TABLE I. Morquio B Mutations Reported in the Literature

Nucletide	Amino acid/mRNA	References
c.Sp2[a]	p.Ins32bp;ex2/3	Ishii et al. [1995]
c.247T > C	p.Y83H	Ishii et al. [1995]
c.248A > G	p.Y83C	Santamaria et al. [2006]
c.808T > G[b]	p.Y270D	Paschke et al. [2001]
c.817-818TG > CT[b]	p.W273L	Oshima et al. [1991]
c.817T > C	p.W273R	Gucev et al.
c.841C > T[b]	p.H281Y	Paschke et al. [2001]
c.1223A > G[b]	p.Q408P	Paschke et al. [2001]
c.1313G > A	p.G438E	Hinek et al. [2000]
c.1331A > G[c]	p.Y444C	Santamaria et al. [2006]
c.1444C > T	p.R482C	Ishii et al. [1995]
c.1452C > A	p.N484K	Hinek et al. [2000]
c.1480G > A[c]	p.G494S	Santamaria et al. [2006]
c.1498A > G	p.T500A	Hinek et al. [2000]
c.1527G > T	p.W509C	Oshima et al. [1991]

[a]G → A transition in the acceptor region of the intron 2.
[b]The corrected base number based on A in the initial ATG as #1.
[c]Heterozygous mutation in one patient [p.Y444C/p.G494S].

between adult G_{M1}-gangliosidosis and Morquio B. The reports of gene mutations in Morquio B disease are summarized in Table I. Some of them may not be specific to this clinical phenotype, as described above for the p.R201H mutation. Based on this short review, we conclude that the molecular pathogenesis of Morquio phenotype is not well elucidated at present.

This case had an abnormal heteroduplex pattern of exon 8, with a previously reported p.H281Y mutation and a novel p.W273R mutation. We attempted an in vitro chaperone experiment for the implementation of a possible therapeutic approach to this patient. The chaperone compound *N*-octyl-4-epi-β-valienamine (NOEV) [Matsuda et al., 2003] was added to culture medium of the patient's skin fibroblasts. Unfortunately, the cells expressing the p.W273R/p.H281Y mutations did not respond to NOEV. In previous studies, cultured cells from Morquio B patients with mutations have responded to NOEV. This phenomenon is a mutation-dependent intracellular event [Iwasaki et al., 2006; Suzuki, 2006]. At present, we have not identified any direct molecular interactions between the chaperone compounds and mutant enzyme proteins. It is possible that the chaperone effect may be induced by additional modification(s) of the experimental conditions, even in the apparently NOEV-nonresponsive mutants. However, at present this patient is not an appropriate candidate for chaperone therapy under the current experimental therapeutic conditions.

ACKNOWLEDGMENTS

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A sensitive and reproducible fluorescent-based HPLC assay to measure the activity of acid as well as neutral β-glucocerebrosidases

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ABSTRACT

The activity of lysosomal acid β-glucocerebrosidase (AGC, EC 3.2.1.45), which hydrolyzes the O-glycosidic linkage between D-glucose and ceramide of glucosylceramide (GlcCer), is a marker for the diagnosis of Gaucher disease because the disease is caused by dysfunction of AGC due to mutations in the gene. The activity of AGC is potently inhibited by conduritol B epoxide (CBE), whereas CBE-insensitive nonlysosomal neutral β-glucocerebrosidase (NGC) activities have been found in various vertebrates, including humans. We report here a new reliable method to determine AGC as well as NGC activities using normal-phase high-performance liquid chromatography (HPLC) and NBD (4-nitrobenzo-2-oxa-1,3-diazole)- or BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-labeled GlcCer as a substrate. The reaction products of the enzymes, C6-NBD-ceramide and C12-BODIPY-ceramide, were clearly separated from the corresponding substrates on a normal-phase column within 5 min using a different solvent system. Reaction products could be detected quantitatively at concentrations ranging from 50 fmol to 50 pmol for C6-NBD-ceramide and from 10 fmol to 5 pmol for C12-BODIPY-ceramide. V_{max}/K_m values of human fibroblast AGC for fluorescent GlcCer were much higher than those for 4-methylumbelliferyl-β-D-glucoside (4MU-Glc), which is used prevalently for Gaucher disease diagnosis. As a result, AGC activity was detected quantitatively using fluorescent GlcCer, but not 4MU-Glc, using 5 μl of human serum or 1×10^4 cultured human fibroblasts. The current method clearly showed the decrease of AGC activities in fibroblasts and serum from the patient with Gaucher disease compared with normal individuals, suggesting that the method is applicable for the diagnosis of Gaucher disease. Furthermore, this method was found to be useful for measuring the activities of nonlysosomal NGC of various cells and tissues in the presence of CBE.

Gaucher disease (GD),[1] the most common lysosomal glycolipid storage disease, is caused by mutations in the gene encoding acid β-glucocerebrosidase (AGC, EC 3.2.1.45, GBA1), resulting in the lysosomal accumulation of glucosylceramide (GlcCer) [1,2]. GD has been divided into three major subtypes—types 1, 2, and 3—although they are possibly a continuum of the disease state [3]. The disease is characterized by a marked decrease of AGC activity in the liver, brain, spleen, fibroblasts, and leukocytes [4]. Enzyme activity is reduced due to mutations that affect the turnover, substrate affinity, or activator binding of the enzyme [5]. AGC is specifically and irreversibly inhibited by conduritol B epoxide (CBE). Recently, two nonlysosomal CBE-insensitive neutral β-glucocerebrosidases (NGCs) have been identified: GBA2, known as a bile acid β-glucosidase [6,7], and GBA3, known as a klotho-related protein (KLrP) [8]. The former is a membrane-bound enzyme, whereas the latter is a cytosolic soluble enzyme, but the physiological roles of the two enzymes remain to be elucidated.

The initial diagnosis of GD is usually performed by measuring the AGC activity of lymphocytes or fibroblasts using 4-methylumbelliferyl-β-D-glucoside (4MU-Glc) as a substrate [9,10]. In the assay, the amount of 4MU released is measured using a microplate reader equipped with a fluorescence detector. This assay

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[1] *Abbreviations used:* GD, Gaucher disease; AGC, acid β-glucocerebrosidase; GlcCer, glucosylceramide; CBE, conduritol B epoxide; NGC, neutral β-glucocerebrosidase; KLrP, klotho-related protein; 4MU-Glc, 4-methylumbelliferyl-β-D-glucoside; FACS, fluorescence-activated cell sorter; HPLC, high-performance liquid chromatography; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; Cer, ceramide; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; Glc, D-glucose; DMEM, Dulbecco's minimal essential medium; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid.

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method is simple and rapid but could not exclude the contamination of other β-glucosidases capable of degrading the artificial substrate but not GlcCer. The assay using radioisotope-labeled GlcCer is specific and sensitive but requires a specific facility and expensive substrates. Furthermore, separation of the digestive product (radioactive ceramide) from the substrate (radioactive GlcCer) by liquid-phase extraction could not exclude the contamination of both radioactive substances. The measurement of AGC activity using a fluorescence-activated cell sorter (FACS) and 5-pentafluorobenzoylamino-fluorescein di-β-D-GlcCer as a substrate has also been reported [11], but it requires specific equipment and experience.

We previously reported the usefulness of the fluorescence-based high-performance liquid chromatography (HPLC) method to determine the activity of synthases for GlcCer as well as lactosylceramide, showing that the method is useful for evaluating both activities in gene-knockdown experiments [12]. In the current study, we extended the fluorescence-based HPLC method to evaluate the activities of β-glucocerebrosidases. We report here a fast, sensitive, and reproducible method to measure the activities of AGC and NGC. The assay of AGC by the current method was found to be useful for the diagnosis of GD.

Materials and methods

Materials

Human fibroblasts and human serum were obtained from the Faculty of Medicine at Tottori University (Japan). C6-NBD-Cer and C12-BODIPY-GlcCer were purchased from Invitrogen (USA). 4MU-Glc and C6-NBD-GlcCer were obtained from Sigma (USA). All other reagents were of the highest purity available.

Cell culture

Human fibroblasts were grown at 37 °C in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum and 60 μg/ml kanamycin in a humidified incubator containing 5% CO_2.

Protein assay

The amount of protein was measured using the bicinchoninic acid protein assay (Pierce, USA) with bovine serum albumin as the standard [13].

Preparation of lysates from human fibroblasts and human serum

To prepare the lysates of human fibroblasts (from three normal individuals and seven GD patients) [14], cells attached to the culture plate were scraped off with a cell scraper and collected in a 1.5-ml tube by centrifugation. The cell pellets were rinsed with phosphate-buffered saline (PBS), suspended in sterile water, and then lysed by sonication. Human serum was prepared by centrifugation (3000*g* for 30 min). Serum from two patients with GD was included.

Fractionation of rat brain

Rat brain was homogenized in 0.25 M sucrose in the presence of 1 mM ethylenediaminetetraacetic acid (EDTA), and the homogenate was centrifuged at 5000*g* for 10 min. The supernatant obtained was further centrifuged at 10,000*g* for 15 min to precipitate the lysosomal fraction. The supernatant was centrifuged at 105,000*g* for 60 min to obtain the cytosol fraction (supernatant) and the microsome fraction (precipitate).

HPLC-based AGC assay using fluorescent GlcCer analogs as the substrate

For AGC assay, 20 μl of reaction mixture contains 0.6% sodium taurocholate, 0.25% Triton X-100, and 2.5 μM C6-NBD-GlcCer or 0.25 μM C12-BODIPY-GlcCer in 50 mM phosphate–citrate buffer (pH 5.0). Standard assays were carried out at 37 °C for 1 h. The reaction was stopped by adding 200 μl of chloroform/methanol (2:1, v/v). After vortexing for a few seconds, the reaction mixture was centrifuged. After the organic phase had been dried up, lipids were dissolved in 200 μl of isopropyl alcohol/*n*-hexane/H_2O (55:44:1) and then transferred to a glass vial in an autosampler (Hitachi L-7200). Fluorescence was determined using a fluorescence detector (Hitachi L-7480) set to excitation and emission wavelengths of 470 and 530 nm, respectively, for the assay using C6-NBD-GlcCer and of 505 and 540 nm, respectively, for the assay using C12-BODIPY-GlcCer. An aliquot of sample (100 μl each) was automatically loaded onto a normal-phase column (Inertsil SIL 150A-5, 4.6 × 250 mm, GL Sciences, USA) and eluted with isopropyl alcohol/*n*-hexane/H_2O (55:44:1) for the assay using C6-NBD-GlcCer as a substrate and with isopropyl alcohol/*n*-hexane/H_2O (55:85:51) for the assay using C12-BODIPY-GlcCer as a substrate at a flow rate of 2.0 ml/min. Column was washed with isopropyl alcohol after runs if necessary. Fluorescent peaks were identified by comparing their retention times with those of standards. C12-BODIPY-Cer was prepared from C12-BODIPY-SM by treatment with *Bacillus cereus* sphingomyelinase.

HPLC-based NGC assay using fluorescent GlcCer analogs as the substrate

For the NGC assay, 20 μl of reaction mixture contained 0.25% sodium cholate, 1 mM CBE, and 2.5 μM C6-NBD-GlcCer in 50 mM Hepes buffer (pH 7.0). Standard assays were carried out at 37 °C for 1 h. The reaction was stopped by adding 200 μl of chloroform/methanol (2:1, v/v). After vortexing for a few seconds, the reaction mixture was centrifuged. After the organic phase had been dried up, lipids were dissolved in 200 μl of isopropyl alcohol/*n*-hexane/H_2O (55:44:1) and then transferred to a glass vial in an autosampler (Hitachi L-7200).

Microplate reader-based AGC assay using 4MU-Glc as a substrate

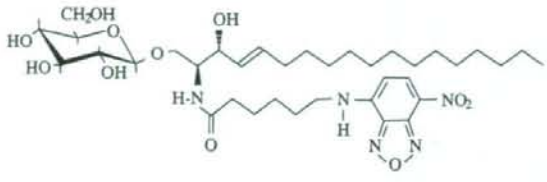
The reaction mixture (20 μl) contained 0.6% sodium taurocholate, 0.25% Triton X-100, and 2.5 mM 4MU-Glc in 50 mM phosphate–citrate buffer (pH 5.0). Standard assays were carried out at 37 °C for 1 h. The reaction was stopped by adding 180 μl of 150 mM glycine/NaOH (pH 10.5). The contents of the tube were transferred to a Microtest 96-well assay plate with a black flat bottom (BD Falcon). Fluorescence was determined using an ARVO MX 1420 multilabel counter (PerkinElmer, USA) set to excitation and emission wavelengths of 340 and 460 nm, respectively.

Results

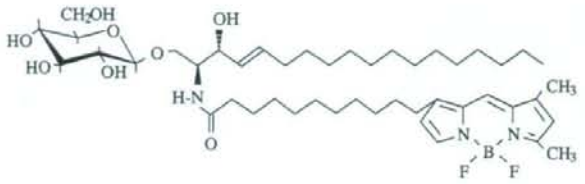
Determination of AGC activity using HPLC and fluorescent substrates

AGC activity is usually measured by a fluorescent microplate reader using 4MU-Glc as a substrate; however, this assay could not exclude contamination by other β-glucosidase activities that hydrolyze the artificial substrate but not GlcCer. Thus, in this study, fluorescent GlcCer analogs were used as a substrate instead of 4MU-Glc. The structure of these fluorescent GlcCer analogs and 4MU-Glc is shown in Fig. 1. To achieve good separation and detection of the fluorescent substrates and products,

C6-NBD-GlcCer



C12-BODIPY-GlcCer



4MU-Glc

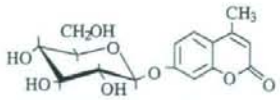


Fig. 1. Structures of C6-NBD-GlcCer, C12-BODIPY-GlcCer, and 4MU-Glc. Shown are the structures of commercially available fluorescent substrates used in this study.

normal-phase HPLC with a fluorescence detector was employed. C6-NBD-GlcCer (substrate) and C6-NBD-Cer (product) were clearly separated on a normal-phase column within 5 min using isopropyl alcohol/*n*-hexane/H_2O (55:44:1, v/v) as a mobile phase at a flow rate of 2.0 ml/min (Fig. 2A), providing suitable conditions for the assay of AGC using C6-NBD-GlcCer as a substrate. Under these conditions, however, C12-BODIPY-GlcCer (substrate) and C12-BODIPY-Cer (product) could not be separated (Fig. 2A). Among several solvents examined, isopropyl alcohol/*n*-hexane/H_2O (55:85:1) was selected as a mobile phase for the assay using

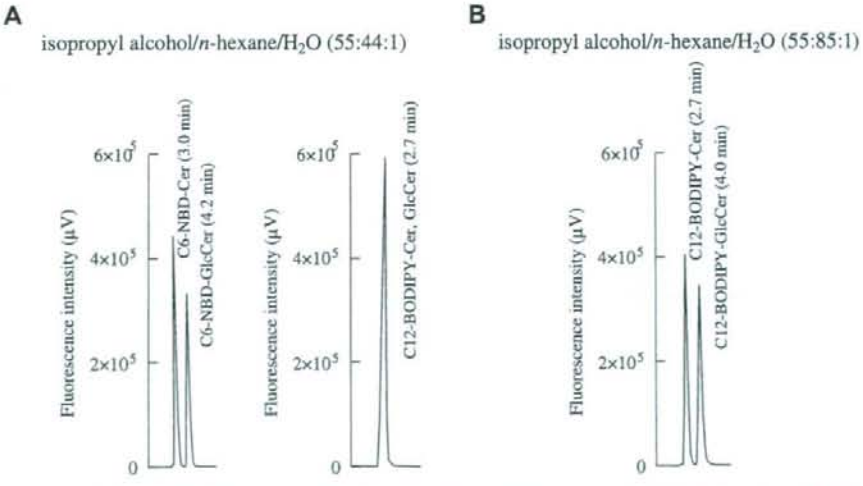


Fig. 2. Detection of fluorescence-labeled Cer and GlcCer analogs on normal-phase HPLC. Shown are profiles of HPLC of C6-NBD-Cer and C6-NBD-GlcCer (50 pmol each) (A) and C12-BODIPY-Cer and C12-BODIPY-GlcCer (5 pmol each) (B). Substrates and products were separated on a normal-phase column (Inertsil SIL 150A-5) using HPLC. Fluorescence was detected with a fluorescent detector. Samples were eluted on HPLC with isopropyl alcohol/*n*-hexane/H_2O (55:44:1) (A) or isopropyl alcohol/*n*-hexane/H_2O (55:85:1) (B) at a flow rate of 2 ml/min at room temperature.

C12-BODIPY-GlcCer as a substrate, by which C12-BODIPY-Cer was clearly separated from C12-BODIPY-GlcCer within 5 min (Fig. 2B).

Dose–response curves using different amounts of fluorescent products

To evaluate the detection limits of assays using 4MU-Glc and fluorescent GlcCer analogs as a substrate, the relationships between the amounts of fluorescent products and fluorescent intensities were examined (see Supplemental Fig. 1 in supplementary material). Fluorescence measured by microplate reader was linear, from 0.1 to 5 nmol of the reaction product 4MU. On the other hand, fluorescent products were detectable by HPLC with a fluorescence detector linearly from 50 fmol to 50 pmol for C6-NBD-Cer and from 10 fmol to 5 pmol for C12-BODIPY-Cer. Collectively, it was concluded that the HPLC-based method using fluorescent substrates provided a sensitive and wide-ranging assay for AGC compared with the microplate reader-based assay using 4MU-Glc as a substrate.

Determination of AGC activity in human fibroblasts using different fluorescent substrates

To evaluate the HPLC-based assay using fluorescent GlcCer analogs, AGC activity in the lysates of human fibroblasts was measured. The decrease of AGC activity in fibroblasts is the primary marker for the diagnosis of GD. First, lysates of human fibroblasts from healthy volunteers were used as an enzyme source of AGC. When the incubation time was fixed at 0.5 h, a linear relationship between the amount of cell lysates and the generation of fluorescent products was observed from 0.25 to 5 μg of total protein for the HPLC-based assay using C6-NBD-GlcCer (Fig. 3A) or C12-BODIPY-GlcCer (Fig. 3B) and from 1 to 20 μg for the microplate reader-based assay using 4MU-Glc (Fig. 3C). The reactions seemed to proceed linearly up to 1 h when total protein was fixed at 2.5 μg and C6-NBD-GlcCer (Fig. 3D) or C12-BODIPY-GlcCer (Fig. 3E) was used as a substrate. On the other hand, linearity was maintained for 1 h using 20 μg protein and 4MU-Glc as a substrate (Fig. 3F). Collectively, the HPLC-based assay using fluorescent GlcCer was found

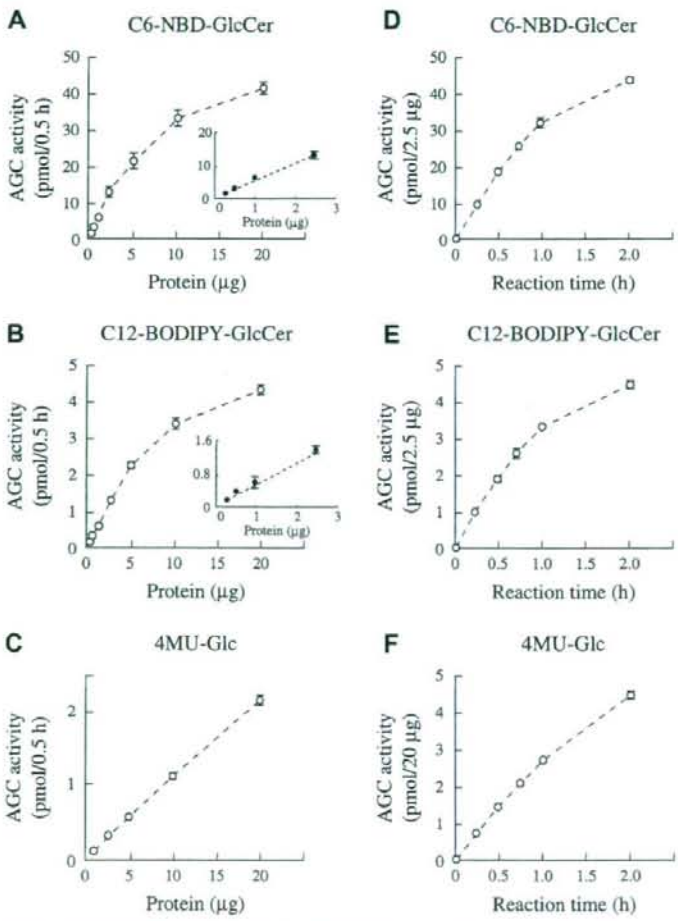


Fig. 3. Determination of the activities of AGC in human fibroblasts using C6-NBD-GlcCer, C12-BODIPY-GlcCer, and 4MU-Glc. In panels A, B, and C, AGC activities were determined by the HPLC-based assay (A, B) or the microplate reader-based assay (C) using various amounts of lysates of human fibroblasts. The enzyme reaction was carried out at 37 °C for 0.5 h. In panels D, E, and F, reaction mixtures containing 2.5 μg total protein (D, E) or 25 μg total protein (F) were incubated at 37 °C for the periods indicated. Values are the averages of three independent experiments with standard deviations.

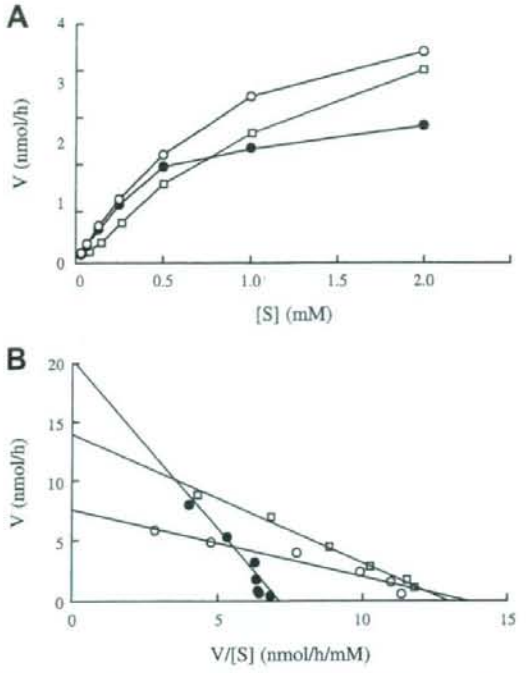


Fig. 4. Kinetic parameters of AGC toward fluorescence substrates. (A) Substrate saturation curves of AGC activity of human fibroblasts were determined using various fluorescence substrates. White circles: C6-NBD-GlcCer; black circles: C12-BODIPY-GlcCer; white squares: 4MU-Glc. Reaction mixtures containing 2.5 μg total protein (C6-NBD-GlcCer and C12-BODIPY-Glc) or 25 μg total protein (4MU-Glc) were incubated at 37 °C for 1 h. (B) Kinetic parameters for three substrates calculated according to Eadie–Hofstee plots. Values are the means of duplicate determinations.

to be more sensitive to determine AGC activity than the microplate reader-based assay using 4MU-Glc as a substrate.

Kinetic parameters of AGC toward three different fluorescent substrates

To examine why the AGC assay using GlcCer analogs seems to be more sensitive than 4MU-Glc substrates, we determined the kinetic parameters of AGC for these fluorescent substrates. Human fibroblast lysates from healthy adults were used as an enzyme source of AGC. Substrate saturation curves for AGC were generated using C6-NBD-GlcCer, C12-BODIPY-GlcCer, and 4MU-Glc as a substrate (Fig. 4A), and kinetics parameters for three substrates were calculated according to Eadie–Hofstee plots (Fig. 4B). Interestingly, the apparent V_{max}/K_m values of AGC for C6-NBD-GlcCer and C12-BODIPY-GlcCer were much higher than those for 4MU-Glc (Table 1), indicating that these fluorescent GlcCer substrates were much more suitable for the AGC assay than was 4MU-Glc.

Table 1
Comparison of K_m and V_{max} values for various fluorescent substrates

	Substrate	K_m (mM)	V_{max} (nmol/h)	V_{max}/K_m (nmol/h/mM)
AGC	C6-NBD-GlcCer	1.10	14.16	12.84
	C12-BODIPY-GlcCer	0.57	7.72	13.60
	4MU-Glc	2.92	20.55	7.03

Note. The kinetic parameters of AGC from lysates of human fibroblasts toward various substrates were calculated using Eadie–Hofstee plots. Values are the means of duplicate determinations.

Measurement of AGC activities in human fibroblasts and serum from normal individuals and GD patients

AGC activities of fibroblasts and serum from normal individuals and GD patients were measured by an HPLC-based method using fluorescent GlcCer analogs and a microplate-based method using 4MU-Glc. As shown in Fig. 5, a decrease of AGC activities in lysates of fibroblasts from GD patients was clearly detected compared with those of normal individuals when either the HPLC-based assay or the microplate reader-based assay was employed. Next, human sera were used as an AGC source instead of fibroblasts because the preparation of serum from patients is much easier and faster than that of lymphocytes or fibroblasts. The AGC assay was carried out at 37 °C for 2 h using 5 μl of serum (~300 μg total protein). Under this condition, AGC activity was clearly detectable by the HPLC-based assay using serum from normal individuals, and the decrease of AGC activity in the sample from GD patients was again clearly confirmed by this method (Fig. 6A and B). On the other hand, the detection of AGC activity in serum from normal individuals by the conventional method using 4MU-Glc is not reliable; that is, some normal individuals showed probable AGC activity, whereas some did not (Fig. 6C).

Collectively, the HPLC-based assay using fluorescent analogs is more reliable to measure AGC activity than is the microplate reader-based assay using 4MU-Glc, which is used prevalently for GD diagnosis.

Determination of NGC activities using C6-NBD-GlcCer as a substrate

To examine whether the HPLC-based assay using fluorescent GlcCer analogs is useful for the determination of NGC activity, we measured the activity of glucocerebrosidase of human fibroblast lysates under acidic (pH 5.0) and neutral (pH 7.0) conditions in either the presence or absence of CBE using C6-NBD-GlcCer as a substrate. The release of C6-NBD-Cer under acidic conditions was strongly inhibited in the presence of CBE, whereas CBE-insensitive activity was detected under neutral conditions. Under both conditions, no C6-NBD-Cer was detected when the enzyme assay was performed using boiled (100 °C for 5 min) lysates (Fig. 7A and B). NGC activity was detected in rat brain (data not shown), mouse brain (data not shown), pig brain, zebrafish embryo, and pufferfish liver (Fig. 7C), but not in lysates of slime mold *Dictyostelium discoideum* (data not shown). Interestingly, NGC activity was detected mainly in cytosol and microsome fractions when rat brains were fractionated using a sucrose gradient. The former activity seems to be derived from KLrP (GBA3), whereas the latter activity seems to be derived from GBA2. Low activity was also found in the lysosome fraction under neutral conditions in the presence of 1 mM CBE, but this could stem from residual activity of GBA1, suggesting that NGC activity was not able to be completely separated from AGC activity under the conditions used in this study. Decreased NGC activity was also found in fibroblast lysates from GD patients when compared with healthy volunteers, and the tendency of the decrease was very similar to that for AGC activity (data not shown). However, it is unclear whether the apparent decrease of NGC activity in fibroblasts under the conditions used was due to the decrease of activity of NGC, AGC, or both. NGC activity was not detected in serum from normal volunteers or GD patients.

Discussion

This article clearly shows that the HPLC-based assay using commercially available fluorescent GlcCer analogs is far supe-

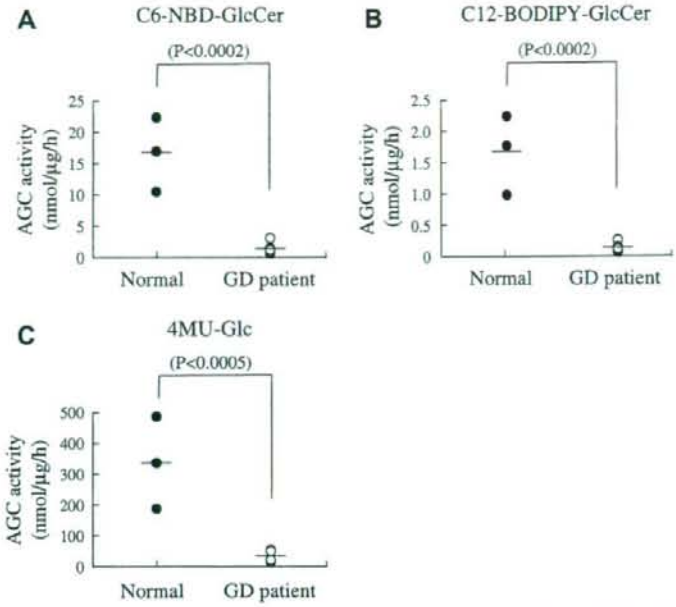


Fig. 5. AGC activities in human fibroblasts from normal individuals and GD patients. AGC activities in the lysates of human fibroblasts from normal individuals ($n = 3$) and GD patients ($n = 7$) were determined using C6-NBD-GlcCer (A), C12-BODIPY-GlcCer (B), and 4MU-Glc (C). Reaction mixtures containing 2.5 μg protein (C6-NBD-GlcCer and C12-BODIPY-Glc) and 15 μg protein (4MU-Glc) were incubated at 37 °C for 30 min and 1 h, respectively.

rior to the conventional microplate reader-based assay using 4MU-Glc, and it suggests that this superiority may stem from the higher V_{max}/K_m value of fluorescent GlcCer analogs compared with 4MU-Glc. As shown in Table 1, V_{max}/K_m values of C6-NBD-GlcCer and C12-BODIPY-GlcCer for AGC are much higher than those of 4MU-Glc when lysates of human fibroblasts were used

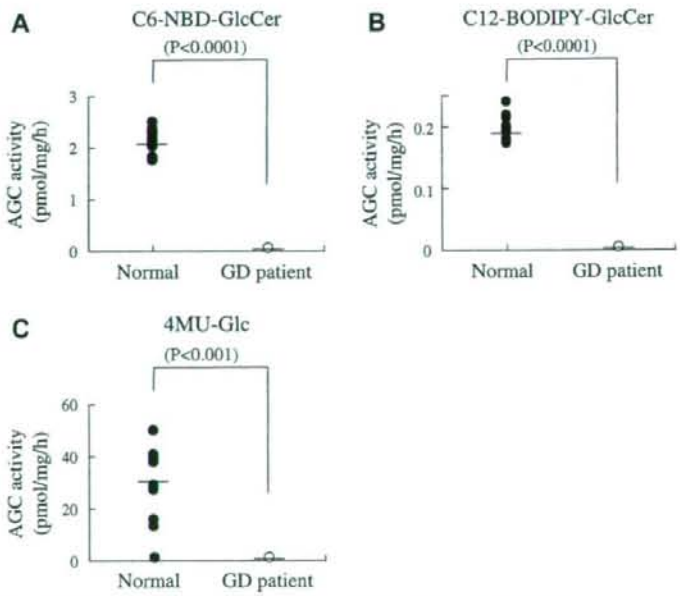


Fig. 6. AGC activities in human serum from normal individuals and GD patients. AGC activities in serum from normal individuals ($n = 10$) and GD patients ($n = 3$) were determined using C6-NBD-GlcCer (A), C12-BODIPY-GlcCer (B), and 4MU-Glc (C). Two of three samples were obtained from the same GD patient but at different periods (6-year interval). Reaction mixtures containing 5 μl of serum (~300 μg protein) were incubated at 37 °C for 2 h.

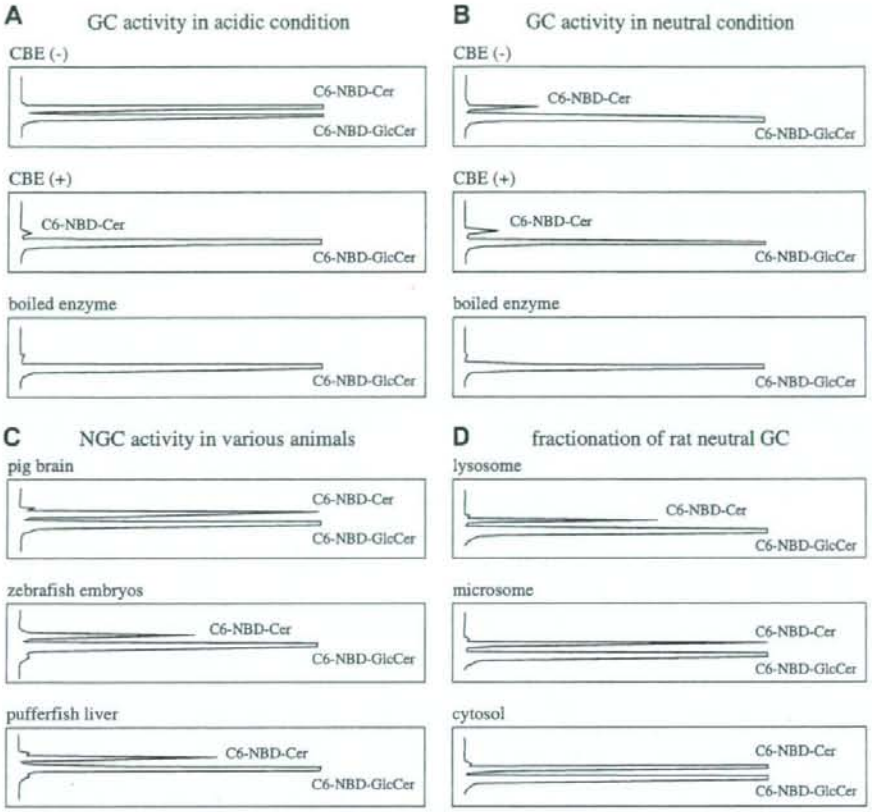


Fig. 7. Determination of the activities of AGC and NGC using C6-NBD-GlcCer. β-Glucocerebrosidase activities, under acidic (A) and neutral (B) conditions, were determined by the HPLC-based assay using lysates of human fibroblasts in the presence or absence of 1 mM CBE. The enzyme reaction was carried out at 37 °C for 1 h using 20 μg total protein as described in Materials and Methods. Shown are assays of NGC activity in lysates of pig brain, zebrafish embryos, and pufferfish liver (C) and rat brain centrifugal fractions (D). Lysates and each fraction were incubated at 37 °C for 1 h with 50 pmol of C6-NBD-GlcCer in 50 mM Hepes buffer (pH 7.0) containing 0.25% sodium cholate in the presence of 1 mM CBE. Fractionation of rat brain was described in Materials and Methods. HPLC runs were performed several times for each experiment, and typical patterns that were very reproducible are shown in panels A–D.

as an AGC source. This may indicate that the acyl side chains of C6-NBD-GlcCer and C12-BODIPY-GlcCer could help substrates to bind better to the enzyme active site. As a result, a decrease of AGC activity was clearly detected using fluorescent GlcCer analogs in fibroblasts as well as serum obtained from patients with GD. It is noteworthy that 4MU-Glc is not suitable to determine a small amount of AGC activity and that the determination of AGC activity in human serum was not reliable when 4MU-Glc was used as a substrate (Fig. 6). Furthermore, GlcCer analogs could be more specific for the determination of AGC and NGC activities than is 4MU-Glc given that the latter substrate is known to be hydrolyzed by various glycosidases, some of which are not able to degrade GlcCer.

An inherited deficiency of GBA1 causes GD, in which GlcCer is accumulated in lysosomes of laden tissue macrophages; however, the accumulation of GlcCer in other cell types is not obvious in patients with GD despite the significant decrease of GBA1 activity, and so participation of other glucocerebrosidases is suspected [1]. The HPLC-based method using fluorescent GlcCer analogs was shown to be useful to determine the activities of not only AGC but also NGC in this study; thus, we compared the NGC activity of healthy volunteers with that of patients with GD. Although CBE-insensitive NGC activity was detected in various tissues of mammals by the current method (Fig. 7), the NGC activity of fibroblasts and sera was very weak, and so the comparison of activity in healthy volunteers and patients with GD was not reliable under the conditions used in this study; thus, the issue of whether NGC participates in GD remains to be elucidated.

Conclusion

The HPLC-based assay using fluorescent GlcCer analogs presented in this study is a rapid, reliable, and specific method to determine the activities of AGC as well as NGC and so should facilitate the study of GlcCer metabolism and GD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2008.07.024.

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Original article

Intracerebral cell transplantation therapy for murine GM1 gangliosidosis

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Abstract

We performed a cell transplantation study to treat the brain involvement in lysosomal storage diseases. We used acid β-galactosidase knock-out mice (BKO) from C57BL/6 as recipients. To minimize immune responses, we used cells derived from transgenic mice of C57BL/6 overexpressing the normal human β-galactosidase. Fetal brain cells (FBC), bone marrow-derived mesenchymal stem cells (MSC), and mixed FBC and MSC cells were prepared and injected into the ventricle of newborn BKO mouse brain. The mice were examined at 1, 2, 4, and 8 weeks and 6 months after injection. In each experiment, the injected cells migrated into the whole brain effectively and survived for at least 8 weeks. Decrease in ganglioside GM1 level was also observed. FBC could survive for 6 months in recipient brain. However, the number of transplanted FBC decreased. In the brains of MSC- or mixed cell-treated mice, no grafted cells could be found at 6 months. To achieve sufficient long-term effects on the brain, a method of steering the immune response away from cytotoxic responses or of inducing tolerance to the products of therapeutic genes must be developed.

Keywords: GM1-gangliosidosis; Cell transplantation; Fetal brain cell; Mesenchymal stem cell

1. Introduction

Enzyme replacement therapy (ERT), hematopoeitic stem cell transplantation (HSCT), and gene transfer have been studied in animals and in humans with lysosomal storage disease (LSD). ERT is now available clinically for Gaucher disease, Fabry disease, Pompe disease, and MPS I, II, and VI in many countries, and has been successful in visceral organs. HSCT is also effective against the somatic involvements in Gaucher disease and MPS I, II, and VI. However, HSCT exhibits little efficacy in conditions such as Fabry disease and Pompe disease, when enzyme secretion from donor cells is poor or the uptake of enzyme proteins by the affected host cells is inadequate. In addition, efficacy in individual organs differs markedly, in both ERT and HSCT, depending on accessibility of blood flow and the density of mannose-6-phosphate receptors. Neither HSCT nor ERT exhibits efficacy against the brain involvement in Gaucher or MPSs because of the poor access due to the blood–brain barrier.

Many experimental studies have been carried out, involving methods such as gene therapy [1–5], cell

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therapy [6–9], or intrathecal administration of enzymes [10,11], for treatment of the brain in LSDs. Such treatments were able to overcome the blood–brain barrier to access brain tissue and exhibit considerable efficacy in brain. However, it is difficult to maintain such efficacy for long periods of time. Repetition of these treatments is not practical because intracranial administration is required for them. On the other hand, the usefulness of intravenous administration is limited because of the blood–brain barrier, except in newborn mice which have an immature barrier. It has been reported that intravenous administration of extremely high doses of enzymes [12–14] or of enzymes that remain in the circulation for long periods [15,16] yielded slight passage through the blood–brain barrier, though with increase in the risk of immune response.

Oral administration of small molecules would be a good and convenient method of treatment of the brain for prolonged periods, such as substrate reduction therapy with *N*-butyldeoxynojirimycin or *N*-butyldeoxygalactonojirimycin for glycosphingolipidoses [17–19] or genistein for mucopolysaccharidoses [20], and chemical chaperone therapy for Fabry disease [21] or GM1-gangliosidosis [22]. However, the efficacy of substrate reduction therapies has thus far been quite limited, and chemical chaperone therapies are not applicable for every type of gene mutation.

GM1 gangliosidosis is an LSD and a progressive neurological disease in humans caused by a genetic defect of lysosomal acid β-galactosidase, which hydrolyses the terminal β-galactosidic residue of ganglioside GM1 and other glycoconjugates. The defects in β-galactosidase activity result in accumulation of ganglioside GM1 in various organs, especially the brain, causing progressive neurodegeneration. In our previous study [2], we injected recombinant adenovirus encoding mouse β-galactosidase cDNA intravenously in β-galactosidase-deficient newborn mice, and showed that vector-mediated β-galactosidase-producing brain cells could reduce ganglioside GM1 accumulation. We showed that β-galactosidase enzyme protein could be secreted as well as taken up by the brain cells and function effectively. However, the efficacy obtained was transient. If sufficient amounts of the defective enzyme could be permanently secreted by cells in the brain, injury of the brain could be prevented. To examine the possibility of long-term cell treatment of the brain in LSDs, we carried out a transplantation experiment in the brain of a GM1-gangliosidosis mouse model (acid β-galactosidase knock-out mouse) using fetal brain cells (FBC) and mesenchymal stem cells (MSC) from bone marrow. These cells used for transplantation were derived from mice of the same genetic background as recipient mice except for possession of the human β-galactosidase gene.

2. Materials and methods

2.1. Knock-out and transgenic mice

A mouse model of GM1 gangliosidosis (BKO mouse) was generated by targeting of the β-galactosidase gene at exon 15 in ES cells as previously described [23]. Newborn mice were obtained by mating heterozygous female mice with homozygous male mice. Identification of newborn mutants was accomplished by quantitative analysis of β-galactosidase activity in tail tip homogenates on the day of birth. Mice with high β-galactosidase activity (TG mice) [24] were generated by introducing the human β-galactosidase gene as a transgene in ES cells obtained from the BKO mouse, which has several copies of the human β-galactosidase gene without the mouse β-galactosidase background. Age-matched wild-type mice of C57BL/6 strain were used as a control.

2.2. Cell preparations for transplantation

Cultured mesenchymal stem cells (MSC) were obtained from the bone marrow of the tibias and femurs of 5–8 month-old TG mice according to the method of Meirelles et al. [25] with some modifications. Dulbecco's modified Eagle's medium (DMEM: Sigma Chemical Co., St Louis, MO) containing 10% fetal bovine serum (Medical and Biological Laboratories, Nagoya, Japan) was used for culture.

Fetal brain cells (FBC) were obtained from the fetal cerebral cortex of TG mice at 13 days of gestation according to the method of Meberg and Miller [26]. The brain tissue was disrupted in a Pasteur glass pipette by gentle stroking several times (uncultured FBC), and then cultured for 4 h in Neurobasal medium (Invitrogen, Carlsbad, CA, No. 12348-07) containing 2 mM glutamine and 10% FBS, followed by two days in Neurobasal medium containing 2 mM glutamine and B27 supplement (Invitrogen, No. 14175-095) (cultured FBC).

2.3. Transplantation of cells into newborn mouse brain

Each BKO mouse received a single injection of 0.5–1.0×10^5 of the cells prepared as described above in the right cerebral ventricle from 24 to 48 hours after birth. Study groups were as follows: uncultured FBC ($n = 18$), cultured FBC ($n = 10$), MSC ($n = 17$), and mixed MSC and FBC (1:1) ($n = 15$). Mice of each experimental group were divided into three subgroups for X-gal staining, β-galactosidase assay and ganglioside GM1 analysis. Mice were examined at one, two, four, and eight weeks and 6 months after injection as shown in Table 1.

For biochemical analysis, mice were anesthetized with diethylether and the blood was washed out with normal saline by perfusion through the heart, and the

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brains were removed and kept at −80 °C until use. For histological studies, the brains were fixed by perfusion through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PB) for 20 min., after washing out the blood with normal saline. To obtain frozen sections, the brains were placed in 0.1 M phosphate buffer pH 7.4 containing 30% sucrose, and frozen in liquid nitrogen.

All surgical and care procedures were carried out in accordance with the Guidelines for Use and Care of Experimental Animals approved by the Animal Committee of Osaka City University School of Medicine.

2.4. X-Gal staining

Frozen sections (16 μm thick) were reacted with X-gal using the β-gal staining Kit (Invitrogen Corp., Carlsbad, CA) to visualize β-galactosidase activity.

2.5. β-Galactosidase assay

β-Galactosidase activity was analyzed in the tissue homogenate with the artificial substrate 2 mM 4-methylumbelliferyl β-galactoside at pH 4.0 in 0.1 M sodium citrate-phosphate buffer according to the method described by Suzuki [27]. Protein was analyzed using the Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA) with the method of Bradford [28].

2.6. Analysis of ganglioside GM1

Amounts of ganglioside GM1 were measured by immunoblot assay using anti-GM1 ganglioside monoclonal antibody (Code: 370685, Seikagaku Corp., Tokyo, Japan) by the method of Michikawa et al. [29] with some modifications.

Brain tissue cells were disrupted by sonication and solubilized in 20 mM Tris–HCl buffer pH 8.0 containing 137 mM NaCl, 10% glycerol, and a protease inhibitor cocktail (Complete, Mini, Cat No. 11836153001, Roche Diagnostics, Mannheim, Germany). Five micrograms of tissue protein was applied onto Trans-Blot Transfer Medium Pure Nitrocellulose Membrane (0.45 μm pore size, Code: 162-0117, Bio-Rad Laboratories) through the slots of a Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories). The membrane was reacted with anti-GM1 ganglioside monoclonal antibody diluted 1:500, after blocking with 5% skim milk in PBS solution for 1 h at room temperature, and then with horseradish peroxidase-linked anti-mouse IgG sheep antibody (Code: NA931, GE Healthcare UK Ltd., Buckinghamshire, UK) diluted 1:1,000. The washing solution used was 0.1 M Tris buffered saline pH 7.5 containing 0.1% Tween 20 (TTBS). Bound antibody was detected using ECL after reaction with ECL™ Western Blotting Detection Reagents (Code: RPN2209, GE Healthcare UK Ltd.) and visualized on X-ray film. Densitometric quantification of immunoreactive signal was performed using the Kodak Digital Science™ EDAS 120 system with 1D Image Analysis software (Eastman Kodak Company, NY). The values obtained were compared with those of quantification of histological immunoreactivity with Leica Control Software as previously described [30], and the same ratios were obtained among the samples (data not shown). The assay was performed three times and in duplicate for each sample independently, and mean values were calculated.

3. Results

3.1. X-Gal staining

Layered staining of the transplanted cells was observed over the entire ventricular surface on both sides of the cerebral hemispheres in treated mice at one week after injection (data not shown). Positive cells had spread into the brain tissue by two weeks (Fig. 1c and f) in the mice treated with cultured FBC ($n = 1$), uncultured FBC ($n = 1$), and MSC ($n = 2$) in the same amounts. The cells had spread further and had reached every part of the brain by 4 weeks in the mice of all experimental groups (Fig. 1d, g and i). Less positive cells were found in the mice treated with MSC ($n = 3$) or mixed MSC and FBC ($n = 3$) (Fig. 1g and i) than in the mice treated with cultured ($n = 3$) or uncultured FBC ($n = 3$) (Fig. 1d). The number of the X-Gal positive cells increased gradually until 4 weeks after injection in every experimental mouse. At 8 weeks after injection, positive cells still existed in the cultured FBC- ($n = 3$) and uncultured FBC-treated ($n = 3$) mice (Fig. 1e) in the same numbers with a similar distribution as at 4 weeks. However, a significant decrease in number of positive cells was found at 8 weeks in the mice treated with MSC ($n = 3$) or mixed MSC and FBC ($n = 3$) (Fig. 1h and j). In the mice treated with mixed MSC and FBC, positive cells existed in higher numbers in deep areas than in the mice treated with MSC alone. In the mice treated with cultured ($n = 2$) and uncultured FBC ($n = 2$), small numbers of positive cells with strong staining still existed in many parts of the brain, especially around the striatum and lateral globs pallidus (Fig. 1k and l), at 6 months after injection. No grafted cells were found in the mice treated with MSC ($n = 1$) or mixed MSC and FBC ($n = 1$) at 6 months. No significant differences were noted among the mice within each experimental group at each stage.

3.2. β-Galactosidase activity

The β-galactosidase activity in FBC and MSC derived from TG mice were 214.5–227.5 nmol/mg/h ($n = 4$) and 143.0–121.4 nmol/mg/h ($n = 3$), respec-

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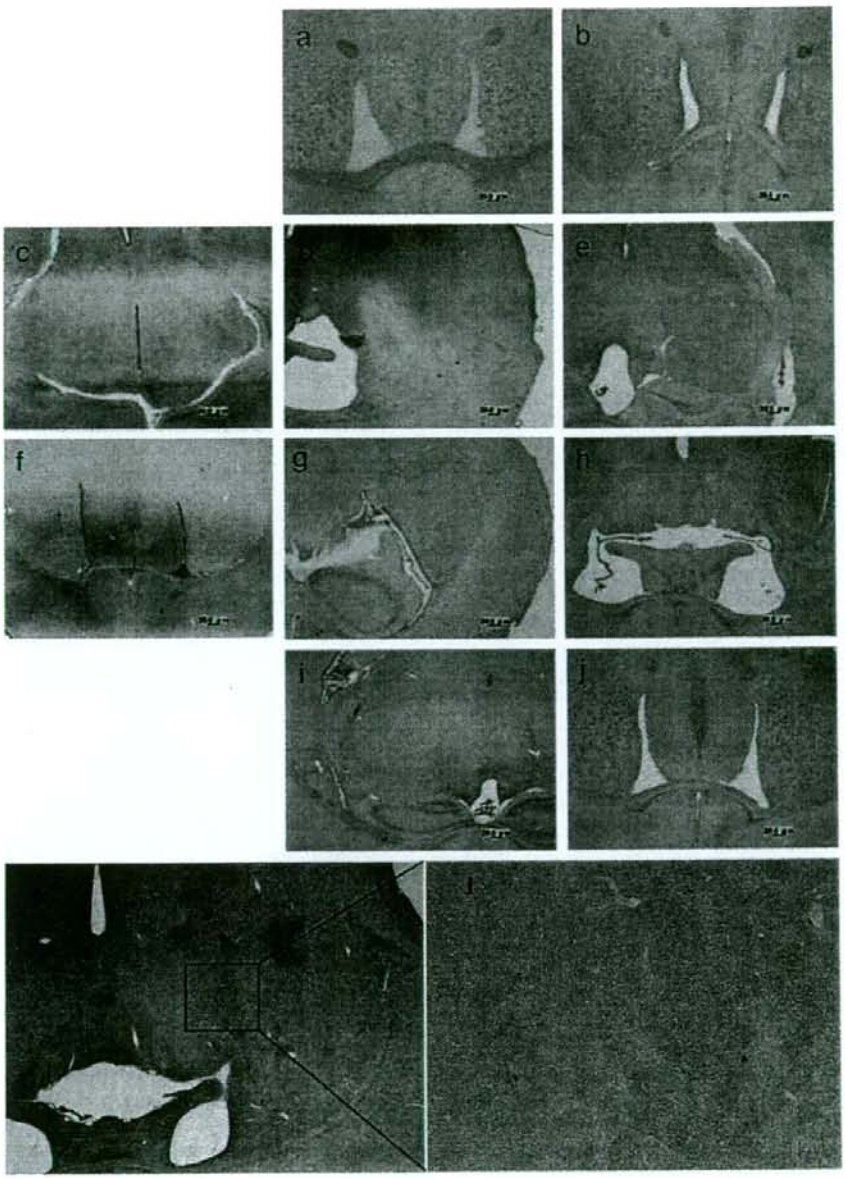

Fig. 1. X-Gal staining of brain coronal sections at +0.8 mm to −2.0 mm of bregma. (a and b) Non-treated BKO mouse at 4 and 8 weeks old, respectively; (c–e) Treated with FBC at 2, 4, and 8 weeks after injection; (f–h) Treated with MSC at 2, 4, and 8 weeks after injection; (i and j) Treated with mixed MSC and FBC at 4 and 8 weeks after injection; (k) FBC-treated brain at 6 months after injection; (l) Magnification of figure k. Positive cells had spread into the brain tissue by two weeks (c and f). The cells had spread further by 4 weeks (d, g and i). Less positive cells were found in the mice treated with MSC or mixed MSC and FBC (g and i) than in the mice treated with FBC (d). At 8 weeks, positive cells still existed in FBC-treated mouse (e) as at 4 weeks (d). A significant decrease in number of positive cells was found at 8 weeks in the mice treated with MSC (h) or mixed MSC and FBC (j). Strong positive staining cells still existed at 6 months in the brain of FBC-treated mouse (k and l).

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tively, while the activity in FBC and in MSC derived from wild-type mice were 54.9–69.1 ($n = 2$) and 63.0 ($n = 1$), respectively.

The results of brain β-galactosidase activity in transplantation experiments are shown in Table 2. Increases in β-galactosidase activity were found in the brains of each experimental group at 4 weeks after injection. Activity in the FBC-treated mice was definitely increased at 4 weeks as well as at 8 weeks, while activity at 8 weeks in the MSC-treated mice and mixed MSC and FBC-treated mice was almost the same level as that in the untreated mice. These findings were consistent with those in the X-Gal staining study.

3.3. Immunoassay of ganglioside GM1

Immunoassay of accumulated ganglioside GM1 was performed for each mouse using anti-GM1 ganglioside monoclonal antibody. Values are ratios to the amounts in age-matched normal control mice. The results are shown in Fig. 2 and Tables 3. At 4 weeks after injection, remarkable decrease in ganglioside GM1 accumulation

Table 1
Mouse numbers used for each experiment.

Time after injection	1 week	2 weeks	4 weeks	8 weeks	6 months
	[X-Gal staining]				
Uncultured FBC	1	1	3	3	2
Cultured FBC	1	1	3	3	2
MSC		2	3	3	1
Mixed MSC and FBC			3	3	1
	[β-galactosidase activity]				
Uncultured FBC			2	2	
Cultured FBC					
MSC			2	2	
Mixed MSC and FBC			2	2	
	[Immunoblot assay of ganglioside GM1 amount]				
Uncultured FBC			1	1	1
Cultured FBC					
MSC			2	2	
Mixed MSC and FBC			2	2	

Table 2
β-Galactosidase activity.

	4 weeks	8 weeks
Age-matched normal control (mean ± SD)	197 ± 61 ($n = 7$)	159 ± 56 ($n = 7$)
Non-treated (mean ± SD)	4.38 ± 0.35 ($n = 5$)	4.10 ± 0.47 ($n = 5$)
Treated with uncultured FBC	Mouse 1	Mouse 7
	Rt: 6.65[a]	Rt: 4.94
	Lt: 5.31[a]	Lt: 6.03[a]
	Mouse 2	Mouse 8
	Rt: 7.36[a]	Rt: 5.58[a]
	Lt: 5.33[a]	Lt: 5.05[a]
Treated with MSC	Mouse 3	Mouse 9
	Rt: 6.30[a]	Rt: 4.13
	Lt: 5.95[a]	Lt: 3.67
	Mouse 4	Mouse 10
	Rt: 5.74[a]	Rt: 4.19
	Lt: 5.12[a]	Lt: 5.05[a]
Treated with mixed MSC and FBC	Mouse 5	Mouse 11
	Rt: 5.80[a]	4.13 (mix of both hemispheres)
	Lt: 5.40[a]	
	Mouse 6	Mouse 12
	Rt: 5.06	Rt: 4.85
	Lt: 4.52	Lt: 5.02

Values are in nmol/mg/h. Each sample was tested in duplicate and results are mean values. Rt, right hemisphere; Lt, left hemisphere.
[a] Increase of activity over mean + 2SD of non-treated mice.

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Table 3
Immunoblot assay of ganglioside GM1 amount.

	4 weeks	8 weeks	6 months
Age-matched non-treated (range)	2.65–3.55 ($n = 3$)	4.98–5.28 ($n = 3$)	7.58 (n = 1)
Treated with uncultured FBC	Mouse I	Mouse VI	Mouse XI
	Rt: 1.42[a]	Rt: 2.30[a]	Rt: 6.18[b]
	Lt: 1.80[a]	Lt: 2.44[a]	Lt: 6.40[b]
Treated with MSC	Mouse II	Mouse VII	
	Rt: 1.82[a]	Rt: 5.30	
	Lt: 1.31[a]	Lt: 5.23	
	Mouse III	Mouse VIII	
	Rt: 1.40[a]	Rt: 4.40[b]	
	Lt: 1.34[a]	Lt: 4.73[b]	
Treated with mixed MSC and FBC	Mouse IV	Mouse IX	
	Rt: 1.33[a]	Rt: 4.55[b]	
	Lt: 1.34[a]	Lt: 4.78[b]	
	Mouse V	Mouse X	
	Rt: 1.78[a]	Rt: 4.45[b]	
	Lt: 1.62[a]	Lt: 4.58[b]	

Values are ratios to those for age-matched control mice. Each sample was tested in duplicate for three times and results are mean values. Rt, right hemisphere; Lt, left hemisphere.

[a] Remarkable decrease.

[b] Slight decrease of ganglioside GM1 compared with non-treated mice.

was found in the mice of every group. However, at 8 weeks, decrease was detected only in the mouse treated with FBC. Efficacy was still noted at 6 months after injection in FBC-treated mouse. These findings were consistent with those for X-Gal staining (Fig. 1) and β-galactosidase activity (Table 2).

4. Discussion

Two therapeutic methods, HSCT and ERT, are clinically available for LSDs. However, neither is markedly effective in the brain. A number of experiments in animal models have been carried out on the treatment of brain in LSDs. Each revealed some efficacy in the brain, though it was transient and incomplete. Sufficient enzyme expression throughout life is needed in the brain. Thus, permanent engraftment of enzyme-secreting cells in the brain, or permanent expression of an exogenous gene with a vector or as an integrated gene might eliminate the brain involvement in LSDs.

However, the immune responses of host animals are among the most difficult problems to overcome in this respect [31–33]. Although the brain, which is sequestered from systemic immune responses, is thought to exhibit little immune response, elimination of cells expressing a therapeutic transgene occurs in the brain. We speculate that innate inflammatory immune responses are stimulated to kill such cells, not necessarily with the induction of a linked adaptive immune response. When host brain cells express a therapeutic transgene mediated by a viral vector, the host cells themselves will be eliminated, possibly resulting in acceleration of neuronal cell death in neurodegenerative disorders. Transplantation of cells having the same genetic information as the host animals with LSD except for expression of a deficient enzyme protein would thus be a good method of treatment for avoiding the elimination of host neuronal cells and curing diseased host cells.

We performed cell transplantation into the brain of β-galactosidase-deficient mice to study the usefulness of long-term engraftment for supplementation of deficient enzyme protein. To minimize the immune responses in the recipient β-galactosidase knock-out mice, we used cells of mice with the same genetic background as the recipient except for possession of copies of the human β-galactosidase gene.

Initially, in the transplantation experiment, we used FBC from transgenic mice expressing the human β-galactosidase gene. The cells could grow in an environment similar to that of the recipient organ in which they were originally growing. The cells spread into the brains and the cell number increased at least until 4 weeks. They grew very successfully for at least 8 weeks and survived for 6 months or more. However, the number of engrafted cells had decreased significantly at 6 months, while the size of the brain had increased. The decrease in ganglioside GM1 accumulation was also marked until 8 weeks after transplantation. However, at 6 months, this decrease was far less pronounced, with re-accumulation of ganglioside GM1. After the cells were engrafted and the cell number was increased by the cell division in the recipient brain, they were depleted. The mechanism of depletion of transplanted cells involved immunological rejection, although the transplanted cells were very similar genetically and physiologically to the recipient.

Next, we performed a transplantation experiment using MSCs obtained from the bone marrow of the

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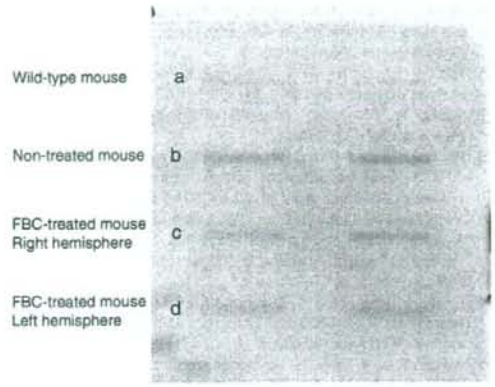

Fig. 2. Immunoblot assay of ganglioside GM1 in brain homogenate at 8 weeks after treatment. Performed in duplicate as shown in two slots for each sample. (a) Wild-type mouse; (b) Non-treated mouse; (c and d) Right and left hemisphere, respectively, of a mouse treated with FBC. The immunoreactivity against ganglioside GM1 antibody in the treated brain (c and d) was less than non-treated brain (d). The accumulated amounts of ganglioside GM1 were calculated in the ratio to the age-matched wild-type mouse (a) from the densitometric quantification signals. These values were shown in Table 3.

same mice expressing the human β-galactosidase gene. MSCs were obtained using the method of plastic adherence. This relatively crude procedure produces a heterogeneous population including multipotential MSCs. These crude cells were used to avoid depletion of potentially important cells and for ease of preparation for clinical application. The cells spread into the brains and the cell number increased similarly to FBC transplantation experiment until 4 weeks. However, decrease in number of engrafted living cells and efficacy in preventing accumulation of ganglioside GM1 were observed in the examination of 8-week-old treated mice.

A number of studies on neural transdifferentiation have been reported [34–37]. Some have reported that neural transdifferentiation of MSCs is induced by cell fusion with host neuronal cells [38–41]. We therefore used mixed FBC and MSC cells to stimulate cell fusion. More engrafted cells were found in the deep areas of the mouse brains treated with mixed cells than in the brains treated with MSC alone. However, no fused cells could be identified. The long-living cells were probably transplanted FBC themselves.

Decrease of ganglioside GM1 was observed even though the increase of the β-galactosidase activity was so small. Similar efficacy was shown previously in our gene therapy experiment [2]. On the other hand, we observed a general depletion of the transplanted cells over time in the BKO mouse brains. The transplanted cells survived in early stage and the number increased by cell division, then, died. This was likely caused by immunological rejection, even though we used fetal brain cells (FBC) from mice with the same genetic background for transplantation. We speculated that immunological reaction occurred because these cells expressed the therapeutic enzyme protein which the host animals did not have. The same has been reported in the transplantation of autogenous cells expressing an exogenous therapeutic gene [33]. The grafted cells were gradually depleted because of immunological rejection by the host animals. To avoid deleterious immune attack and to achieve sufficient long-term efficacy in brain, development of methods to steer the immune response away from cytotoxic responses or to induce tolerance to the products of therapeutic genes is needed [42,43].

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Perspectives in Medicinal Chemistry, in press

Chaperone Therapy for Neuronopathic Lysosomal Diseases: Competitive Inhibitors as Chemical Chaperones for Enhancement of Mutant Enzyme Activities

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Abstract

Chaperone therapy is a newly developed molecular approach to lysosomal diseases, a group of human genetic diseases causing severe brain damage. We found two valienamine derivatives, *N*-octyl-4-epi-β-valienamine (NOEV) and *N*-octyl-β-valienamine (NOV), as promising therapeutic agents for human β-galactosidase deficiency disorders (mainly G_{M1}-gangliosidosis) and β-glucosidase deficiency disorders (Gaucher disease), respectively. We briefly reviewed the historical background of research in carbasugar glycosidase inhibitors. Originally NOEV and NOV had been discovered as competitive inhibitors, and then their paradoxical bioactivities as chaperones were confirmed in cultured fibroblasts from patients with these disorders. Subsequently G_{M1}-gangliosidosis model mice were developed and useful for experimental studies. Orally administered NOEV entered the brain through the blood-brain barrier, enhanced β-galactosidase activity, reduced substrate storage, and improved neurological deterioration clinically. Furthermore, we executed computational analysis for prediction of molecular interactions between β-galactosidase and NOEV. Some preliminary results of computational analysis of molecular interaction mechanism are presented in this article. NOV also showed the chaperone effect forward several β-glucosidase gene mutations in Gaucher disease. We hope chaperone therapy will become available for some patients with G_{M1}-gangliosidosis, Gaucher disease, and potentially other lysosomal storage diseases with central nervous system involvement.

Keywords:

Chaperone; Valienamine; Lysosomal disease; Lysosomal enzyme; β-Galactosidase; β-Glucosidase; G_{M1}-gangliosidosis; Gaucher disease.

Abbreviations: