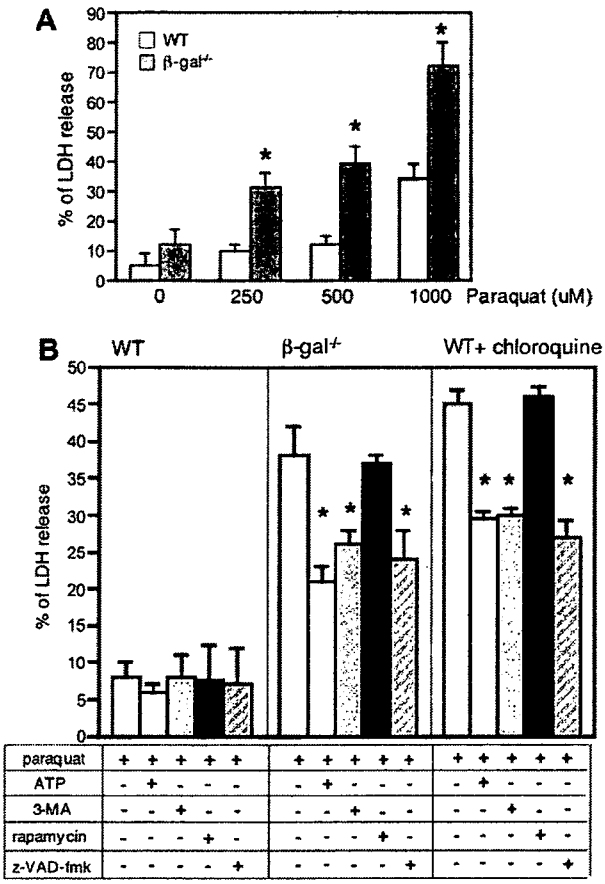

Fig. 4. Effects of ATP, 3-MA, rapamycin and a pan-caspase inhibitor on oxidative stress-induced cell death of astrocyte. (A) Lactate dehydrogenase (LDH) release assay. Astrocytes were cultured with or without paraquat for 24 h and the medium was collected for LDH release assay. Values were expressed as relative to the values from cells treated with 1% Tween 20. Each bar represents the mean (SEM) from three independent experiments. $^{*}p < 0.01$ significantly differ from the value of WT cells. (B) Both WT, β-gal$^{-/-}$ and chloroquine-treated WT astrocytes were treated with indicated drugs. LDH release assay were performed after 24 h treatment. Each bar represents the mean (SEM) from three independent experiments. $^{*}p < 0.01$ significantly differ from the value of paraquat-treated cells.

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HUMAN MUTATION Mutation in Brief #991 (2007) Online

MUTATION IN BRIEF

Novel Mutations of the GLA Gene in Japanese Patients with Fabry Disease and Their Functional Characterization by Active Site Specific Chaperone

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Fabry disease is an X-linked recessive inborn metabolic disorder caused by a deficiency of the lysosomal enzyme α-galactosidase A (EC 3.2.1.22). The causative mutations are diverse, include both large rearrangements and single-base substitutions, and are dispersed

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throughout the 7 exons of the α-galactosidase A gene (*GLA*). Mutation hotspots for Fabry disease do not exist. We examined 62 Fabry patients in Japan and found 24 *GLA* mutations, including 11 novel ones. A potential treatment reported for Fabry disease is active site specific chaperone (ASSC) therapy using 1-deoxygalactonojirimycin (DGJ), an inhibitor of α-galactosidase A, at subinhibitory concentrations. We transfected COS-7 cells with the 24 mutant *GLA*s and analyzed the α-galactosidase A activities. We then treated the transfected COS-7 cells with DGJ and analyzed its effect on the mutant enzyme activities. The activity of 11 missense mutants increased significantly with DGJ. Although ASSC therapy is useful only for misfolding mutants and therefore not applicable to all cases, it may be useful for treating many Japanese patients with Fabry disease. © 2007 Wiley-Liss, Inc.

KEY WORDS: α-galactosidase A; GLA; lysosomal storage disease; 1-deoxygalactonojirimycin; chaperone therapy

INTRODUCTION

Fabry disease (FD; MIM# 301500) is a pan-ethnic, X-linked, lysosomal storage disorder caused by a deficiency in the lysosomal enzyme α-galactosidase A (EC 3.2.1.22) (Brady et al., 1967). Human α-galactosidase A is a homodimeric glycoprotein with three N-linked oligosaccharide chains on each subunit. Enzyme deficiency results in a systemic lysosomal accumulation of glycolipids, primarily globotriosylceramide (Gb3), in the vascular endothelium and other tissues. In the classical form of the disease, the patient develops angiokeratoma, hypohidrosis, and episodic pain crises in the extremities during childhood or adolescence. With advancing age, the morbidity of renal failure, cardiac disease, and early onset of stroke increases. The severity of the clinical manifestations depends on the amount of residual α-galactosidase A activity. Hemizygous male patients with no or very low α-galactosidase A activity usually have severe clinical symptoms and die as young adults. Heterozygous female Fabry patients exhibit a wide range of severity, from a virtually symptom-free course (Marguery et al., 1993) to one comparable to that of their male counterparts (Whybra et al., 2001), although they usually have no symptoms or very mild manifestations.

We examined Fabry patients in Japan and sequenced the patients' *α-galactosidase A* gene (*GLA*) (MIM# 300644). To analyze the mutant α-galactosidase A activities, we transfected COS-7 cells with the mutant *GLA*s. In addition, since 1-deoxygalactonojirimycin (DGJ) stabilizes the α-galactosidase A conformation and improves its stability (Asano et al., 2000; Ishii et al., 2000; Yam et al., 2006), we added DGJ to the incubation medium and examined its effect on the mutant α-galactosidase A activities.

MATERIALS AND METHODS

Patients

We examined 62 Fabry patients from 31 unrelated families. Diagnosis was based on reduced or absent α-galactosidase A activity and typical signs and symptoms of the disease. We received approval to use the patients' DNA for this study from The Ethics Committee on Genetics of the Niigata University School of Medicine, and obtained informed consent from the patients.

Mutation analysis

Blood samples obtained from patients with Fabry disease were transferred into blood-collecting tubes containing ethylene diamine tetraacetic acid (EDTA) and stored at 4°C. Unlike heparin, EDTA does not inhibit the activity of reverse transcriptase, which is used for the reverse transcriptase polymerase chain reaction (RT-PCR). The human *GLA* consists of 7 exons. We found it difficult to obtain full-length *GLA* cDNA (RefSeq BC_002689.2) by performing a single RT-PCR. We therefore obtained exon 1 and exon 7 by PCR from genomic DNA, and the region between exons 1 and 7, containing the full-length exons 2-6 by RT-PCR, from total RNA.

White blood cells were collected using Lymphoprep (Axis-Shield PoC, Oslo, Norway). The genomic DNA of the white blood cells was isolated using an automatic isolation system (NA1000; Kurabo, Osaka, Japan) within 1 week after blood sampling and stored at -20°C until use.

To determine the sequence of exons 1 and 7 of *GLA*, we performed PCR on genomic DNA with the following specific primers: Sense Ex1-1(+), 5'-CCAGTTGCCAGAGAAACA-3'; Antisense Ex1-2(-), 5'-GAGACTCTCCAGTTCCC-3'; Sense Ex7-5(+), 5'-ACAAGTGCTTGATAGTTCTGA-3'; Antisense Ex7-6(-), 5'-CAGGAAGTAGTAGTTGGCAA-3'. The PCR protocol consisted of 2 min at 94°C, then 30 cycles of 15 sec at 94°C, 30 sec at 59°C, and 30 sec at 68°C with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The lengths of the expected products were 390 bp for exon 1 and 420 bp for exon 7. The PCR products were analyzed by 2% NuSieve GTG agarose (Takara, Shiga, Japan) gel electrophoresis and then eluted using the Gene Clean Spin Kit (Q-BIOgene, Irvine, CA, U.S.A.).

To determine the sequence of the cDNA region of *GLA* that included exons 2 to 6, we first amplified it by RT-PCR. We generated first-strand cDNA by RT (ReverTra Ace -α-, Toyobo) using random 9-mers. The reaction consisted of 10 min at 30°C, 20 min at 42°C, 5 min at 99°C, and 5 min at 4°C. To amplify the cDNA of *GLA*, we performed the first PCR with specific primers as follows: 5' #1 primer: 5'-TATGCTGTCCGGTCACC-3', #66 (AG66) primer: 5'-TTAAAGTAAGTCTTTTAATGACAT-3'. The PCR protocol consisted of 2 min at 94°C, then 30 cycles of 15 sec at 94°C, 30 sec at 55°C, and 1 min 30 sec at 68°C with KOD-Plus DNA polymerase (Toyobo). The first PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). The length of the expected products was 1.3 kbp. To amplify the *GLA* cDNA, we performed a second (nested) PCR with the following specific primers: cDNA#1, 5'-TTGGCAAGGACGCCTAC-3'; cDNA#2, 5'-TGCGATGGTATAAGAGCG-3'. The PCR protocol consisted of 2 min at 94°C, then 30 cycles of 15 sec at 94°C, 30 sec at 55°C, and 1 min at 68°C with KOD-Plus DNA polymerase (Toyobo). The length of the expected product was 1 kbp. The PCR products were analyzed by 2% NuSieve GTG agarose (Takara) gel electrophoresis and then eluted using the Gene Clean Spin Kit (Q-BIO gene).

We analyzed both strands of the *GLA* sequence in the PCR and RT-PCR products by direct sequencing using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster, CA, U.S.A.).

Construction of pKSCX-*GLA*

We constructed plasmid pKSCX-*GLA* by inserting the normal human *GLA* cDNA in pCXN2Gal (Ishii et al., 1993) into the unique EcoRI site of the pKSCX expression vector, which bears the cytomegalovirus immediate-early enhancer/chicken β-actin hybrid promoter, and the kanamycin-resistance gene for selection (Niwa et al., 1991). We confirmed the pKSCX-GLA construct by sequencing both DNA strands.

Construction of pKSCX-mutant-*GLA*

To create point mutations or to delete or insert single or multiple amino acids, we used the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) (Yasuda et al., 2004). Because pKSCX contains many GC-rich sequences, we constructed plasmid pCI-*GLA* by inserting the normal human *GLA* cDNA of pCXN2Gal (Ishii et al., 1993) into the unique *Eco*RI site of the pCI mammalian expression vector (Promega, Madison, WI, U.S.A.). We then used pCI-*GLA* as the mutagenesis template. We designed specific primer pairs according to each mutation. To clone the mutant-*GLA* cDNA derived from patients with Fabry disease, we incorporated the mutation into the pCI-*GLA* cDNA using the site-directed mutagenesis kit with the specific primer pairs. We confirmed the mutant *GLA*s by sequencing both DNA strands. We digested the pCI-mutant-*GLA* with *Eco*RI, eluted the *Eco*RI-*Eco*RI fragment containing the mutant-*GLA* cDNA, and ligated it into the like-digested cloning site of pKSCX, yielding pKSCX-mutant-*GLA*.

Cell culture and transfection

We prepared pKSCX-*GLA* or pKSCX-mutant-*GLA* using the Qiagen EndoFree plasmid Giga kit (Qiagen GmbH) (Maruyama et al., 2000), as described previously, and then subjected it to ethanol precipitation.

COS-7 cells (Originator, Gluzman Y; Riken Cell Bank, RCB 0539) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, U.S.A.) containing 10% fetal bovine serum (FBS; Gibco) (Yasuda et al., 2003). The cells were harvested by trypsin treatment, washed in 10 ml of DMEM containing 10% FBS, and resuspended at 5×10^6 cells/ml in DMEM containing 10% FBS. The cells were

transfected by the LipofectAMINE 2000 method (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's instructions. The cells were cultured in DMEM containing 10% FBS at 37°C and 5% CO_2. The COS-7 cells were transfected with pKSCX plasmid *in vitro* using the LipofectAMINE2000 cationic lipid reagent. Briefly, 8 μg of DNA (4 μg pKSCX-mutant-*GLA* and 4 μg pKSCX-luciferase) were mixed with 20 μL of LipofectAMINE2000 reagent and added to the medium in 60-mm dishes. After the transfection, the transformants were cultured in 5 ml of DMEM containing 10% FBS with or without 10 μM DGJ (Sigma Chemical, St. Louis, MO, U.S.A.) at 37°C for 3 days before the enzyme assay. The cells were harvested by trypsin treatment, rinsed with phosphate-buffered saline, and homogenized with 0.2 ml of water with a Physcotron (NS-310E, Niti-on, Chiba, Japan). The sample was then subjected to centrifugation at 10000×g for 5 min. The water-soluble extract was used as the enzyme source.

α-Galactosidase A activity assay

The α-Galactosidase A activity of the water-soluble extract was determined using an artificial substrate, 4-methyl-umbelliferyl α-D-galactopyranoside (Nacalai Tesque, Kyoto, Japan), as described previously (Fan et al., 1999). The protein content of the water-soluble extract was measured by the method of Lowry (DC protein assay; Bio-Rad, Hercules, CA, U.S.A.).

Luciferase assay

Briefly, 20 μl of the water-soluble extract was added to 100 μl of firefly luciferin solution (PicaGene, Toyo Ink, Tokyo, Japan). The luciferase activity was measured for 10 sec using a luminometer (GENE LIGHT, Microtec Nition, Chiba, Japan), according to the manufacturer's instructions.

Statistical analysis

The data are presented as the mean values ± the standard deviation of the mean. We analyzed all data using the Statcel2 Microsoft Office Excel Add-in Software (Hisae Yanai, Department of Mathematics, Faculty of Science Saitama University). Statistical significance was evaluated using the unpaired *t*-test. We considered P values of < 0.05 to be statistically significant.

RESULTS AND DISCUSSION

We detected 24 mutations in 62 Fabry patients from 31 unrelated families in Japan (Table 1). The patients' clinical characteristics are shown in Table 2; the numbers correspond to those of the mutant *GLA*s in Table 1. The mutations consisted of 17 missense substitutions (p.M42V, p.E66Q, p.M76T, p.D93V, p.R112H, p.S148N, p.P205T, p.D231V, p.S235F, p.G258V, p.G260A, p.T282A, p.K308N, p.Q312R, p.G328R, p.L403S, p.T410P), 3 nonsense substitutions (p.S102X, p.R227X, p.W399X), 2 small deletions (c.718_719delAA, c.1020delG), 1 insertion (c.323_324insCAGA), and 1 double missense mutation (p.E66Q, p.R112C). Importantly, 11 of the mutations (p.M76T, p.D231V, p.S235F, p.G258V, p.T282A, p.K308N, p.Q312R, p.L403S, p.T410P, p.S102X, c.323_324insCAGA) were novel. The mutant *GLA* p. E66Q, which is a G-to-C transversion at nucleotide 196 (cDNA) of exon 2, was observed in seven putatively unrelated families, and the mutant *GLA* c.1020Gdel-deletion, was observed in two putatively unrelated families.

To date, more than 20 mutant *GLA*s have been reported in Japan (Takata et al., 1997; Okumiya et al., 1995; Miyamura et al., 1996). Of the 24 mutant *GLA*s we examined, 4 (p. E66Q, p. G260A, p. G328R, c. 718_719delAA) are the same as the known mutant *GLA*s in Japan, and 20 are different. Therefore, in the present study, we examined over half the mutant *GLA*s in Japan.

To examine the activities of the α-galactosidase A produced by these mutant genes, COS-7 cells were transfected with the mutant *GLA*s and cultured with or without 10 μM DGJ for 3 days. All the mutant-α-galactosidase A activities were low, with 0.1% to 50% of the mean normal activity. We examined the relationship between the enzyme activity and clinical symptoms. As expected from previous studies, a one-seventh value of normal α-galactosidase A activity was sufficient to suppress clinical symptoms, and 8 of the 24 mutations reached an activity at least one-seventh that of normal α-galactosidase A activity with DGJ treatment (Table 2) (Nakao et al., 1995; Nakao et al., 2003; Yoshitama et al., 2001). The patients with a one-seventh value of normal α-galactosidase A activity or more did not exhibit the classic symptoms of the disease (angiokeratoma, hypohidrosis,

and episodic pain crises), nor did they develop renal failure and/or cardiac disease later in life (Nakao et al., 1995; Nakao et al., 2003; Yoshitama et al., 2001).

Some mutant-α-galactosidase A variants form aggregates in the endoplasmic reticulum (ER), due to their failure to fold properly during synthesis, and they may lose their enzyme activity. Such mutant-α-galactosidase A variants may be degraded by the ER quality control system (Yam et al., 2006). Moreover, misfolded α-galactosidase A variants appear to stay within the ER regardless of their catalytic competence, leading to their possible degradation or aggregate formation. As a result, mutant α-galactosidase A variants with defective folding tend to be retained in the ER and not reach the lysosomes, where the enzyme functions. DGJ functions as an active-site-specific chaperone (ASSC) to stabilize the conformation of mutant-α-galactosidase A variants and preserve at least some of their enzyme activity. In some cases, DGJ allows mutant-α-galactosidase A variants to pass through the ER quality control system and be sorted into lysosomes, resulting in an increase in the mutant's α-galactosidase A activity. This suggests that the stability of mutant α-galactosidase A is a critical factor that determines its further transportation and function as residual enzyme activity. However, DGJ does not have a measurable effect on all mutant α-galactosidase A activities, particularly those with frame-shift or early termination mutations.

In this study, the activities of 11 of the 24 α-galactosidase A mutants significantly increased after incubation with 10 μM DGJ for 3 days (Table 1). The activity of 11 missense mutants significantly increased with DGJ treatment, while that of the other missense, and the nonsense, insertion, and deletion mutants did not increase. Because the No. 7 mutation causes the incorrect synthesis of most of the protein, including the active site, the apparent enzyme activity of No. 7, 4% of the normal enzyme activity, may be experimental noise. Therefore, some of the other mutations with less than 4% enzyme activity need to be interpreted carefully. Nevertheless, these findings indicate that although ASSC therapy is not applicable to all patients with Fabry disease, many Japanese patients might respond to such treatment. Importantly, a preliminary study of DGJ use in transgenic mice showed no general signs of toxicity (Fan et al., 1999), supporting this compound's promise as a treatment for human Fabry disease.

Our findings support the potential usage of ASSC as an alternative treatment for Fabry disease. It is likely that by correcting the trafficking defect with DGJ, the misfolded α-galactosidase A variants are directed to lysosomes, thereby increasing their residual enzymatic activities. Our results showed that DGJ specifically affects misfolded α-galactosidase A, since it reproducibly increased the activity of different *GLA* variants. We also showed that DGJ causes normal α-galactosidase A activity to increase, although the reason is not known. Therefore, DGJ may be a useful treatment for every heterozygous patient.

Recently, enzyme replacement therapy (ERT) was approved for patients with Fabry disease: two recombinant glycoprotein products, Replagal® (Transkaryotic Therapies, Cambridge, MA, U.S.A.) and Fabrazyme® (Genzyme, Cambridge, MA, U.S.A.), are now available (Goi et al., 2005; Hoffmann et al., 2006). Because kidney and heart involvements are important causes of the morbidity and mortality in patients with Fabry disease, they should be the main targeted organs in therapies designed to treat it. However, many investigations have reported that in Fabry disease the kidney and heart are highly resistant to ERT with either enzyme treatment (Brady et al., 2001; Ioannou et al., 2001). Furthermore, most of the injected enzyme is delivered to the liver and has difficulty reaching other organs, such as the kidney and heart (Lee et al., 2003; Sakuraba et al., 2006). In addition, the Gb3 in the Fabry mouse kidney is highly resistant to ERT by systemic intravenous injection. In ERT of the Fabry mouse, most (65~70%) of the injected enzyme is recovered in the liver, and less than roughly 1% of the injected dose is detected in the kidney and heart (Lee et al., 2003). An examination of ERT in the Fabry mouse also showed that only a very small quantity of the recombinant enzyme remains in tissues hours after the infusion.

DGJ treatment, in contrast, increased the enzyme activity of normal α-galactosidase A and of several mutant-α-galactosidase A variants (Table1, No.1, 4, 8, 10, 13, 15, 16, 17,18, 19, 23) enough to extend the persistence of enzyme activity *in vitro*. Using DGJ as an ASSC may resolve the problems caused by mutant α-galactosidase A effectively. Increasing the activity of both normal and mutant α-galactosidase A with DGJ treatment may prove clinically useful.

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Table 1. Mutations in the *GLA* genes of Fabry patients

No.	Number of Patients (hemizygote/ heterozygote)	Exon	Nucleotide Change (RefSeq BC_002689.2)	Amino acid change (RefSeq AAH02689.1)	GLA activity		GLA DGJ (-) /normal GLA	GLA DGJ (+) /normal GLA	GLA DGJ (+) /GLA DGJ (-)
					DGJ (-)	DGJ (+)			
					nmol/h/ml/10^7RLU		%	%	ratio
normal GLA					3770 ± 1410	*6840 ± 1980		181.0	1.8
1	1 / 0	1	c.124A<G	p.M42V	160 ± 52	*848 ± 245	4.2	22.5	5.3
2	8 / 8	2	c.196G<C	p.E66Q	1883 ± 102	2280 ± 841	49.8	60.5	1.2
3	1 / 0	2	c.196G<C	p.E66Q	121 ± 53	129 ± 30	3.2	3.4	1.1
		2	c.334C<T	p.R112C					
4	0 / 1	2	c.227T<C	p.M76T	108 ± 49	*222 ± 31	2.9	5.9	2.1
5	1 / 0	2	c.278A<T	p.D93V	45 ± 7	56 ± 7	1.2	1.5	1.2
6	0 / 1	2	c.305C<G	p.S102X	71 ± 27	74 ± 24	1.9	2.0	1.1
7	1 / 0	2	c.323_324insCAGA		89 ± 60	150 ± 119	2.4	4.0	1.7
8	1 / 0	2	c.335G<A	p.R112H	24 ± 3	*2470 ± 716	0.6	65.5	102.0
9	2 / 1	3	c.443G<A	p.S148N	5 ± 6	49 ± 7	0.1	1.3	9.1
10	0 / 1	4	c.613C<A	p.P205T	321 ± 108	*5640 ± 776	8.5	150.0	17.6
11	1 / 0	5	c.679C<T	p.R227X	81 ± 33	104 ± 29	2.1	2.8	1.3
12	1 / 0	5	c.692A<T	p.D231V	128 ± 32	160 ± 33	3.4	4.2	1.3
13	1 / 0	5	c.704C<T	p.S235F	120 ± 23	*305 ± 63	3.2	8.1	2.5
14	3 / 0	5	c.718_719delAA		19 ± 11	20 ± 13	0.5	0.5	1.0
15	1 / 1	5	c.773G<T	p.G258V	73 ± 12	***385 ± 24	1.9	10.2	5.3
16	1 / 0	5	c.779G<C	p.G260A	406 ± 65	***2230 ± 257	10.8	59.2	5.5
17	1 / 1	6	c.844A<G	p.T282A	9 ± 2	*223 ± 62	0.2	5.9	25.3
18	1 / 0	6	c.924A<T	p.K308N	175 ± 38	***1770 ± 125	4.6	46.9	10.1
19	2 / 0	6	c.935A<G	p.Q312R	381 ± 57	***1640 ± 197	10.1	43.5	4.3
20	1 / 0	6	c.982G<A	p.G328R	8 ± 12	9 ± 6	0.2	0.2	1.1
21	1 / 2	7	c.1021delG		27 ± 10	78 ± 11	0.7	2.1	2.9
22	1 / 3	7	c.1196G<A	p.W399X	76 ± 31	86 ± 28	2.0	2.3	1.1
23	2 / 0	7	c.1208T<C	p.L403S	857 ± 262	*1440 ± 176	22.7	38.2	1.7
24	6 / 5	7	c.1228A<C	p.T410P	6 ± 3	8 ± 3	0.2	0.2	1.3

*, $p < 0.05$ **, $p < 0.005$ ***, $p < 0.0005$.
The α-galactosidase A activity of COS-7 origin is subtracted from the results of the α-galactosidase A activity assay.

Table 2. Clinical characteristics of the study patients

No*		serum GLA activity (nmol/h/mg)	age at onset	neuropathic pain	hypohidrosis	Angiokeratoma	corneal opacity	urine abnormality	ECG
1	hemizygote	unknown	10	-	-	+	+	protein2+,occult blood1+	left ventricular hypertrophy
2	hemizygote	3.4	26	-	-	-	-	-	normal
	hemizygote	2.3	45	-	-	-	-	-	left ventricular hypertrophy
	hemizygote	1	-	-	-	-	-	-	normal
	hemizygote	3.5	-	-	-	-	-	-	normal
	hemizygote	unknown	-	-	-	-	-	-	normal
	hemizygote	unknown	50	-	-	-	-	chronic renal failure on HD	left ventricular hypertrophy
	heterozygote	unknown	-	-	-	-	-	-	normal
	heterozygote	unknown	-	-	-	-	-	-	normal
	heterozygote	unknown	-	-	-	-	-	-	unknown
	heterozygote	unknown	50	-	-	-	-	chronic renal failure on HD	-
	heterozygote	2.5	44	-	-	-	-	-	unknown
	heterozygote	4.3	47	-	-	+	-	unknown	unknown
	heterozygote	4.5	21	-	-	-	-	unknown	unknown
	heterozygote	4	24	-	-	-	-	unknown	unknown
	heterozygote	unknown	40	-	-	-	-	chronic renal failure on HD	left ventricular hypertrophy
	heterozygote	4	40	-	-	-	-	chronic renal failure	left ventricular hypertrophy
3	hemizygote	unknown	8	+	+	-	-	protein2+	left ventricular hypertrophy
4	hemizygote	unknown	20	-	-	-	-	protein2+	normal
5	heterozygote	11.1	unknown	-	-	-	-	unknown	normal
6	heterozygote	8.4	11	+	-	-	+	-	normal
7	hemizygote	0.2	9	+	-	+	+	protein2+	left ventricular hypertrophy
8	hemizygote	unknown	50	-	-	-	-	chronic renal failure on HD	left ventricular hypertrophy
9	hemizygote	0.8	childhood	+	-	+	+	-	normal
	hemizygote	1.6	childhood	+	-	+	unknown	protein2+	normal
	heterozygote	11.4		+	-	+		-	left ventricular hypertrophy

No*		serum GLA activity (nmol/h/mg)	age at onset	neuropathic pain	hypohidrosis	Angiokeratoma	corneal opacity	urine abnormality	ECG
10	heterozygote	unknown	40	unknown	-	unknown	unknown	-	left ventricular hypertrophy
11	hemizygote	unknown	11	+	+	-	-	protein3+,occult blood+-	left ventricular hypertrophy
12	hemizygote	3.7	3	+	+	+	+	protein+-	aortic regurgitation 3
13	hemizygote	unknown	14	+	+	+	-	unknown	normal
14	hemizygote	2	9	+	unknown	-	-	-	normal
	hemizygote	2.2	10	+	unknown	-	+	-	normal
	hemizygote	2.5	13	+	unknown	-	-	-	normal
15	heterozygote	4.3	16	-	-	-	+	-	normal
	hemizygote	3.4		+	+	+	-	protein2+	left ventricular hypertrophy
16	hemizygote	1.2	7	+	+	-	+	protein+,occult blood+	left ventricular hypertrophy
17	heterozygote	5.25	45	-	-	-	-	-	mitral regurgitation 3
	hemizygote	3	12	+	+	-	-	-	left ventricular hypertrophy
18	hemizygote	unknown	childhood	+	-	unknown	unknown	protein2+	left ventricular hypertrophy
19	hemizygote	0.4	-	-	-	-	-	-	-
	hemizygote	1.8	-	-	-	-	-	-	-
20	hemizygote	5	unknown	-	-	-	-	-	normal
21	hemizygote	0.4	25	-	-	-	-	protein2+	left ventricular hypertrophy
	heterozygote	6.3	10	+	-	-	+	protein3+	left ventricular hypertrophy
	heterozygote	11.7	10	+	-	-	+	protein3+	left ventricular hypertrophy
22	hemizygote	0.3	8	+	+	-	-	-	-
	heterozygote	unknown	unknown	-	-	-	-	unknown	unknown
	heterozygote	unknown	unknown	-	-	-	-	unknown	unknown
	heterozygote	unknown	unknown	-	-	-	-	unknown	unknown
23	hemizygote	3.1	unknown	-	-	-	-	protein+	left ventricular hypertrophy
	hemizygote	unknown	unknown	-	-	-	-	-	left ventricular hypertrophy
24	hemizygote	2.4	10	+	+	+	-	protein3+	sick sinus syndrome
	heterozygote	unknown	8	+	+	+	+	chronic renal failure on HD	left ventricular hypertrophy

No*		serum GLA activity (nmol/h/mg)	age at onset	neuropathic pain	hypohidrosis	Angiokeratoma	corneal opacity	urine abnormality	ECG
	heterozygote	5	20	+	-	+	+	-	-
	heterozygote	3.1	10	+	+	+	+	-	-
	hemizygote	2.9	childhood	+	+	+	+	chronic renal failure on HD	chronic heart failure
	hemizygote	2	13	+	+	+	-	-	-
	heterozygote	3.8	8	+	+	-	-	-	-
	heterozygote	3	12	+	-	-	-	-	-
	hemizygote	3	7	+	+	-	-	-	-
	heterozygote	4.2	12	+	-	-	-	-	-
	hemizygote	unknown	12	+	+	+	+	chronic renal failure on HD	-

*The mutation numbers in Table 2 correspond to those in Table 1.
Case numbers in bold italic denote α-galactosidase A activity greater than one-seventh of normal, following DGJ treatment.

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Gaucher 病

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key words: Gaucher 病，D409H 変異，水頭症

画像診断のポイント

❶ 通常の神経型 Gaucher 病では脳萎縮が主体の中枢神経画像を示すのみで特異的な所見はない。
❷ III型 Gaucher 病の D409H 変異を有する患者の一部は水頭症を呈する。

●疾患の概念

Gaucher 病はリソソーム酵素のひとつであるグルコセレブロシダーゼ活性の遺伝的欠損により，その基質であるグルコセレブロシドが主として肝，脾，骨髄などの細網内皮系に蓄積する常染色体劣性遺伝形式をとるリピドーシスのひとつである。Gaucher 病は神経症状の有無および重症度により**表 1** のように 3 つの臨床型に分類されているが，いずれの病型においても臨床症状に著しい多様性が存在することが本症の特徴である。

I 型 Gaucher 病(慢性非神経型，成人型)は神経症状を伴わない病型で，肝脾腫，汎血球減少症，骨症状を主徴とする。発症年齢，骨合併症の有無，肝脾腫の程度などの点において臨床的異質性が認められる病型である。II 型 Gaucher 病(急性神経型，乳児型)は乳児期に発症し，肝脾腫に加え重篤な神経症状を呈し，急速に退行し 2 歳頃までに死亡する予後不良の病型である。III型 Gaucher 病(亜急性神経型，若年型)は肝脾腫に加え，神経症状を伴うがその発症は II 型に比較して遅く，またその進行も緩徐な病型である。本病型は IIIa，IIIb，IIIc の 3 つの亜型に分類されている。IIIa 型は古典的なIII型であり，肝脾腫に加えて若年発症の神経症状(小脳失調，ミオクローヌス，けいれん，知能低下など)を呈する病型である。IIIb 型は核上性上方注視麻痺を唯一の神経症状とし，それに加えて重篤な臓器症状を示すので，早期発症の I 型と鑑別が難しい病型である。IIIc 型は水頭症，角膜混濁，心弁膜の石灰化などきわめてユニークな臨床症状を呈する病型である。1995 年，Abrahamov らに

表 1　Gaucher 病の臨床病型

	I 型	II 型	III型
発症時期	幼少～成人	乳児	乳児～学童期
肝脾腫	(+)～(+++)	(+)	(+)～(+++)
骨症状	(−)～(+++)	(−)	(−)～(++)
神経症状	(−)	(+++)	(+)～(+++)

(−)：なし，(+)：軽度あり，(++)：中等度あり，(+++)：著明にあり

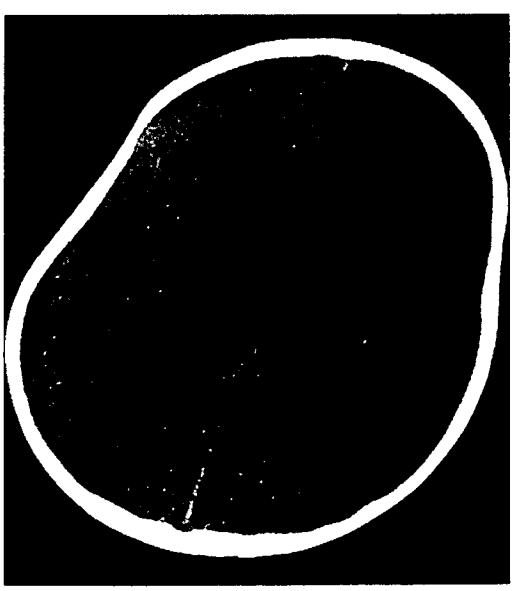

図　IIIc 型 Gaucher 病の CT 像

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表2　水頭症を呈したGaucher病の6例のまとめ

症例	診断時年齢	遺伝子型	転帰	心合併症	眼合併症
1	1歳3カ月	D409H/?	7歳時死亡	＋	－
2	29歳	D409H/D409H	31歳時死亡	＋	＋
3	3歳1カ月	D409H/?	不明	－	－
4	1歳7カ月	D409H/?	15歳時死亡	＋	＋
5	1歳9カ月	D409H/R120W	生存(6歳)	－	－
6	8カ月	D409H/RecNcil	6歳時死亡	－	－

よってこの臨床亜型は初めて報告され[1]，遺伝子型は全例D409H変異のhomozygoteであった。当初，アラブ人にのみ認められると考えられていたが，類似した症例がアラブ以外の地域でも報告されている。

●画像所見

I型は神経症状を呈さないので，当然，中枢神経系画像に異常所見を示すことはない。II型，IIIa型，IIIb型においては非特異的な脳萎縮像を示すのみである。前述したようにIIIc型は中枢神経画像上，水頭症を示す。**図**に当科で経験したIIIc型の脳CT写真を示す。著明な脳質拡大は認めるが，脳実質の減少や脳回の拡大は目立たず，水頭症の所見を呈している。当科で把握している水頭症を呈したGaucher病症例のまとめを**表2**に示す。当院で確定診断あるいは遺伝子診断した日本人Gaucher病は現在まで117症例で，うち25例が初診時からIII型と診断されていた。この25例中6例が水頭症を呈していた。遺伝子変異に注目すると全例D409H変異を有していたが，homozygoteは1例のみであった。このhomozygoteはAbrahamovらによって報告された症例に酷似していた[1]。6例中，心合併症は3例に，眼合併症は2例に認められた。

Shiiharaらは，水頭症を呈したIII型Gaucher病の症例を臨床的および病理学的に検討した。その報告によれば，MRI所見および脳槽造影所見からこの水頭症は交通性と確認されている(表2，症例3)。また，脳室-腹腔シャント手術にて水頭症は改善し，その手術の際得られた髄膜の病理所見は著明な線維性肥厚を示しており，この病理学的変化が水頭症の原因と考えられている[2]。Inuiらは，水頭症に加えて心弁膜症を呈したGaucher病症例において肝臓および脾臓の被膜に同様な著明な肥厚を認めている(表2，症例4)[3]。これら結合組織の線維性肥厚は，D409H変異を有するGaucher病症例に特徴的と考えられている。

●確定診断の方法

水頭症を呈している症例で，肝脾腫，心弁膜症，角膜混濁を認めた場合，IIIc型Gaucher病を疑うべきである。酸性ホスファターゼ値，アンギオテンシン転換酵素値の上昇は補助診断の助けとなる。最終的には，培養皮膚線維芽細胞のβ-グルコシダーゼ活性の低下を証明することによって確定診断される。D409H変異が同定できれば診断に有用である。反対に水頭症を伴うGaucher病で，D409H変異が同定されれば心合併症および眼合併症の発症に注意を払うべきである。

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Chemical Chaperone Therapy: Clinical Effect in Murine G_{M1}-Gangliosidosis

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Certain low-molecular-weight substrate analogs act both as in vitro competitive inhibitors of lysosomal hydrolases and as intracellular enhancers (chemical chaperones) by stabilization of mutant proteins. In this study, we performed oral administration of a chaperone compound *N*-octyl-4-epi-β-valienamine to G_{M1}-gangliosidosis model mice expressing R201C mutant human β-galactosidase. A newly developed neurological scoring system was used for clinical assessment. *N*-Octyl-4-epi-β-valienamine was delivered rapidly to the brain, increased β-galactosidase activity, decreased ganglioside G_{M1}, and prevented neurological deterioration within a few months. No adverse effect was observed during this experiment. *N*-Octyl-4-epi-β-valienamine will be useful for chemical chaperone therapy of human G_{M1}-gangliosidosis.

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G_{M1}-gangliosidosis (OMIM 230500) is a hereditary human disorder with progressive central nervous system damage, visceromegaly, and skeletal dysplasias in children and adults, caused by mutations of the gene *GLB1* (3p21.33) coding for lysosomal β-galactosidase (EC 3.2.1.23) that catalyzes hydrolysis of ganglioside G_{M1} and related compounds.[1]

In 2003, we proposed chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis.[2] The first original studies in this direction had been published on mutant α-galactosidase A in Fabry's disease, using galactose[3] and 1-deoxygalactonojirimycin.[4] We then found *N*-octyl-4-epi-β-valienamine (NOEV) as a potent stabilizer of mutant β-galactosidase activity in G_{M1}-gangliosidosis.[2] It increased mutant β-galactosidase activity in cultured fibroblasts from more than 30% of patients.[5]

On the other hand, we developed a novel method to assess neurological alterations in G_{M1}-gangliosidosis model mice by modifying neurological tests in human infants and young children.[6] This technique was applied to monitor their clinical course under chaperone treatment. We found that NOEV prevents neurological deterioration in this animal model.

Materials and Methods

G_{M1}-Gangliosidosis Model Mice

We maintained a C57BL/6-based congenic knock-out (KO) mouse strain with β-galactosidase deficiency[7] and a transgenic (Tg) mouse strain overexpressing R201C mutant human β-galactosidase.[2] Care of experimental animals was performed in accordance with the Guidelines on Animal Experimentation of International University of Health and Welfare (Otawara, Japan). Wild-type (WT) mice (C57BL/6Cr) were purchased from Japan SLC (Shizuoka, Japan).

Neurological Assessment

Quantitative neurological assessment consisted of 11 test items.[6] Each item was scored in four grades (0–3) based on increasing severity of abnormality. The total scores were periodically followed. Reliability and reproducibility of this test method have been established.[6]

N-Octyl-4-epi-β-valienamine Administration and Determination

Tg or WT mice were provided 1mM aqueous solution of NOEV hydrochloride ad libitum. The average daily intake of NOEV was 75μg/gm (75mg/kg) body weight. The NOEV concentration was determined by combined liquid chroma-

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tography and tandem mass spectrometry system (Fig 1). For neurological assessment, 16 Tg mice were given NOEV from 2 months of age, and they were compared clinically with the other 16 Tg mice without NOEV treatment.

General Pathology, Neuropathology, and Quantitative Immunohistochemistry

The mice were perfused through the heart with 4% phosphate-buffered paraformaldehyde, and tissues were used for pathology and immunohistochemistry.[2,8] We further performed immunohistochemical quantitation of ganglioside G_{M1} in the brain by confocal fluorometry (Fig 2).

Enzyme Assay

β-Galactosidase and α-galactosidase A were assayed with 4-methylumbelliferyl derivatives (Nacalai Tesque, Kyoto) as substrates[9] and galactosylceramidase with 6-hexadecanoylamino-4-methylumbelliferyl β-galactoside (Erasmus MC, Rotterdam, the Netherlands).[10] Protein was determined with Micro TP-Test Wako (Wako Pure Chemical Industries, Osaka, Japan).

Blood Chemistry and Urinalysis

Blood was collected by cardiac puncture and centrifuged. Plasma was analyzed using FUJI DRI-CHEM 3000V (Fuji Film, Tokyo, Japan) for 14 test items, including glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, and others, as indicated by this analysis kit. Urine was performed by collection by external pressure or direct puncture of the bladder, using Uro-Labstix SG-L (Bayer Medical, Tokyo, Japan).

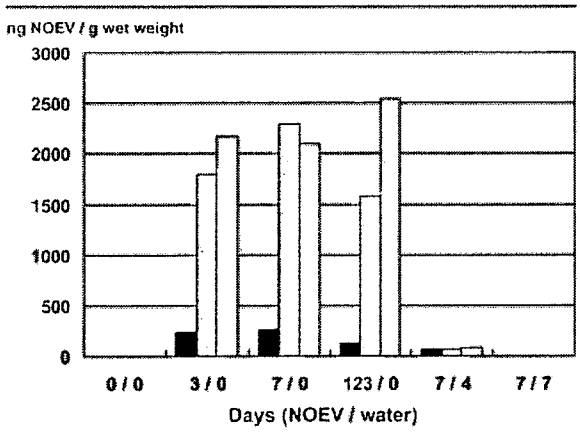

Fig 1. N-*Octyl-4-epi-β-valienamine (NOEV) concentrations in mouse tissues.* Black bars *indicate brain;* gray bars *indicate liver;* white bars *indicate kidney. Tissue content is measured in ng/gm wet weight. 0/0: water only (n = 2); 3/0: NOEV for 3 days (n = 2); 7/0: NOEV for 7 days (n = 2); 123/0: NOEV for 123 days (n = 1); 7/4: NOEV for 7 days, followed by water for 4 days (n = 2); 7/7: NOEV for 7 days, followed by water for 7 days (n = 2).*

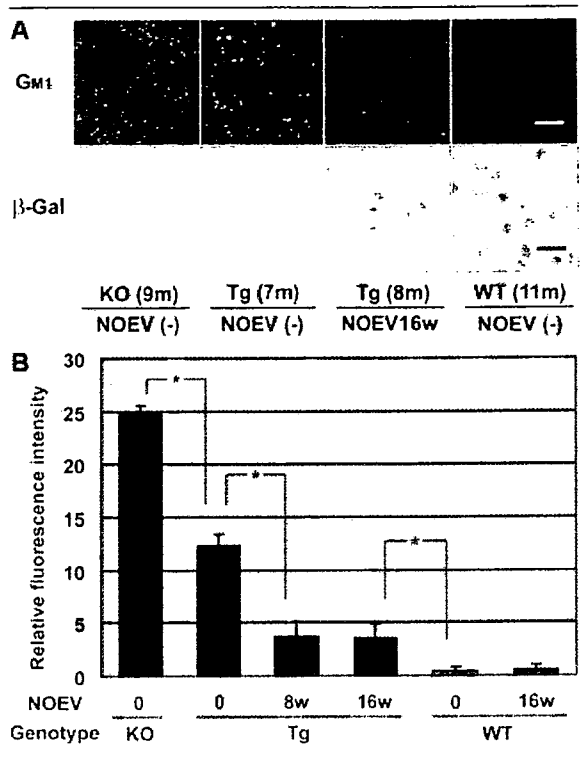

Fig 2. Immunohistochemical analysis of the R201C mouse brain. (A) Histochemical stain of G_{M1} and β-galactosidase. (B) Quantitative confocal immunohistochemistry of G_{M1}. Each column *indicates the mean of relative fluorescence intensity in the mouse brain (*vertical bar = *standard error of the mean). **p < 0.05. KO (0w) = KO mouse; water only (n = 2; age: 7 and 9 months). Tg (0w) = Tg mouse; water only (n = 3; age = 7, 11, and 15 months). Tg (8w) = Tg mouse;* N-*octyl-4-epi-β-valienamine (NOEV) for 8 weeks (n = 2; age = 10 and 11 months). Tg (16w) = Tg mouse; NOEV for 16 weeks (n = 1; age = 9 months). WT (0w) = WT mouse; water only (n = 1; age = 11 weeks). WT (16w) = WT mouse; NOEV for 16 weeks (n = 1; age = 9 months). KO = knock-out; Tg = transgenic; WT = wild type. See supplementary material for additional methodology details.*

Results

N-Octyl-4-epi-β-valienamine Concentration in Mouse Tissues

The NOEV concentration increased in the brain, liver, and kidney of WT mice within 3 days immediately after starting treatment, remained at the same level for as long as 123 days of continuous administration, decreased rapidly within 4 days after discontinuation of treatment, and completely disappeared within 7 days (see Fig 1). The concentration was almost the same in the liver and kidney, and about 10% to 15% in the brain compared with the two extraneural tissues. Tissue concentrations remained the same after 8 to 16 weeks of NOEV administration in Tg mice (data not shown).

Pathology and Immunohistochemistry

There were no specific changes in the liver, spleen, kidney, lung, heart, thymus, pancreas, or skeletal muscle of NOEV-treated mice. Bleeding, hemostasis, leukocyte infiltration, or cytoplasmic vacuolation was observed in some sporadic WT, Tg, or KO mice with or without treatment (data not shown). Immunohistochemical stain showed a marked decrease in G_{M1} storage and increase in the enzyme activity in almost all areas of the brain after 8 to 16 weeks of NOEV treatment (see Fig 2A). This observation was confirmed quantitatively by confocal fluorometry, indicating a significant decrease of G_{M1} in the NOEV-treated Tg mouse brain (see Fig 2B).

Enzyme Activities

β-Galactosidase activity increased remarkably during NOEV treatment for 8 to 16 weeks in Tg mice, particularly in the liver and spleen (data not shown). In the brain, the enzyme activity in Tg mice reached 30% to 40% of that in WT mice. Galactosylceramidase and α-galactosidase A activities did not change in this experiment.

Neurological Assessment

We first compared the three genotypes without NOEV treatment (Fig 3A). The total score remained low (<5) in the WT mouse until 24 months. It was high (almost 10) in the KO mouse already at 5 months (middle symptomatic stage), and increased to 25 at 9 to 10 months (late stage). The Tg mouse showed slower progression than the KO mouse. However, even at 2 to 4 months (early symptomatic stage), the mean of total score was significantly greater than that of the WT mouse.

NOEV treatment was started at 2 months of age (see Fig 3B). There was no significant difference for the first 2 months between the two groups with or without treatment. Then a definite statistical difference was detected at 5 to 7 months of age, although the score increased gradually also in the treatment group. This clinical benefit was not evident when the treatment was started at 5 months over the ensuing 5 months (data not shown).

Blood Chemistry and Urinalysis

Glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase were high in some WT, Tg, or KO mice examined. However, they were not related to the genotype, clinical course, age, or NOEV treatment. Urinalysis was normal in all mice examined.

Discussion

In this study, we investigated the clinical effect of the chemical chaperone NOEV after our first report on laboratory data in G_{M1}-gangliosidosis mouse model.[2]

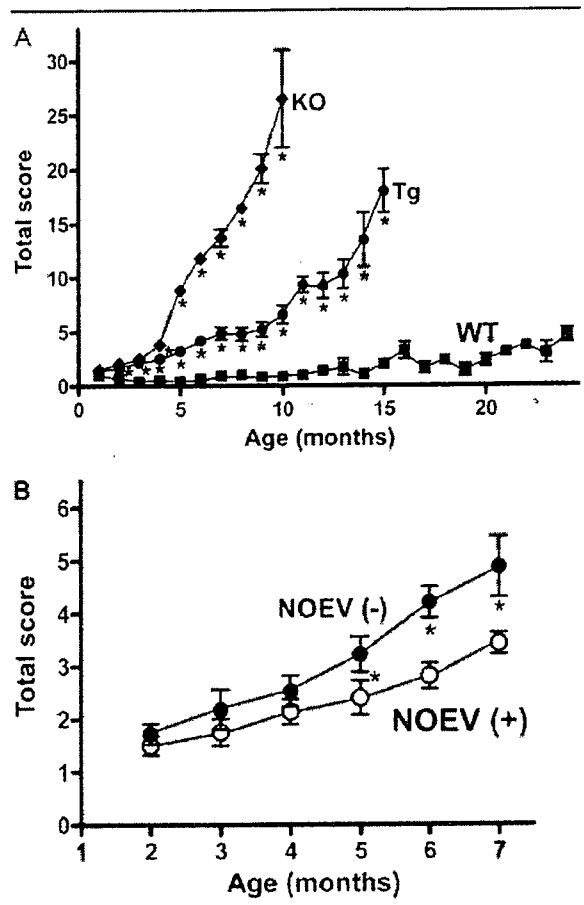

Fig 3. Neurological assessment scores in G_{M1}-gangliosidosis mouse model. (A) Clinical course in three mouse genotypes without N-*octyl-4-epi-β-valienamine (NOEV) treatment. The quantitative neurological assessment consisted of the following 11 test items: gait, posture/forelimb, posture/hind limb, posture/trunk, posture/tail, avoidance response, rolling over, body righting acting on head, parachute reflex, horizontal wire netting (stepping through interstice), and vertical wire netting (clinging and holding body).*[6] *The mice were scored in four grades based on increasing severity of abnormality for each test item: 0 (normal), 1 (slightly abnormal), 2 (moderately abnormal), and 3 (severely abnormal). The highest score was 33 for those with the most extensive neurological abnormalities. We used GraphPad Prism 4 (GraphPad Software, San Diego, CA) for unpaired Student's* t *test.* Black squares *indicate wild-type (WT) mouse;* black circles *indicate transgenic (Tg) mouse;* black diamonds *indicate knock-out (KO) mouse.* Vertical bars *indicate standard error of the mean (SEM).* $^*p < 0.05$ *(WT vs Tg, and Tg vs KO). n = 10, 10, 11, 24, 28, 17, 21, 13, 2 for KO (2–10 months); n = 32, 11, 19, 18, 29, 17, 17, 18, 18, 17, 11, 6, 4, 2 for Tg (2–15 months); n = 11, 5, 12, 12, 9, 18, 21, 21, 16, 19, 18, 8, 10, 9, 11, 9, 8, 9, 9, 10, 7, 2, 3 for WT (2–24 months). (B) Clinical effect of NOEV therapy in Tg mice. The experimental conditions were the same as Figure 3A.* Black circles *indicate Tg mouse, nontreated;* white circles *indicate Tg mouse, treated with NOEV.* Vertical bars *indicate SEM.* $^*p < 0.05$. *n = 16 for both treated and nontreated mice.*

NOEV is an epimer of *N*-octyl-β-valienamine,[11,12] a potent inhibitor of β-galactosidase in vitro[13,14] and a potent inducer to express mutant β-galactosidase activity in human and murine fibroblasts and tissues.[2] NOEV was effective in almost all patients with juvenile G_{M1}-gangliosidosis and in some with infantile G_{M1}-gangliosidosis.[5] Most patients were compound heterozygotes. We expect a successful therapeutic effect if one of the mutant genes is responsive to NOEV. The efficacy of enhancement varied among different mutations. Eight human mutant enzymes responded positively to NOEV, including known common mutations (K. Higaki and colleagues, unpublished data). The optimal NOEV concentration was 0.2μM for R457Q and 2μM for R201C and R201H.[5] We estimate that NOEV therapy will be successful in at least one-third of patients with G_{M1}-gangliosidosis.

This study indicates that orally administered NOEV entered the central nervous system from the bloodstream across the blood–brain barrier. The compound did not accumulate in the tissues examined during oral administration for 4 months. The increase of β-galactosidase activity and reduction of G_{M1} reflected changes of NOEV concentration in mouse tissues. We did not analyze urinary oligosaccharides.

In this study, we tried two new approaches for quantitative evaluation of the NOEV effect in murine G_{M1}-gangliosidosis: immunohistochemistry and clinical assessment. Quantitative confocal fluorometry demonstrated a remarkable decrease of G_{M1} in the mouse brain after NOEV treatment. The neurological assessment scores corresponded well with laboratory data.

Early chaperone therapy resulted in a positive clinical effect within a few months, although complete arrest or prevention of disease progression was not achieved under the current experimental conditions. The latency before the clinical effect was longer if the therapy was started in the late symptomatic stage. We conclude that early treatment at the early stage of disease is mandatory for prevention of brain damage. We do not know the optimal dose of NOEV at present in murine G_{M1}-gangliosidosis.

No significant adverse effect was observed during NOEV administration up to 6 months. Random increases of plasma glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase concentrations were not related to genotype or NOEV treatment. Blood collection by direct cardiac puncture after ethyl ether anesthesia and thoracotomy may have partly contributed to abnormal release of intracellular enzymes into the extracellular fluid. We did not observe excessive enzyme enhancement in the course of NOEV treatment, but it may cause some metabolic derangement in human and mouse tissues.

So far we demonstrated effectiveness of chemical chaperone therapy in G_{M1}-ganglosidosis,[2,5] Gaucher's disease,[15,16] and Fabry's disease.[4] A short-term effect was reported on a Fabry's disease patient with galactose,[17] and other investigators confirmed the effectiveness of chaperone therapy in Gaucher's disease fibroblasts.[18] In addition, the effect of chaperone treatment has been reported in G_{M2}-gangliosidosis[19] and Pompe's disease.[20] Theoretically, this principle can be applied to other lysosomal diseases, if a specific chaperone compound becomes available for each target enzyme. Furthermore, other neurogenetic diseases may be considered for chemical chaperone therapy. We expect that studies in this direction will open a new aspect of molecular therapy for inherited metabolic diseases with central nervous system involvement in the near future.

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Original article

Motor and reflex testing in G_{M1}-gangliosidosis model mice

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Abstract

A large number of genetic disease model mice have been produced by genetic engineering. However, phenotypic analysis is not sufficient, particularly for brain dysfunction in neurogenetic diseases. We tried to develop a new assessment system mainly for motor and reflex functions in G_{M1}-gangliosidosis model mice. Two genetically engineered model mouse strains were used for this study: the β-galactosidase-deficient knockout mouse representing infantile G_{M1}-gangliosidosis (severe form), and transgenic mouse representing juvenile G_{M1}-gangliosidosis (mild form). We modified human child neurology techniques, and selected eleven tests for motor assessment and reflex testing. The test results were scored in four grades: 0 (normal), 1 (slightly abnormal), 2 (moderately abnormal), and 3 (severely abnormal). Both disease model mouse strains showed high scores even at the apparently pre-symptomatic stage of the disease, particularly with abnormal tail and hind limb postures. Individual and total test scores were well correlated with the progression of the disease. This method is simple, quick, and reproducible. The testing is sensitive enough to detect early neurological abnormalities, and will be useful for monitoring the natural clinical course and effect of therapeutic experiments in various neurogenetic disease model mice, such as chemical chaperone therapy for G_{M1}-gangliosidosis model mice.
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Keywords: G_{M1}-gangliosidosis; Genetic engineering; Disease model mouse; Motor assessment; Reflex testing; Mouse neurology

1. Introduction

The recent advance of molecular technology has made it possible to produce a large number of disease model animals, particularly genetically engineered mice. Many of them present with progressive or non-progressive central nervous system manifestations of various severities. At present the neurological status is assessed mainly by gross clinical observations or with sophisticated instruments mainly for evaluation of cortical functions, such as memory, learning, and behavior. The past clinical experience taught us that clinical impression was not always supported by neuropathologic or neurochemical analysis, particularly for rapidly progressive neurological diseases. Sometimes brain pathology was far more severe or extensive than expected by clinically recognizable minimal cerebral dysfunction. Accordingly the neurological course has not been well delineated in many neurogenetic diseases in the mouse species.

For more than 15 years we performed molecular analyses of β-galactosidase deficiency disorders (β-galactosidosis) [1]: cDNA cloning [2], mutation analyses [3,4],

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