ganglioside in CNS in human patients with GM1 gangliosidosis ([Yamanaka et al., 1987](#); [Kaye et al., 1992](#)). Therefore, the concentration of CSF GM1 ganglioside was considered a good biomarker for evaluating neurodegeneration in both human and canine GM1 gangliosidosis.

We previously reported that the concentration of CSF GM2 ganglioside in a dog (Yamato et al., 2002b) and a cat (Yamato et al., 2004c) with GM2 gangliosidosis variant 0 was markedly higher than that in control animals and the CSF GM2 ganglioside concentration can be utilized for diagnosis of this disease (Yamato et al., 2004d). Therefore, GM2 ganglioside, a major storage material in GM2 gangliosidosis, might also be a useful biomarker showing the neurodegeneration in GM2 gangliosidoses including Tay-Sachs disease, Sandhoff disease and GM2 activator protein deficiency. However, there is a disadvantage in that the analysis of GM1 and GM2 gangliosides in CSF requires complex procedures and specific techniques (Izumi et al., 1993; Satoh et al., 2004; Yamato et al., 2004d).

In the present study, we assessed fourteen biochemical components in CSF to identify reliable biomarkers that can be analyzed more easily than GM1 ganglioside (Table 1). As a result, it was found that the activities of AST and LDH were significantly higher in affected dogs than in control dogs. Therefore, the activities of AST and LDH were further evaluated in the following study using CSF collected periodically from affected and control dogs (Figs. 3 and 4). Consequently, the changes in AST and LDH activities in CSF mostly showed a positive correlation with age until 7 months of age in affected dogs although these enzyme activities did not increase with age after 7 months of age.

It was reported that CSF AST activity is elevated in human patients with Alzheimer's disease (Riemenschneider et al., 1997), Niemann-Pick disease and GM2 gangliosidosis (Aronson et al., 1958), and brain insult (Osuna et al., 1992). Particularly in Alzheimer's disease, the increase of CSF AST activity has been noted because of a very few clinico-pathologic characteristics for definitive diagnosis of this disease (Riemenschneider et al., 1997; Esmonde 1998; Tapiola et al., 1998). Impairment of glucose metabolism in the brain

is another feature of Alzheimer's disease, and this may result in the use of glucogenic amino acids as alternative sources of energy and subsequently the increased activity of aminotransferases including AST ([Riemenschneider et al., 1997](#)). Unlike Alzheimer's disease, the increase in CSF AST activity in patients with lysosomal diseases including Niemann-Pick disease and gangliosidoses, might be due to a leakage from neurons or other cells in CNS tissues as that occurs in patients with brain insult ([Osuna et al., 1992](#)) or stroke ([Parakh et al., 2002](#)).

LDH is also a stable cytoplasmic enzyme expressed in all cells including neurons and rapidly released into the cell culture supernatant when the plasma membrane is damaged. Therefore, it is regarded as a biochemical index of cytotoxicity ([O'Neill et al., 2004](#)). Release of LDH into the CSF occurs under various conditions causing acute brain cell damage, and CSF LDH activity increases in CNS infections, head trauma, vascular accidents, intracerebral lymphoma, organophosphate poisoning, Creutzfeldt-jakob disease, and metastatic CNS disease ([Schmidt et al., 1999](#)). From these observations, the increased activity of CSF LDH observed in the present study is also thought to be due to leakage from neuronal cells damaged by the accumulation of specific storage materials. Supporting our hypotheses, there was no elevation of AST and LDH activities in CNS tissues in affected dogs compared with those in control dogs. The elevation of AST and LDH in CSF in affected dogs may be due to the release from CNS tissues that are originally rich in these enzyme activities. The AST and LDH activities in CSF seem to be very useful biomarkers at least in GM1 gangliosidosis because they can not only show CNS degeneration but also be measured easily and at a low cost.

In some reports, it was suggested that massive neuronal storage of GM1 ganglioside

results in neuronal apoptosis (Tessitore et al., 2004), abnormal axoplasmic transport (van der Voorn et al., 2004) and other processes. These contribute to the pathogenesis or disease progression in GM1 gangliosidosis. In shiba dogs with GM1 gangliosidosis, deficit of myelin as well as swelling and subsequent disappearance of neuronal cells is observed in CNS tissues, and these findings are progressive with age in months (unpublished data). Therefore, we determined the concentrations of NSE and MBP, which are used as general biomarkers to evaluate CNS damage including the neuronal loss and demyelination. As a result, the NSE and MBP concentrations increased markedly, becoming significantly higher in affected dogs than in control dogs (Figs. 5 and 6).

The CSF NSE concentration was one of the good biomarkers because its change was similar to those of AST and LDH activities (Figs. 3-5). NSE is specific for neurons and is present in cell bodies as well as in axons, and has been shown to be elevated in patients with hypoxic brain injury, meningeal hemorrhage, subarachnoid hemorrhage, acute head injury, hydrocephalus, and other neurological disorders (Orlino et al., 1997). Also in animal models, NSE increases in head trauma, stroke and focal ischemia (Orlino et al., 1997), and it has been shown that there is a reasonable correlation between the volume of infarction and the CSF NSE level (Hatfield and McKernan, 1992). In the present study, the CSF NSE concentration seemed to be well related to the degree or extent of CNS degeneration in dogs with GM1 gangliosidosis.

In contrast, the CSF MBP concentration started to increase at 9 months of age in affected dogs (Fig. 6) when pathological alteration in the CNS tissues becomes marked. It is suspected that the elevation of MBP concentration in affected dogs might be due to deficit of myelin, which generally results from axonal loss, dysplasia of myelin (Folkerth, 1999) or abnormal

axoplasmic transport ([van der Voorn et al., 2004](#)), but this hypothesis has not been completely proven.

In the present study, we preliminarily treated an affected shiba dog with prednisolone for 6 months and examined how the clinical course and symptoms were changed by the treatment. In addition, we evaluated the efficacy of this treatment using CSF biomarkers determined in the present study. However, this trial did not affect either the clinical symptoms or the CSF biomarkers compared with those of untreated dogs with GM1 gangliosidosis (Figs. 2-6). Therefore, we concluded that low-dose prednisolone therapy is not effective for GM1 gangliosidosis.

Many reports have indicated that inflammatory reaction in the CNS is associated with the pathogenesis of neurodegenerative diseases including GM1 and GM2 gangliosidosis ([Gehrmann et al., 1995](#); [Bianca et al., 1999](#); [Wada et al., 2000](#); [Myerowitz et al., 2002](#); [Jeyakumar et al., 2003](#); [Yamaguchi et al., 2004](#)). Actually, depression of CNS degeneration and amelioration of clinical features were observed in mice with GM2 gangliosidosis by suppression of the inflammatory reaction ([Wu et al., 2004](#)) or administration of non-steroidal anti-inflammatory drugs either alone or in combination with *N*-butyldeoxynojirimycin, which can inhibit the synthesis of a storage material ([Jeyakumar et al., 2004](#)). However, glucocorticoid therapy in the present study did not ameliorate the clinical features of affected dog. This may be because inflammatory reaction does not contribute to the pathogenesis or disease progression in GM1 gangliosidosis compared with those in other neurodegenerative lysosomal diseases, or because the dose of prednisolone used in this study was not sufficient to suppress intracranial inflammatory reaction. Further study will be required to evaluate the efficacy of anti-inflammatory therapy in GM1 gangliosidosis, but this kind of therapy does

not seem to be useful for the rescue of patients with GM1 gangliosidosis.

In conclusion, the results in the present study suggested that GM1 ganglioside, AST, LDH, NSE and MBP could be utilized as CSF biomarkers showing the CNS degeneration in experimental dogs with GM1 gangliosidosis to evaluate the efficacy of novel therapies proposed for this disease. This canine model and these CSF biomarkers are expected to contribute to the development of new therapeutic modalities for human GM1 gangliosidosis.

4. Experimental procedures

4.1. Animals and sample collection

In the present study, we used 10 homozygous affected shiba dogs with GM1 gangliosidosis and 3 heterozygous carrier shiba dogs from a closed breeding colony maintained by the Graduate School of Veterinary Medicine, Hokkaido University (Yamato et al., 2003). The genotypes of these dogs were determined using a DNA-based assay (Yamato et al., 2004a). One of the affected dogs died at birth as a result of dystocia (Yamato et al., 2000). The other affected dogs were humanely euthanized by excessive administration of sodium pentobarbital (Dainippon Pharmaceutical Co., Osaka, Japan) by 15 months of age, which is the terminal period of this disease. All the affected dogs were provided with adequate food and water and received nursing care to prevent bedsores during their lifetime. The heterozygous carriers were used only for collection of CSF and venous blood until 13 months of age as controls for affected dogs. CSF was also collected from 6 clinically normal beagle dogs aged 9 months to 5 years as controls. The CNS and visceral organ tissues used as control in the present study were obtained from a 5-month-old beagle dog and a 6-year-old mixed-breed dog and stored at -80 °C, which had been utilized in past studies (Yamato et al., 2000 and 2002a).

CSF was collected by cisternal puncture under general anesthesia. Venous blood was occasionally collected from affected and carrier dogs for hematological examinations including complete blood counts and serum chemistry. All the samples including CSF, serum and tissues, were frozen at -80 °C until use. All experimental procedures using experimental animals were performed in accordance with the guidelines regulating animal use at the Graduate School of Veterinary Medicine, Hokkaido University.

4.2. Analysis of GM1 ganglioside in CNS tissues

Total lipids containing gangliosides were extracted from CNS tissues by the method of [Folch et al. \(1957\)](#). The tissue (0.1-0.2 g) was homogenized with 5 ml of chloroform-methanol (2:1, vol/vol) using a generator shaft type homogenizer. The suspension was centrifuged at 1,600 g for 10 min at 20 °C and a clear supernatant was separated. The pellet was reextracted with 5 ml of chloroform-methanol (2:1, vol/vol), and the two supernatants were then combined. Two ml of 0.5 % potassium chloride was added to the combined sample, and the solution was thoroughly mixed and centrifuged at 2,200 g for 10 min. The gangliosides contained within the upper-phase lipids were isolated using a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) as described by [Williams and McCluer \(1980\)](#). The gangliosides trapped in the cartridge were eluted with more than 15 ml of chloroform-methanol (2:1, vol/vol). The eluate was evaporated in vacuo and dissolved in an appropriate amount of deionized water. The total lipid-bound NANA was determined as a content of total gangliosides by the method of [Jourdain et al. \(1971\)](#). Purified NANA (Wako Pure Chemical Industries, Osaka, Japan) was used as a standard for determination of total lipid-bound NANA. Total ganglioside contents in these tissues were expressed as nmol NANA. After determination of total ganglioside content,

an aliquot of the sample for chromatographic estimation was evaporated in vacuo and frozen at -80 °C until use.

Individual gangliosides were separated by the high performance thin-layer chromatography (HPTLC) method on a plate precoated with superfine silica gel 60 (Merck, Darmstadt, Germany) (Williams and McCluer, 1980). Briefly, the ganglioside sample was dissolved in chloroform-methanol (2:1, vol/vol). A part of the sample containing 2 µg of NANA was applied to an HPTLC plate, then the plate was developed with chloroform-methanol-0.2 % CaCl₂ (58:42:10, vol/vol/vol). The ganglioside spots were visualized by spraying the plate with resorcinol-HCl reagent and heating it at 95 °C for 15 min (Jourdain et al., 1971). The percentage of individual gangliosides was determined by densitometric scanning (ATTO AE-6920M densitometer, ATTO, Tokyo, Japan), and the absolute amount of individual gangliosides was calculated from the total ganglioside content. Purified GM2, GM1, GD1a, GD1b and GT1b gangliosides derived from bovine brain (Sigma Chemical, St. Louis, MO, USA) were used as the standard for densitometric analysis. Simultaneously, the total protein concentration of original CNS tissues was measured with a commercial kit (Bio-Rad Protein Assay, Bio-Rad laboratories) using the method of Bradford (1976), and GM1 ganglioside concentrations in CNS tissues were expressed as nmol NANA per mg protein.

4.3. Analysis of GM1 ganglioside in CSF

The total gangliosides were extracted from 250 µl of CSF by the previously described method (Izumi et al., 1993; Yamato et al., 2004d) with a slight modification. Briefly, CSF gangliosides were extracted 3 times using organic solvents, each time with a

chloroform-methanol mixture, comprising a 2-ml (2:1, vol/vol) upper phase, 1.5-ml methanol supernatant, and 2-ml (1:2, vol/vol) supernatant. The combined lipid extract was evaporated in vacuo, dissolved in approximately 0.7 ml of deionized water, and then dialyzed using a cellulose ester membrane tube with a molecular weight cutoff value of 500 (Spectra/Por, Houston, TX, USA) against a sufficient volume of deionized water at 4 °C for 2 days. After dialysis, the solution was collected from the tube and evaporated in vacuo and then dissolved in a small amount of chloroform-methanol (2:1, vol/vol). The clear supernatant was evaporated in vacuo and the dry matter was frozen at -80 °C until use.

GM1 ganglioside concentration in CSF was determined using TLC-enzyme immunostaining as described in the previous report ([Sato et al., 2004](#)). The ganglioside extract was redissolved in chloroform-methanol (2:1, vol/vol), and part of the sample, which corresponded to 50 or 100 µl of CSF, was applied to a TLC plate (Polygram Sil G, Macherey-Nagel, Düren, Germany), then the plate was developed with chloroform-methanol-12 mM MgCl₂-15 M NH₄OH (60:40:7.5:3, vol/vol/vol/vol). After the plate was dried under a stream of warm air, it was again developed with chloroform-methanol-12 mM MgCl₂ (58:40:9, vol/vol/vol) in the same manner, then dried. The plate was soaked in solution A (10 mM phosphate-buffered saline [PBS], pH 7.4, containing 1 % egg albumin and 1 % polyvinylpyrrolidone [K-30, molecular weight 40,000]) at room temperature for 30 min after it was developed with solution A diluted 10-fold with PBS in the same direction as the first 2 times. The plate was treated with biotin-conjugated cholera toxin B (List Biological Laboratories, Campbell, CA, USA) in solution A (1:500 dilution ratio) at 37 °C overnight. After the first reaction, the plate was washed with 10 mM PBS containing 0.1 % Tween 20 (ICN Biomedicals, Aurora, OH, USA) and soaked in

solution A at 37 °C for 15 min. The plate then was treated with horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in 10 mM PBS containing 3 % polyvinylpyrrolidone (1:1000 dilution ratio) at 37 °C for 2 h and washed with 10 mM PBS.

The band of GM1 ganglioside was observed by a peroxidase reaction involving 4-chloro-1-naphthol as a chromogenic substrate. Purified GM1 ganglioside derived from bovine brain (ALEXIS Biochemicals, San Diego, CA, USA), which was quantitatively determined by the method of [Jourdian et al. \(1971\)](#), was used as the standard for densitometric scanning (GS-800 Calibrated Densitometer, Bio-Rad Laboratories, Hercules, CA, USA). The analysis of the densitometric scan data was performed by an appropriate software (Quantity One, Bio-Rad Laboratories), and the GM1 ganglioside concentration was calculated on the basis of the densities of GM1 ganglioside standards.

4.4. Measurement of biochemical components in CSF

The number of nucleated cells was estimated using Bürker-Türk hemocytometer (Kayagaki Irika Kogyo Co., Tokyo, Japan). The total protein concentration in CSF was measured with a commercial kit (Bio-Rad laboratories) as described above. The concentrations of sodium, potassium and chloride ions were measured using an automated biochemical analyzer (Fuji DRI-CHEM 800, Fujifilm Medical Co., Tokyo, Japan). The concentrations of calcium, inorganic phosphorus, glucose and creatinine, and the activities of alkaline phosphatase, alanine aminotransferase, AST, creatinine kinase and LDH, were measured using an automated serum biochemical analyzer (COBAS MIRA plus, Hoffmann-La Roche, Basel, Switzerland) without pretreatment. The concentrations of NSE and MBP were determined

using commercial kits (NSE: CanAg Diagnostics AB, Gothenburg, Sweden and MBP: COSMIC CORPORATION, Tokyo, Japan) according to the manufacturer's protocols, respectively.

4.5. Glucocorticoid therapy

A shiba dog with GM1 gangliosidosis received orally an anti-inflammatory dose of prednisolone (Prednisolone powder 1 %, Takeda Pharmaceutical Co., Osaka, Japan) at a dose of 0.5 mg/kg of body weight every other day. The administration started at 5 months of age and continued for 6 months until the dog reached the terminal stage at 11 months of age. CSF was collected from the dog every month, and all CSF biomarkers described above were analyzed and compared with those of untreated affected dogs and control dogs. To estimate the efficacy and side-effect of this therapy, this treated dog was also evaluated by observation of clinical symptoms, body weight and hematological examinations.

4.6. Statistical analysis

In the present study, statistical analysis was performed using Student *t* test or 1-way factorial analysis of variance (ANOVA) with post hoc tests (Tukey method). These analyses were carried out on a computer using a statistical software package (SYSTAT, Evanston, IL, USA). Values of $P < 0.05$ were considered significant. The analytical method and *P*-values are shown in each figure and table.

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REFERENCES

- Aronson, S.M., Saifer, A., Perle, G., Volk, B.W., 1958. Cerebrospinal fluid enzymes in central nervous system lipidoses (with particular reference to amaurotic family idiocy). *Proc. Soc. Exp. Bio. Med.* 97, 331-334.
- Asano, N., Ishii, S., Kizu, H., Ikeda, K., Yasuda, K., Kato, A., Martin, O.R., Fan, J.Q., 2000. In vitro inhibition and intracellular enhancement of lysosomal α -galactosidase A activity in Fabry lymphoblasts by 1-deoxygalactonojirimycin and its derivatives. *Eur. J. Biochem.* 267, 4179-4186.
- Bianca, V.D., Dusi, S., Bianchini, E., Dal Pra, I., Rossi, F., 1999. β -amyloid activates the O₂ forming NADPH oxidase in microglia, monocytes, and neutrophils – a possible inflammatory mechanism of neuronal damage in Alzheimer’s disease. *J. Biol. Chem.* 274, 15493-15499.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
- Esmonde, T., 1998. Diagnosis of Alzheimer’s disease with cerebrospinal fluid tau protein and aspartate aminotransferase. *Lancet* 351, 63.

- Fan, J.Q., Ishii, S., Asano, N., Suzuki, Y., 1999. Accelerated transport and maturation of lysosomal α -galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat. Med.* 5, 112-115.
- Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226, 497-509.
- Folkerth, R.D., 1999. Abnormalities of developing white matter in lysosomal storage diseases. *J. Neuropathol. Exp. Neurol.* 58, 887-902.
- Gehrmann, J., Matsumoto, Y., Kreutzberg, G.W., 1995. Microglia: Intrinsic immuneffector cell of the brain. *Brain Res. Brain Res. Rev.* 20, 269-287.
- Hahn, C.N., del Pilar Martin, M., Schröder, M., Vanier, M.T., Hara, Y., Suzuki, K., Suzuki, K., d'Azzo, A., 1997. Generalized CNS disease and massive GM1-ganglioside accumulation in mice defective in lysosomal acid β -galactosidase. *Hum. Mol. Genet.* 6, 205-211.
- Hatfield, R.H., McKernan, R.M., 1992. CSF neuron-specific enolase as a quantitative marker of neuronal damage in a rat stroke model. *Brain Res.* 577, 249-252.
- Huang, J.Q., Trasler, J.M., Igdoura, S., Michaud, J., Hanai, N., Gravel, R.A., 1997. Apoptotic cell death in mouse models of GM2 gangliosidosis and observations on human Tay-Sachs and Sandhoff diseases. *Hum. Mol. Genet.* 6, 1879-1885.
- Ichinomiya, S., Watanabe, H., Maruyama, K., Toda, H., Iwasaki, H., Kurosawa, M., Matsuda, J., Suzuki, Y. 2006. Motor and reflex testing in GM1-gangliosidosis model mice. *Brain Dev.* in press.
- Izumi, T., Ogawa, T., Koizumi, H., Fukuyama, Y., 1993. Normal developmental profiles of CSF gangliotetraose-series gangliosides from neonatal period to adolescence, *Pediatr. Neurol.* 9, 297-300.

- Jeyakumar, M., Smith, D.A., Williams, I.M., Borja, M.C., Neville, D.C.A., Butters, T.D., Dwek, R.A., Platt, F.M., 2004. NSAIDs increase survival in the Sandhoff disease mouse: synergy with *N*-butyldeoxynojirimycin. *Ann. Neurol.* 56, 642-649.
- Jeyakumar, M., Thomas, R., Elliot-Smith, E., Smith, D.A., van der Spoel, A.C., d'Azzo, A., Hugh Perry, V., Butters, T.D., Dwek, R.A., Platt, F.M., 2003. Central nervous system inflammation is a hallmark of pathogenesis in mouse models of GM1 and GM2 gangliosidosis. *Brain* 126, 974-987.
- Jourdian, G.W., Dean, L., Roseman, S., 1971. The sialic acids XI. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. *J. Biol. Chem.* 246, 430-435.
- Kaye, E.M., Ullman, M.D., Kolodny, E.H., Krivit, W., Rischert, J.C., 1992. Possible use of CSF glycosphingolipids for the diagnosis and therapeutic monitoring of lysosomal storage diseases. *Neurology* 42, 2290-2294.
- Lassmann, H., Bancher, C., Breitschopf, H., Wegiel, J., Bobinski, M., Jellinger, K., Wisniewski, H.M., 1995. Cell death in Alzheimer's disease evaluated by DNA fragmentation in situ. *Acta Neuropathol.* 89, 35-41.
- Matsuda, J., Suzuki, O., Oshima, A., Ogura, A., Naiki, M., Suzuki, Y., 1997. Neurological manifestations of knockout mice with β -galactosidase deficiency. *Brain Dev.* 19, 19-20.
- Matsuda, J., Suzuki, O., Oshima, A., Yamamoto, Y., Noguchi, A., Takimoto, K., Itoh, M., Matsuzaki, Y., Yasuda, Y., Ogawa, S., Sakata, Y., Nanba, E., Higaki, K., Ogawa, Y., Tominaga, L., Ohno, K., Iwasaki, H., Watanabe, H., Brady, R.O., Suzuki, Y., 2003. Chemical chaperone therapy for brain pathology in GM1-gangliosidosis. *Proc. Natl. Acad. Sci. USA.* 100, 15912-15917.

- Myerowitz, R., Lawson, D., Mizukami, H., Mi, Y., Tiffit, C.J., Proia, R.L., 2002. Molecular pathophysiology in Tay-Sachs and Sandhoff diseases as revealed by gene expression profiling. *Hum. Mol. Genet.* 11, 1343-1350.
- O'Neill, K., Chen, S., Diaz Brinton, R., 2004. Impact of the selective estrogen receptor modulator, tamoxifen, on neuronal outgrowth and survival following toxic insults associated with aging and Alzheimer's disease. *Exp. Neurol.* 188, 268-278.
- Orlino, E.N., Jr., Olmstead, C.E., Lazareff, J.A., Peacock, W.J., Fisher, R.S., Fluharty, A.L., 1997. An enzyme immunoassay for neuron-specific enolase in cerebrospinal fluid. *Biochem. Mol. Med.* 61, 41-46.
- Osuna, E., Perez-Carceles, M.D., Luna, A., Pounder, D.J., 1992. Efficacy of cerebro-spinal fluid biochemistry in the diagnosis of brain insult. *Forensic Sci. Int.* 52, 193-198.
- Parakh, N., Gupta, H.L., Jain, A., 2002. Evaluation of enzymes in serum and cerebrospinal fluid in cases of stroke. *Neurol. India* 50, 518-519.
- Riemenschneider, M., Buch, K., Schmolke, M., Kurz, A., Guder, W.G., 1997. Diagnosis of Alzheimer's disease with cerebrospinal fluid tau protein and aspartate aminotransferase, *Lancet* 350, 784.
- Satoh, H., Yamato, O., Asano, T., Yamasaki, M., Maede, Y., 2004. Increased concentration of GM1-ganglioside in cerebrospinal fluid in dogs with GM1- and GM2-gangliosidoses and its clinical application for diagnosis. *J. Vet. Diagn. Invest.* 16, 223-226.
- Schiffmann, R., Brady, R.O., 2002. New prospects for the treatment of lysosomal storage diseases. *Drugs* 62, 733-742.
- Schmidt, H., Otto, M., Niedmann, P., Cepek, L., Schröter, A., Kretzschmar, H.A., Poser, S., 2004. CSF lactate dehydrogenase activity in patients with creutzfeldt-jakob disease

- exceeds that in other dementias. *Dement. Geriatr. Cogn. Disord.* 17, 204-206.
- Su, J.H., Anderson, A.J., Cummings, B.J., Cotman, C.W., 1994. Immunohistochemical evidence for apoptosis in Alzheimer's disease. *Neuroreport* 5, 2529-2533.
- Suzuki, Y., Oshima, A., Nanba, E., 2001. β -Galactosidase deficiency (β -galactosidosis): GM1 gangliosidosis and Morquio B disease. In: Scriver, C.R., Beaudet, A.L., Valle, D. Sly, W.S. Childs, B. Kinzler, K.W., Vogelstein, B. Ed., *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York, pp. 3775-3809.
- Tapiola, T., Lehtovirta, M., Pirttilä, T., Alafuzoff, I., Riekkinen, P., Soininen, H., 1998. Increased aspartate aminotransferase activity in cerebrospinal fluid and Alzheimer's disease. *Lancet* 352, 287.
- Tessitore, A., del Pilar Martin, M., Sano, R., Ma, Y., Mann, L., Ingrassia, A., Laywell, E.D., Steindler, D.A., Hendershot, L.M., d'Azzo, A., 2004. GM1-ganglioside-mediated activation of the unfolded protein response causes neuronal death in a neurodegenerative gangliosidosis. *Mol. Cell.* 15, 753-766.
- van der Voorn, J.P., Kamphorst, W., van der Knaap, M.S., Powers, J.M., 2004. The leukoencephalopathy of infantile GM1 gangliosidosis: Oligodendrocytic loss and axonal dysfunction. *Acta Neuropathol. (Berl)* 107, 539-545.
- Wada, R., Tiffet, C.J., Proia, R.L., 2000. Microglial activation precedes acute neurodegeneration in Sandhoff disease and is suppressed by bone marrow transplantation. *Proc. Natl. Acad. Sci. USA.* 97, 10954-10959.
- Williams, M.A., McCluer, R.H., 1980. The use of Sep-pak C18 cartridges during the isolation of gangliosides. *J. Neurochem.* 35, 266-269.
- Wu, Y.P., Proia, R.L., 2004. Deletion of macrophage-inflammatory protein 1 α retards

- neurodegeneration in Sandhoff disease mice. *Proc. Nat. Acad. Sci. USA.* 101, 8425-8430.
- Yamaguchi, A., Katsuyama, K., Nagahama, K., Takai, T., Aoki, I., Yamanaka, S., 2004. Possible role of autoantibodies in the pathophysiology of GM2 gangliosidoses. *J. Clin. Invest.* 113, 200-208.
- Yamanaka, T., Hirabayashi, Y., Koketsu, K., Higashi, H., Matsumoto, M., 1987. Highly sensitive analysis of gagliosides in human cerebrospinal fluid with neurological diseases. *Jpn. J. Exp. Med.* 57, 131-135.
- Yamato, O., Endoh, D., Kobayashi, A., Masuoka, Y., Yonemura, M., Hatakeyama, A., Satoh, H., Tajima, M., Yamasaki, M., Maede, Y., 2002a. A novel mutation in the gene for canine acid β -galactosidase that causes GM1-gangliosidosis in Shiba dogs. *J. Inherit. Metab. Dis.* 25, 525-526.
- Yamato, O., Masuoka, Y., Yonemura, M., Hatakeyama, A., Kobayashi, A., Satoh, H., Nakayama, M., Ochiai, K., Umemura, T., Maede, Y., 2003. Clinical and clinico-pathologic characteristics of Shiba dogs with a deficiency of lysosomal acid β -galactosidase: A canine model of human GM1-gangliosidosis. *J. Vet. Med. Sci.* 65, 213-217.
- Yamato, O., Jo, E.O., Shoda, T., Yamasaki, M., Maede, Y., 2004a. Rapid and simple mutation screening of GM1-gangliosidosis in Shiba dogs by direct amplification of deoxyribonucleic acid from various forms of canine whole-blood specimens. *J. Vet. Diagn. Invest.* 16, 469-472.
- Yamato, O., Kobayashi, A., Satoh, H., Endoh, D., Shoda, T., Masuoka, Y., Hatakeyama, A., Jo, E.O., Asano, T., Yonemura, M., Yamasaki, M., Maede, Y., 2004b. Comparison of polymerase chain reaction-restriction fragment length polymorphism assay and enzyme assay for diagnosis of GM1-gangliosidosis in Shiba dogs. *J. Vet. Diagn. Invest.* 16,

299-304.

Yamato, O., Matsuki, N., Satoh, H., Inaba, M., Ono, K., Yamasaki, M., Maede, Y., 2002b.

Sandhoff disease in a golden retriever dog. *J. Inherit. Metab. Dis.* 25, 319-320.

Yamato, O., Matsunaga, S., Takata, K., Uetsuka, K., Satoh, H., Shoda, T., Baba, Y.,

Yasoshima, A., Kato, K., Takahashi, K., Yamasaki, M., Nakayama, H., Doi, K., Maede, Y.,

Ogawa, H., 2004c. GM2-gangliosidosis variant 0 (Sandhoff-like disease) in a family of

Japanese domestic cats. *Vet. Rec.* 155, 739-744.

Yamato, O., Ochiai, K., Masuoka, Y., Hayashida, E., Tajima, M., Omae, S., Iijima, M.,

Umemura, T., Maede, Y., 2000. GM1 gangliosidosis in shiba dogs. *Vet. Rec.* 146,

493-496.

Yamato, O., Satoh, H., Matsuki, N., Ono, K., Yamasaki, M., Maede, Y., 2004d. Laboratory

diagnosis of canine GM2-gangliosidosis using blood and cerebrospinal fluid. *J. Vet. Diagn.*

Invest. 16, 39-44.

Figure legends

Fig. 1 – Changes in GM1 ganglioside concentration in the cerebrum (closed circles), cerebellum (closed triangles) and spinal cord (closed squares) in shiba dogs with GM1 gangliosidosis. GM1 ganglioside concentration was expressed as nmol of *N*-acetylneuraminic acid (NANA) per mg of protein.

Fig. 2 – Changes in GM1 ganglioside concentration in cerebrospinal fluid (CSF) in affected dogs (n=4, solid lines with closed circle), control dogs (n=3, dotted lines with closed squares) and an affected dog treated with prednisolone (solid lines with open circles). Vertical bars represent standard deviations. **P* < 0.05, ***P* < 0.01, †*P* < 0.005 and ††*P* < 0.001 vs control by 1-way factorial ANOVA with post hoc tests.

Fig. 3 – Changes in aspartate aminotransferase (AST) activity in cerebrospinal fluid (CSF) in affected dogs (n=4, solid lines with closed circle), control dogs (n=3, dotted lines with closed squares) and an affected dog treated with prednisolone (solid lines with open circles). Vertical bars represent standard deviations. **P* < 0.05, ***P* < 0.01, †*P* < 0.005 and ††*P* < 0.001 vs control by 1-way factorial ANOVA with post hoc tests.

Fig. 4 – Changes in lactate dehydrogenase (LDH) activity in cerebrospinal fluid (CSF) in affected dogs (n=3, solid lines with closed circle), control dogs (n=3, dotted lines with closed squares) and an affected dog treated with prednisolone (solid lines with open circles). Vertical bars represent standard deviations. **P* < 0.05, ***P* < 0.01 and †*P* < 0.005 vs control by 1-way factorial ANOVA with post hoc tests.

Fig. 5 – Changes in neuron-specific enolase (NSE) concentration in cerebrospinal fluid (CSF) in affected dogs (n=4, solid lines with closed circle), control dogs (n=3, dotted lines with closed squares) and an affected dog treated with prednisolone (solid lines with open circles). Vertical bars represent standard deviations. * $P < 0.05$, ** $P < 0.01$, † $P < 0.005$ and †† $P < 0.001$ vs control by 1-way factorial ANOVA with post hoc tests.

Fig. 6 – Changes in myelin basic protein (MBP) concentration in cerebrospinal fluid (CSF) in affected dogs (n=4, solid lines with closed circle), control dogs (n=3, dotted lines with closed squares) and an affected dog treated with prednisolone (solid lines with open circles). Vertical bars represent standard deviations. * $P < 0.05$, ** $P < 0.01$, † $P < 0.005$ and †† $P < 0.001$ vs control by 1-way factorial ANOVA with post hoc tests.

Table 1. Candidate biomarkers of neuronal degeneration in cerebrospinal fluid in dogs with GM1 gangliosidosis.

Candidate biomarkers	Affected dogs ^a	Normal dogs ^b
Nucleated cell count (/μl)	7.4 ± 7.2 ^c	2.0 ± 1.1
Total protein (mg/dl)	19.2 ± 7.8	17.8 ± 2.4
Sodium (mmol/l)	151.6 ± 3.0	152.3 ± 2.2
Potassium (mmol/l)	3.0 ± 0.2	3.1 ± 0.1
Chloride (mmol/l)	113.8 ± 3.0	114.0 ± 1.3
Calcium (mg/dl)	5.8 ± 1.8	5.2 ± 0.2
Inorganic phosphorus (mg/dl)	1.4 ± 0.4	0.7 ± 0.7
Glucose (mg/dl)	74.0 ± 8.8	66.3 ± 5.2
Creatinine (mg/dl)	0.5 ± 0.1	0.5 ± 0.0
Alkaline phosphatase (U/l)	2.6 ± 0.9	1.8 ± 0.4
Alanine aminotransferase (U/l)	3.0 ± 0.7	3.0 ± 1.7
Aspartate aminotransferase (U/l)	154.8 ± 29.8 ^{††}	16.8 ± 4.5
Creatinine kinase (U/l)	13.2 ± 10.9	3.8 ± 3.3
Lactate dehydrogenase (U/l)	23.2 ± 17.6 [*]	2.0 ± 1.2

^a Mean ± standard deviation of 5 affected shiba dogs (4 to 10 months old).

^b Mean ± standard deviation of 6 normal beagle dogs (9 months to 5 years old).

^c Data of nucleated cell count in affected dogs were collected from three dogs.

^{*} $P < 0.05$ and ^{††} $P < 0.001$ between the two groups by Student *t*-test.











