16) Matsuda J, Suzuki O, Oshima A, Ogura A et al.: β -Galactosidase-deficient mouse as an animal model for GM1-gangliosidosis. Glycoconjugate J 14: 729-736, 1997

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第 20 回日本小児脂質研究会のお知らせ

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会　　長　獨協医科大学小児科学教授　有阪　治
特別講演　「Developmental Origins of Health and Disease（DOHaD）の視点からみた周産期医療」
昭和大学医学部小児科学　板橋家頭夫
教育講演　「摂食，エネルギー代謝調節と視床下部・全身臓器連携」
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ORIGINAL ARTICLE

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Six novel mutations detected in the GALC gene in 17 Japanese patients with Krabbe disease, and new genotype–phenotype correlation

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Abstract Krabbe disease is an autosomal recessive leukodystrophy. It is pathologically characterized by demyelination of the central and peripheral nervous systems and the accumulation of globoid cells in brain white matter. It is caused by a deficiency of galactocerebrosidase (GALC) activity. We investigated mutations of the GALC gene in 17 Japanese patients with Krabbe disease, the largest subject number of Japanese patients to date, and found 27 mutations. Of these mutations, six were novel, including two nonsense mutations, W115X and R204X, two missense mutations, S257F and L364R, a small deletion, 393delT, and a small insertion, 1719-1720insT. Our findings, taken with the reported mutations in Japanese patients, confirm several mutations common to Japanese patients, the two most frequent being 12Del3Ins and I66M + I289V, which account for 37% of all mutant alleles. With two additional mutations, G270D and T652P, these account for up to 57% of genetic mutations in Japanese patients. Distribution of the mutations within the GALC gene indicated some genotype–phenotype correlation. I66M + I289M, G270D, and L618S contributed to a mild phenotype. Screening for these mutations may provide an effective method with which to predict the clinical phenotype.

Keywords Globoid cell leukodystrophy · Krabbe disease · Galactocerebrosidase · Mutation analysis · Genotype–phenotype correlation

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Introduction

Krabbe disease (globoid cell leukodystrophy: GLD, MIM 245200) is an autosomal recessive neurodegenerative disorder caused by a deficiency of galactocerebrosidase (GALC) (EC 3.2.1.46). It was first reported by Krabbe (1916) under the title "A new familial, infantile form of diffuse brain sclerosis". Approximately 90% of patients have the early infantile form, first manifesting symptoms before 6 months old and experiencing rapid disease progression over the ensuing 1 or 2 years (Wenger et al. 2001). The remaining 10% have late-onset Krabbe disease and are classified into one of three classes, late-infantile type, juvenile type, or adult type, depending on the onset period and the rate of progression of the disease. Molecular cloning of the human GALC gene by Chen et al. (1993) (GenBank Accession No. L23116) and Sakai et al. (1994) (GenBank Accession No. L38544, L38559), has led to molecular-level analyses of Krabbe disease.

More than 60 GALC mutations, all with molecular heterogeneity, have been reported worldwide (Wenger et al. 1997). Whereas several papers (Tatsumi et al. 1995; Furuya et al. 1997; Kukita et al. 1997-98; Satoh et al. 1997; Fu et al. 1999) have reported Krabbe disease mutations in Japanese patients, clear genotype–phenotype correlations remain obscure, because of the small number of subjects studied. We evaluated the GALC gene in 17 Japanese patients, classifying mutations according to clinical phenotype. Here, we report the common mutations and the correlation between such mutations and their clinical severity.

Materials and methods

Patients

We studied 17 unrelated Japanese patients with Krabbe disease, originating from different regions of Japan.

There were no consanguineous marriages between the patients' parents. The subjects included three (patients A1, A2, and A13) included in a previous study who were shown to have only one single mutant allele. The clinical information is summarized in Table 1. Diagnoses were determined in our laboratory as reduced GALC activity in either fibroblasts or leukocytes, and by other characteristic clinical and laboratory findings. According to the age at onset, patients were classified into one of four clinical phenotype groups: infantile onset, aged up to 6 months, nine patients; late-infantile onset, 7 months to 2 years, two patients; juvenile onset, 3–8 years, four patients; and adult onset, over 9 years, two patients.

Amplification of genomic DNA

After informed consent, genomic DNA was prepared, by standard methods, from patients' peripheral blood leukocytes and/or cultured skin fibroblasts and used for the subsequent studies. PCR reactions were conducted in 25-μL reaction volumes containing approximately 100 ng genomic DNA, 1×PCR reaction buffer (50 mmol L^{-1} KCl, 10 mmol L^{-1} Tris-HCl), 1.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTP, 0.2 μmol L^{-1} of each primer, and 1.25 U Taq DNA polymerase (Promega, Madison, USA). The thermal profile consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 51°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 7 min. Each PCR mixture (5 μL) was run on agarose gel to ensure that only the specific product was amplified. Seventeen pairs of primer sequences for amplication of exons and exon–intron boundaries of GALC gene are listed Table 2.

Screening for 12Del3Ins and I66M + I289V by restriction enzyme digestion

For screening of 12Del3Ins (635-646del/insCTC resulting in 212-216 del(NLWES)/ins(TP)), a previously reported and relatively common mutation (Tatsumi et al. 1995; Fu et al. 1999), genomic DNA samples were amplified with the primer pair of exon 7 and the product was digested with *Hin*fI (Fig. 1a). I66M + I289V, first reported by Furuya et al. (1997), is a unique mutation identified in the Japanese population. Only when two single-nucleotide substitutions (I66M, I289V) resided on the same allele was their combination (I66M + I289V) proved to be a pathogenic mutation (Furuya et al. 1997). To detect I66M (198A > G), genomic DNA samples were amplified using a sense primer of exon 2 (Table 2) and a mismatch-antisense primer (5′-TCATTACCTTAAAGAGATAATCCGA-3′). The product was digested with *Eco*RV (Fig. 1b). To detect I289V (865A > G), genomic DNA samples were amplified with a sense primer of exon 9 (Table 2) and a mismatch-antisense primer (5′-ACTAGCCACTAAATTCCAGTCGA-3′) and product was digested with *Sal*I (Fig. 1c). All the digested fragments were subjected to electrophoresis in 3% NuSieve 3:1 agarose gel (BioWhittaker Molecular Applications, Rockland, ME, USA). The PCR amplification of three fragments was performed under the same conditions described above. When both I66M and I289V were heterozygous in a patient, mutation analysis of the patient's parents with screening for I66M and I289V was necessary to clarify whether I66M and I289V resided on the same allele. For all observed digestion patterns different from the normal control, the corresponding fragments were re-amplified for direct sequencing analysis.

Screening for the 30 kb large deletion mutation

To screen for the 30 kb deletion mutation, a previously reported and common mutation in Caucasians, genomic DNA samples were amplified using three primers in accordance with the method described by Luzi et al. (1995).

Denaturing high performance liquid chromatography (DHPLC)

For patients A1–A11, all of the 17 exons and exon–intron boundaries were amplified by polymerase chain reaction (PCR) as described above. DHPLC analysis was performed with the Wave DNA fragment analysis system equipped with a DNASep Column (Transgenomic Omaha, NE, USA). Before mutation analysis the PCR products for each exon were denatured at 94°C for 5 min, followed by gradual re-annealing at 94–25°C over 45 min to enable formation of heteroduplexes. All samples were run at three different oven temperatures, listed in Table 2. PCR-amplified products with a heteroduplex profile were re-amplified and used for direct sequencing analysis.

Reverse-transcription PCR and direct sequencing

For patients with no mutations or only one mutation by common mutation screening or DHPLC, GALC mutation analysis was performed by sequencing cDNA. Total RNA was extracted from cultured skin fibroblasts or lymphocytes and first-strand cDNA synthesis was performed with MMLV reverse transcriptase (Gibco BRL) according to the manufacturer's recommendations. The coding region was amplified by PCR in two overlapping fragments from first-strand cDNA, as described elsewhere (Fu et al. 1999). PCR products were directly sequenced using the BigDye Terminator V1.1 cycle-sequencing kit (Applied Biosystems, Warrington, UK) and the Applied Biosystems 3730 DNA analyzer.

Screening for S257F and L364R in healthy individuals

To screen for two novel missense mutations (S257F, L364R), PCR amplification of genomic DNA from 100

Table 1 Summary of clinical information and mutations identified in Japanese patients with Krabbe disease

Patient no.	Genotype	Phenotype	Age at onset	GALC activity		CSF protein (mg dL^{-1})	MCV (m s^{-1})	Refs.
				Lymphocytes (nmol mg^{-1} h^{-1})	Skin fibroblasts (nmol mg^{-1} h^{-1})			
A1[b]	**W115X**/P302A[a]	Infantile	6 months	Not described	Not described	ND	ND	Tatsumi et al. (1995)
A2	**R204X**/12Del3Ins[a]	Infantile	< 6 months	Not described	Not described	ND	ND	Tatsumi et al. (1995)
A3	**R204X**/I234T	Infantile	< 6 months	0.08	ND	ND	ND	
A4	**L364R**/T652P	Infantile	5 months	0	0.1	81	Undetectable	
A5	12Del3Ins/–	Infantile	6 months	ND	0.27	ND	ND	
A6	T652P/T652P	Infantile	4 months	0.07	0.3	244	Undetectable	
A7	12Del3Ins/T652P	Infantile	6 months	0.1	ND	169	ND	
A8	**393delT**/–	Infantile	4 months	0	ND	185	12	
A9	**S257F**/–	Infantile	6 months	0.3	0.4	236	18	
A10	P302A/L618S	Late-infantile	8 months	0	0.3	119	25	
A11	G270D/R515H	Late-infantile	2 years	0.03	0.08	71	50	
A12	I66M + I289V/ 12Del3Ins	Juvenile	5 years	0.09	0.14	95	24	
A13	I66M + I289V / W647X[a]	Juvenile	3 years	0.6	ND	124	Low	Fu et al. (1999)
A14	I66M + I289V/ **1719-1720insT**	Juvenile	3.5 years	0.09	0.23	75	Low	
A15	12Del3Ins/–	Juvenile	3.5 years	0.12	0.5	34	ND	
A16	G270D/G270D	Adult	69 years	0.2	0.8	ND	ND	
A17	I66M + I289V/ I66M + I289V	Adult	59 years	0.29	0.23	51	17	

Patient no.	Genotype	Phenotype	Age at onset	CSF protein (mg dL^{-1})	MCV (m s^{-1})	Refs.
B1	12Del3Ins/12Del3Ins	Infantile	4 months	ND	ND	Tatsumi et al. (1995)
B2	12Del3Ins/12Del3Ins	Infantile	3 months	106	Low	Tatsumi et al. (1995)
B3	S52F/W410G	Infantile	< 6 months	ND	ND	Fu et al. (1999)
B4	12Del3ins/T652P	Infantile	4 months	ND	13–18	Fu et al. (1999)
B5	R515H/R515H	Infantile	5 months	80	Low	Fu et al. (1999)
B6	T262I/12Del3Ins	Infantile	1 years	42	36	Fu et al. (1999)
B7	G270D/G270D	Adult	10–20 years	Not described	Not described	Furuya et al. (1997)
B8	I66M + I289V/ I66M + I289V	Adult	10–20 years	Not described	Not described	Furuya et al. (1997), Kukita et al. (1997-98)
B9	I66M + I289V/Y354X	Adult	10–20 years	Not described	Not described	Furuya et al. (1997), Kukita et al. (1997-98)
B10	L618S/IVS6 + 5G > A	Adult	10–20 years	Not described	Not described	Furuya et al. (1997), Kukita et al. (1997-98)
B11	L618S/L618S	Adult	51 years	Not described	Not described	Satoh et al. (1997)

Bold print in Genotype column denotes a novel mutation identified in this study
Normal ranges of GALC activity in lymphocytes and skin fibroblasts are 2.1 ± 0.29 and 4.5 ± 1.2 nmol mg^{-1} h^{-1}, respectively
"A" in patient no. column represents the subjects investigated in this study; "B" in patient no. column represents subjects reported previously
"–" in the Genotype column indicates no mutation was found in the second allele in our experiment
CSF cerebrospinal fluid, MCV motor nerve conduction velocity, ND not done
[a]Previously detected mutant allele
[b]The patient was previously reported as late-infantile type. This has, however, been corrected to infantile type on the basis of detailed clinical information

Table 2 Primer sequences used for PCR amplification of GALC gene, and DHPLC oven temperatures

Exon	Forward primer (5′ > 3′)	Reverse primer (5′ > 3′)	Size (bp)	Predicted temperature (°C)	DHPLC oven temperature (°C)
1	GGAGTCATGTGACCCACACA	CGCGTATCCCCGCAGCTT	242	56	55/56/57
2	GGTGTGCGTGAACACTGTAGA	CTATGGTGAAATTCACCATCC	215	54.8	54/55/56
3	GGATGGTGAATTTCACCATAG	TCACAGTCCATATGCTGAGGT	333	54.9	54/55/56
4	GGTCCTAGGAAGTACCATTCATG	CACCAACACGATTCAGAATTTAA	190	56.4	55/58/61
5	GTTTTATTTTCAATAGCGCCAGC	CCTCATGGCATAAAATGGTTAGTC	312	57	55/57/59
6	AATGGTATCGTAACGATAATCTG	TTTCTGTGTTAGGAACCATAAGG	190	54.5	54/55/56
7	CTATTATCCAGAACGCTGATTTG	GTAATCAAATGGGGAGAAGGC	335	54.4	53/55/56
8	GAAAACCTTGGAGAAGACTCGTA	GGCTGGAAGAATAAGGAATTCC	354	53.2	54/56/58
9	CTCCAGGTTTTTAGACATTTAC	CTGCTTTGTCTCTTAGAGAAGA	251	55.7	52/55/58
10	CAGACTCAAATTGATATACAGCT	GGCATCTGTCTGTATGCTTAT	337	53.1	54/56/58
11	TTCTGTTAATCTTGGGCATTAAC	CAGGGCCTCTGTCAATTCATA	317	54.4	52/54/56
12	CATTGGTACATTCTTGCTGGTACT	GTCACCATCCACCAAGACAAA	413	54.5	53.5/54.5/56.5
13	ATTCCAAGGGCCTTGATATTG	TTTGACAGCCACTCCATCATG	413	55.7	54/56/60
14	AATATCAGCAAGGAGAGCTTCTG	GGAGGACCATTGAAAACTCTTC	339	56.7	54/56/58
15	GCATGTGCTGTGAAATGACATA	CCCACAAATAACAAGTAGGTGCT	325	55.1	55.1/56.1/57.1
16	CAGATGCCACTCAAGAACCCC	CCCCTCCTATTTTATAACAG	251	55.1	52/54/55
17	CCATACATGATCAAGAAACAGAC	GAAACAAGAATTGGCTCTGAA	301	57.9	56.9/57.9/58.9

healthy individuals was performed with the primer pair for exon 8 and exon 10 (Table 2) and the product was digested with *Eco* 57I and *Aci* I, respectively. The normal allele of 257S should be digested by *Eco* 57I and the mutant allele of 364R should be digested by *Aci* I.

Results

All 27 mutations detected in this study are listed in Table 1. The analytical procedures for GALC mutation detection are described in detail below.

Screening for 12Del3Ins and I66M + I289V with restriction enzyme digestion

DNA isolated from peripheral blood leukocytes or cultured skin fibroblasts was tested for mutation detection. Screening for 12Del3Ins mutation by restriction enzyme digestion revealed that five patients (A2, A5, A7, A12, A15), including one patient (A2) reported previously, were heterozygous. As a result of screening for I66M + I289V, I66M was found to be homozygous in one patient (A17), heterozygous in three patients (A12, A13, and A14), and I289V was found to be homozygous

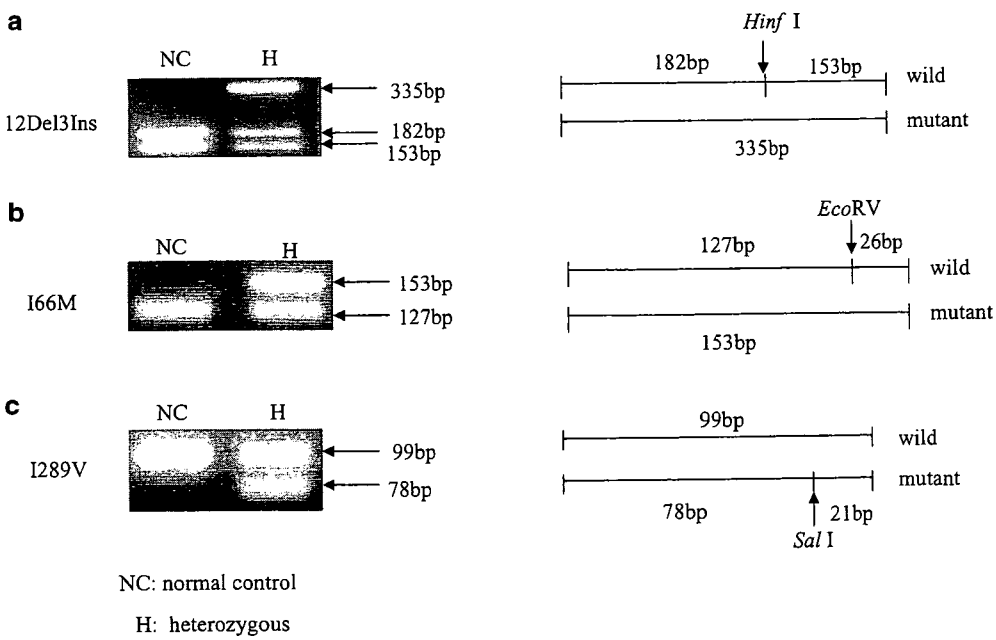

Fig. 1 Mutation detection for 12Del3Ins and I66M + I289V with restriction enzyme digestion. **a** Genomic DNA samples were amplified with the primer pair of exon 7; the product was digested with *Hin*fI and subjected to 3% Nusieve gel. Fragments with 12Del3Ins were not digested with *Hin*fI. **b** For detection of I66M, amplified fragments using a sense primer of exon 2 and a mismatch-antisense primer (5′-TCATTACCTTAAAGAGATAATCCGA-3′) were digested with *Eco*RV. **c** To detect I289V, amplified fragments with a sense primer of exon 9 and a mismatch-antisense primer (5′-ACTAGCCACTAAATTCCAGTCGA-3′) were digested with *Sal*I

in A14 and A17, and heterozygous in A12 and A13. In patients 14 and 17, I66M and I289V were easily confirmed to reside on the same allele. To confirm that I66M and I289V resided on the same allele in patients A12 and A13, their parents' DNA was also analyzed for I66M and I289V. In A12 and A13, both I66M and I289V were proved to reside on the same allele inherited from the father of A12 and the mother of A13, respectively. I289V substitution without I66M in patient A14 was regarded as a polymorphism. These mutations were also confirmed by direct sequencing analysis.

Screening for the 30 kb large deletion mutation

Shortened PCR products from the deleted allele were not detected in any of our patients. The most frequent mutation in Caucasians was not found in our Japanese patients.

Mutation screening with DHPLC and mutation detection with direct sequencing

DHPLC analysis was performed for 11 patients (A1–11). Seventeen abnormal DHPLC elution profiles (A1, exons 4, 9; A2, exon 7; A3, exon 7; A4, exons 10, 12, 17; A5, exon 7; A7, exons 5, 7, 17; A8, exon 4; A9, exons 14, 15; A10, exons 9, 16; A11, exon 14) were found. The corresponding amplicons were characterized by direct sequencing. As a result, four polymorphisms (A7, IVS5+71G>A; A9, IVS14+60C>T; A4, 1254C>T (S418S); and A9, 1637T>C (I546T)) and 15 mutations (A1, W115X, P302A; A2, R204X, 12Del3Ins; A3, R204X, I234T; A4, L364R, T652P; A5, 12Del3Ins; A7, 12Del3Ins, T652P; A8, 393delT; A10, P302A, L618S; A11, R515H) were identified in the above 17 amplicons by direct sequencing. Within four polymorphisms, two (IVS5+71G>A and IVS14+60C>T) were novel intronic polymorphisms and the other two (1254C>T (S418S) and 1637T>C (I546T)) were exonic polymorphisms which have previously been reported (Sakai et al. 1994; Kukita et al. 1997-98). Within 15 mutations, 11 mutations were first identified by DHPLC; the exceptions were two mutations (A1, P302A; A2, 12Del3Ins) identified in the previous study (Tatsumi et al. 1995) and two mutations (A5, A7, 12Del3Ins) already detected in the screening step for 12Del3Ins. Four different novel mutations (W115X, R204X, L364R, and 393delT) were found in this step.

Reverse-transcription PCR and direct sequencing

Two mutations were identified in nine patients (A1, 2, 3, 4, 7, 10, 12, 13, 17) by use of the screening methods described above. Only one mutation (12Del3Ins, 393delT, R515H, I66M+I289V, and 12Del3Ins) was found in five patients (A5, 12Del3Ins; A8, 393delT; A11, R515H; A14, I66M+I289V; A15, 12Del3Ins). In the remaining three patients (A6, 9, 16), no mutations were found. To identify other mutations, mutation analysis by RT-PCR and direct sequencing were performed for eight patients (A5, 6, 8, 9, 11, 14, 15, 16), and four different mutations, the exceptions being the mutations detected by use of the above methods, were detected in five patients (T652P homozygous in A6, S257F heterozygous in A9, G270D heterozygous in A11, 1719-1720insT heterozygous in A14, and G270D homozygous in A16). In patient A14, I289V was also confirmed on the same allele as 1719-1720insT; I289V is regarded as a polymorphism, however, because substitution of I289V without I66M has been proven to be polymorphism (Furuya et al. 1997) and the 1719-1720insT resulted in frame shift leading to a premature stop codon. Two mutations (S257F and 1719-1720insT) were novel. These mutations were also confirmed by direct sequencing of genomic DNA.

Screening for S257F and L364R in healthy individuals

Of the six novel mutations (W115X, R204X, S257F, L364R, 393delT, and 1719-1720insT), two missense mutations (S257F, L364R) were screened by restriction enzyme digestion. These mutations were not detected in 100 healthy controls.

Genotype–phenotype correlation

The detected mutations are summarized in Table 1 with reference to reported mutations in Japanese patients. Table 1 includes other clinical information about the patients. Fourteen different mutations identified in this study were found in 27 detected alleles of 17 patients, including nine missense mutations (I66M+I289V, I234T, S257F, G270D, P302A, L364R, L618S, T652P), two nonsense mutations (W115X and R204X), one small deletion (393delT), one small insertion (1719-1720T), and one deletion/insertion (12Del3Ins). Of these mutations, six were novel (W115X, R204X, S257F, L364R, 393delT, and 1719-1720insT). All GALC mutations in Japanese patients, with their frequencies, including those previously reported, are summarized in Table 3.

The distribution of the clinical phenotype for each mutation is summarized in Table 4. For rare mutations, those detected less than twice, the genotype–phenotype correlation remains indeterminate; for rather common mutations, however, the tendency between genotype and phenotype was observed. The phenotype for 12Del3Ins, T652P, and R515H was observed mostly in infantile-type Krabbe disease whereas I66M+I289V, G270H, and L618S were mostly detected in the adult type and never in the infantile form of the disease. Concerning the regional distribution of patients, there was no specific region for each mutation.

Table 3 Summary of mutations identified in 28 Japanese patients including 11 patients previously characterized

No.	Exon/intron	Nucleotide change	Amino acid change	Allele frequency	Allele number
1	2	198A > G +865A > G	I66M + I289V	0.15	8
2	2	155C > T	S52F	0.02	1
3	4	**344G > A**	**W115X**	0.02	1
4	4	**393delT**	**L130fs; 154X**	0.02	1
5	IVS6	IVS6 + 5G > A	179–191 skipping	0.02	1
6	7	**610C > T**	**R204X**	0.04	2
7	7	635-646 del/ins CTC (12Del3Ins)	212-216 del (NLWES)/ins (TP)	0.22	11
8	7	701T > C	I234T	0.02	1
9	8	**770C > T**	**S257F**	0.02	1
10	8	785C > T	T262I	0.02	1
11	8	809G > A	G270D	0.1	5
12	9	904C > G	P302A	0.04	2
13	10	1062C > G	Y354X	0.02	1
14	10	**1091T > G**	**L364R**	0.02	1
15	12	1228T > G	W410G	0.02	1
16	14	1544G > A	R515H	0.06	3
17	15	**1719-1720insT**	**R574X**	0.02	1
18	16	1853T > C	L618S	0.08	4
19	17	1941G > A	W647X	0.02	1
20	17	1954A > C	T652P	0.1	5
Total				1	52

Bold print denotes novel mutations identified in this study. "fs" indicates a frameshift starting after the respective codon

Discussion

In this work we detected 27 mutant alleles in 17 patients. We found six novel mutations of the GALC gene in Japanese patients with Krabbe disease. Two, W115X and R204X, were nonsense mutations whereas 393delT and 1719-1720insT resulted in frame shifts. It was obvious all four were pathogenic mutations. The final two, S257F and L364R, were missense mutations that were not detected in 100 healthy controls. These two loci are well conserved in different species, including monkey, dog, and mouse (Luzi et al. 1997). This evidence, when taken together, suggests that these two missense mutations may be regarded as causative of Krabbe disease.

Table 4 Correlation between genotype and phenotype in 28 Japanese subjects

Mutation	Infantile	Late-infantile	Juvenile	Adult	Total allele
12Del3Ins	9	0	2	0	11
T652P	5	0	0	0	5
R515H	2	1	0	0	3
R204X	2	0	0	0	2
P302A	1	1	0	0	2
S52F	1	0	0	0	1
W115X	1	0	0	0	1
393delT	1	0	0	0	1
I234T	1	0	0	0	1
S257F	1	0	0	0	1
T262I	1	0	0	0	1
L364R	1	0	0	0	1
W410G	1	0	0	0	1
1719-1720insT	0	0	1	0	1
W647X	0	0	1	0	1
IVS6 + 5G > A	0	0	0	1	1
Y354X	0	0	0	1	1
I66M + I289V	0	0	3	5	8
G270H	0	1	0	4	5
L618S	0	1	0	3	4
Total allele	27	4	7	14	52

In four patients, only one mutant allele was detected. Because our screening method covered the coding region and the exon–intron boundary, undetected mutations may lie outside the analyzed region, for example in promoter and enhancer regions. We also did not analyze large-scale genomic rearrangement, other than the screened 30 kb deletion.

As reported in the literature, mutations of the GALC gene in Krabbe disease are very heterogeneous. In Caucasians, the 30 kb large deletion reported by Rafi et al. (1995) and Luzi et al. (1995) was widespread within the patient population, having a frequency of 40–50%. Other mutations followed heterogeneous patterning. As in Caucasians, to date, examination of Japanese patients revealed 14 mutations with a very heterogeneous distribution. It was considered hard to screen the GALC mutation, because there is no common mutation in Japanese patients; as our results show, however, there are several common mutations. The most common mutation in Japanese patients is 12Del3Ins with a 0.22 allele frequency. The second most frequent mutation, I66M + I289V, has 0.15 prevalence. To date, these two mutations have been identified in Japanese patients only. These two mutations are also unique and difficult to conceive as recurrently occurring mutations. They might be derived from a founder; parents' samples necessary for haplotype analysis were unavailable from most of the families, however.

Referring to Tables 1 and 4, the most common mutation (12Del3Ins) and the two other mutations

(T652P and R515H) in the homozygous state (B1, B2, A6, and B5) resulted in the classic infantile phenotype. The second most common mutation, I66M + I289V, contributed to late-onset-type Krabbe disease, because the homozygous state of this mutation was found only in the adult type (patients A17 and B8), the mildest form of the disease, whereas the heterozygous state was detected in the juvenile or adult form (patients A12, A13, A14, and B9). This concordance strongly suggests that the existence of this mutation leads to the mild clinical phenotype. Because screening for this mutation might directly reveal a mild phenotype of Krabbe disease in Japanese patients, we propose a viable screening method using restriction enzyme digestion with PCR fragments for I66M (Fig. 1b) and I289V (Fig. 1c). For the missense mutations G270D and L618S, similar concordance is shown in Tables 1 and 4, demonstrating that it will be effective to screen for this mutation to estimate the mild phenotype.

For most enzyme-deficiency diseases it is generally believed there is a correlation between residual enzyme activity and clinical severity; this does not appear to be true for Krabbe disease, however. The expression experiment did not always reveal better residual activity in late-onset patients. Harzer et al. (2002) analyzed substrate specificity for several mutations and, although the G270D mutation led to loss of enzymatic activity for galactocerebroside as its natural substrate, nearly normal activity for psychosine, its second substrate, was preserved. This paper supports the idea that measurement enzyme activity with one substrate does not necessarily lead to identification of an essential defect. It might be important to analyze the substrate specificity of L618S and I66M + I289V to elucidate genotype–phenotype correlation.

In a previous study, Furuya et al. (1997) investigated I66M + I289V allele expression, and found reduced enzymatic activity only when these two amino acid changes occurred on the same allele. We likewise confirmed that I66M and I289V occurred on the same strand and same peptide in all four patients. That I66M and I289V reside on the same strand suggests that each amino acid contributes to a different function, for example reaction center or substrate binding. Analysis of the structure of the crystallized protein might be important for future understanding of the mechanism of this mutation.

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References

Chen YQ, Rafi MA, de Gala G, Wenger DA (1993) Cloning and expression of cDNA encoding human galactocerebrosidase, the enzyme deficient in globoid cell leukodystrophy. Hum Mol Genet 2:1841–1845

Fu L, Inui K, Nishigaki T, Tatsumi N, Tsukamoto H, Kokubu C, Muramatsu T, Okada S (1999) Molecular heterogeneity of Krabbe disease. J Inherit Metab Dis 22:155–162

Furuya H, Kukita Y, Nagano S, Sakai Y, Yamashita Y, Fukuyama H, Inatomi Y, Saito Y, Koike R, Tsuji S, Fukumaki Y, Hayashi K, Kobayashi T (1997) Adult onset globoid cell leukodystrophy (Krabbe disease): analysis of galactosylceramidase cDNA from four Japanese patients. Hum Genet 100:450–456

Harzer K, Knoblich R, Rolfs A, Bauer P, Eggers J (2002) Residual galactosylsphingosine (psychosine) beta-galactosidase activities and associated GALC mutations in late and very late onset Krabbe disease. Clin Chim Acta 317:77–84

Krabbe K (1916) A new familial, infantile form of diffuse brain sclerosis. Brain 39:74–114

Kukita Y, Furuya H, Kobayashi T, Sakai N, Hayashi K (1997-98) Characterization of the GALC gene in three Japanese patients with adult-onset Krabbe disease. Genet Test 1:217–223

Luzi P, Rafi MA, Wenger DA (1995) Characterization of the large deletion in the GALC gene found in patients with Krabbe disease. Hum Mol Genet 4:2335–2338

Luzi P, Rafi MA, Victoria T, Baskin GB, Wenger DA (1997) Characterization of the rhesus monkey galactocerebrosidase (GALC) cDNA and gene and identification of the mutation causing globoid cell leukodystrophy (Krabbe disease) in this primate. Genomics 42:319–324

Rafi MA, Luzi P, Chen YQ, Wenger DA (1995) A large deletion together with a point mutation in the GALC gene is a common mutant allele in patients with infantile Krabbe disease. Hum Mol Genet 4:1285–1289

Sakai N, Inui K, Fujii N, Fukushima H, Nishimoto J, Yanagihara I, Isegawa Y, Iwamatsu A, Okada S (1994) Krabbe disease: isolation and characterization of a full-length cDNA for human galactocerebrosidase. Biochem Biophys Res Commun 198:485–491

Satoh JI, Tokumoto H, Kurohara K, Yukitake M, Matsui M, Kuroda Y, Yamamoto T, Furuya H, Shinnoh N, Kobayashi T, Kukita Y, Hayashi K (1997) Adult-onset Krabbe disease with homozygous T1853C mutation in the galactocerebrosidase gene. Unusual MRI findings of corticospinal tract demyelination. Neurology 49:1392–1399

Tatsumi N, Inui K, Sakai N, Fukushima H, Nishimoto J, Yanagihara I, Nishigaki T, Tsukamoto H, Fu L, Taniike M, Okada S (1995) Molecular defects in Krabbe disease. Hum Mol Genet 4:1865–1868

Wenger DA, Rafi MA, Luzi P (1997) Molecular genetics of Krabbe disease (globoid cell leukodystrophy): diagnostic and clinical implications. Hum Mutat 10:268–279

Wenger DA, Suzuki Ku, Suzuki Y, Suzuki Ki (2001) Galactosylceramide lipidosis: globoid cell leukodystrophy (Krabbe disease). In: Scriver CR, Beaudet AL, Sly WS,Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw–Hill, New York, pp 3669–3694

Ⅷ. 酵素補充療法

Gaucher病

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要旨

Gaucher病の酵素補充療法は，臨床的な効果が確認された初めての酵素補充療法である．日本においては，全病型に健康保険適用が認められている．副作用もほとんどなく，貧血，血小板減少，肝脾腫などには著明な効果を示す．一方，神経症状，骨症状の改善には課題を残している．酵素補充療法の効果と限界を知り，患者さんの利益を考慮して酵素補充療法を行うことが重要である．

Key Words
Gaucher病
酵素補充療法

Gaucher病とは

Gaucher病は，ライソゾーム酵素であるグルコセレブロシダーゼ活性の遺伝的酵素活性低下により，その基質であるグルコセレブロシドという糖脂質が，主として肝臓，脾臓，骨髄などの細網内皮系に蓄積するため，肝脾腫，骨症状を主症状とし，検査学的には貧血，血小板減少，酸性ホスファターゼ高値，アンギオテンシン変換酵素高値などを特徴とする．神経症状の有無とその重症度により，1型（非神経型），2型（急性神経型），3型（亜急性神経型）に分類されている．いずれの病型においても，その臨床表現型に異質性が存在するのが特徴である．

Gaucher病に対する酵素補充療法の歴史

Gaucher病などのライソゾーム病においては，1960年代に酵素欠損が次々に明らかにされた．1970年代に入り，欠損酵素を補充してこれらライソゾーム病を治療する試みが行われたが，その有効性は認められなかった．この要因は，酵素の臓器移行性に関する概念が欠如していたためである．すなわち，酵素を静脈内投与しても罹患臓器にターゲットすることができなかったのである．

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1980年代になり，ライソゾーム酵素の生化的分析が行われ，臓器移行性には酵素の糖鎖末端が重要な働きをすることが明らかとなった．さらにグルコセレブロシダーゼの大量精製法が開発された．これらの知見により，胎盤から抽出した天然型グルコセレブロシダーゼの糖鎖末端を，高マンノース型に修飾した製剤が1990年に開発された．そして，実際に患者さんに投与したところ肝脾腫，貧血，血小板減少は著明に改善した．

この報告は，酵素補充療法が臨床的に有効であることを示す画期的な結果であった．大量生産が可能であること，非加熱製剤におけるウイルスの混入が避けられることなどの利点があるので，現在では，リコンビナント技術を用いた酵素製剤が用いられている（商品名：セレザイム®）．

Gaucher病に対する酵素補充療法の実際

Gaucher病は，前述したように1型，2型，3型に分類されているが，わが国では，どの病型においても酵素補充療法は健康保険収載されており，適用がある．

初期投与量は年齢にかかわらず，60単位/kg/回を2週間ごとに投与するのが標準的である．1アンプルは200単位であり，必要投与量を100 mlの生理的食塩水で溶解して，1時間かけて点滴静注する．蛋白製剤なのでフィルターを用いて夾雑物を除去すること，酵素製剤なので失活を防ぐため投与直前に溶解すること，同様に失活を防ぐため溶解時に泡立てないようにすることは，実際上の留意点である．

酵素製剤は，高分子蛋白なのでアレルギー反応が問題になることがあるが，Gaucher病の酵素補充療法においてはごくまれである．じんま疹，発熱，悪寒などのアレルギー反応を呈する患者さんに対しては，点滴速度を1/2に落とすか，アタラックスP®などの抗ヒスタミン薬，あるいはソルコーテフ®のようなステロイド薬を適宜，静注する．

症状の改善により初期投与量を漸減できるが，骨症状の改善には少なくとも2年以上，初期投与量を維持することが重要である．この骨症状の評価には単純X線では不十分で，大腿骨頭を含めたMRIによる評価が必須である．酵素製剤の骨への取り込みは，肝臓，脾臓に比較して不良なので，安易な酵素量の減量は病的骨折，骨クリーゼなどの骨合併症の発症をきたすので，留意する必要がある（後述）．また，Gaucher病は臨床表現型において異質性が強く，また酵素製剤が高額のため，その至適投与量については議論が多い（後述）．

Gaucher病の治療費はきわめて高額なので，患者さんが18歳未満であれば小児慢性特定医療の，18歳以上であれば難病医療費助成の認可が得られた後，投与を開始すべきである．また，過量投与は健康保険の査定対象になるので，症状詳記を記載することが重要である．

Gaucher病に対する酵素補充療法の効果

図1に60単位/kg/回，2週間ごと投与を3年間継続した自験例の酵素補充療法の効果を示す．患者さんは比較的重症で，著明な肝脾腫，貧血，血小板減少を呈していたが，貧血は治療開始後，6カ月程度で，肝脾腫，血小板減少は3年の経過で改善している．

比較的多数の国際的な症例の結果を報告しているWeinrebのデータを図2に示す[1]．図2は，脾摘していない1型Gaucher病に対する効果をヘモグロビン値，血小板値，脾容積を指標にして検討した結果であるが，いずれの指標も酵素補充療法により改善している．ただし，2年間

の治療にもかかわらず，平均血小板値，平均脾容積は正常化していない．この研究では投与酵素量に関する記載がなく，投与酵素量を含めた投与方法が，この結果に影響を及ぼしていると考えられる．

神経症状に対する酵素補充療法の効果のまとめを表1に示す．これによると2型，3型ともに神経症状は進行しているか，改善がない[2]．3型Gaucher病21例に対する酵素補充療法の効果についてまとめたAltarescuらの報告[3]でも，ほとんどの例で肝脾腫，骨症状，血液学的異常などの全身症状は改善していた．しかし多くの患者で，神経学的退行は認めなかったものの改善は認められなかった．そのため，肺および中枢神経症状を改善させるためには，新たな治療方策が必要であると結論している．またVellodiらは，神経型Gaucher病に対する勧告治療ガイドラインを提唱している（表2）．しかしながら，患者さんの臨床的異質性，およびわが国での健康保険システムなどの問題があり，実際には，わが国での治療方針として採用するには問題が多い．

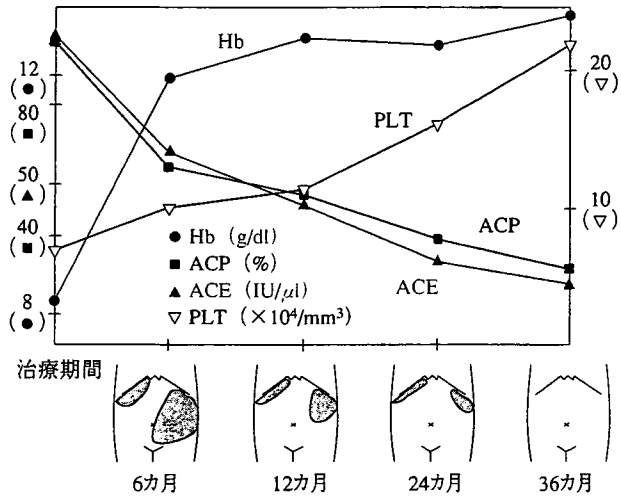


図1　重症1型Gaucher病に対する酵素補充療法の効果（自験例）

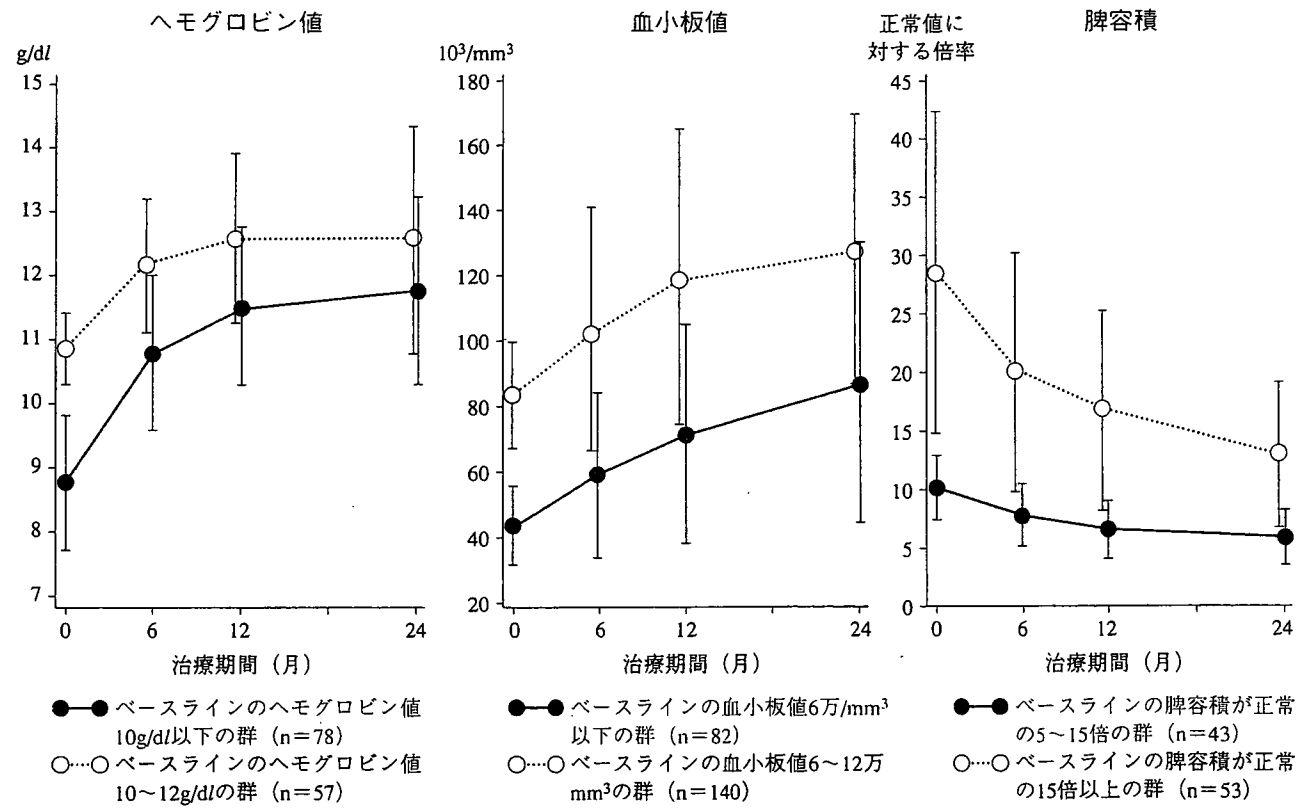


図2　1型Gaucher病の酵素補充療法の効果（文献1）より引用）

Gaucher病に対する酵素補充療法の課題

前述したように，Gaucher病は臨床表現型に異質性が強く，また酵素製剤が高価なため，その至適投与量あるいは漸減法の設定が一つの課題である．国際的には60単位/kg/回を2週間ごとに投与する方法（120単位/kg/月），2.3単位/kg/回を週に3回投与する方法（30単位/kg/月），1.15単位/kg/回を週に3回投与する方法（15単位/kg/月）が提唱されている．これらの治療法を考えるうえで，遺伝子型を含めた各症例の重

表1　神経型Gaucher病に対する酵素補充療法の効果のまとめ（文献2）より引用）

(1) 2型Gaucher病

報告	治療開始時期	投与量	転　帰
1	生後1日	120単位/kg × 1/週	生後7カ月時に神経学的進行 （生後11カ月で生存）
2	生後4日	35単位/kg × 1/週	脳幹機能の退行 （生後15カ月で死亡）
3	生後1カ月 （生後9カ月から）	220単位/kg/月を静注 （160単位/月 × 2/週を随腔内投与）	改善なし
4	生後5カ月 （生後6カ月から）	400単位を隔日投与 （400単位× 2/週に減量）	発達の改善あり，しかし球麻痺症状の改善なく生後7カ月で治療中止 （生後13カ月で死亡）
5	生後7カ月 （生後12カ月から）	120 ～ 240単位/kg/月を静注 （10単位/kg/月を髄腔内投与）	神経学的改善あり，ただし重症心身障害児 （生後39カ月で生存）
6	生後7カ月	60単位/kg/月，隔週投与	生後11カ月で神経学的退行
7	生後9カ月	120 ～ 240単位/kg/月	臨床的改善なし （生後15カ月で死亡）
8	生後14カ月	60 ～ 75単位/kg × 3/週	生後18カ月より退行 （生後25カ月で死亡）
9	2歳	60単位/kg/月，隔週投与	改善なし （生後2歳6カ月で死亡）

(2) 3型Gaucher病

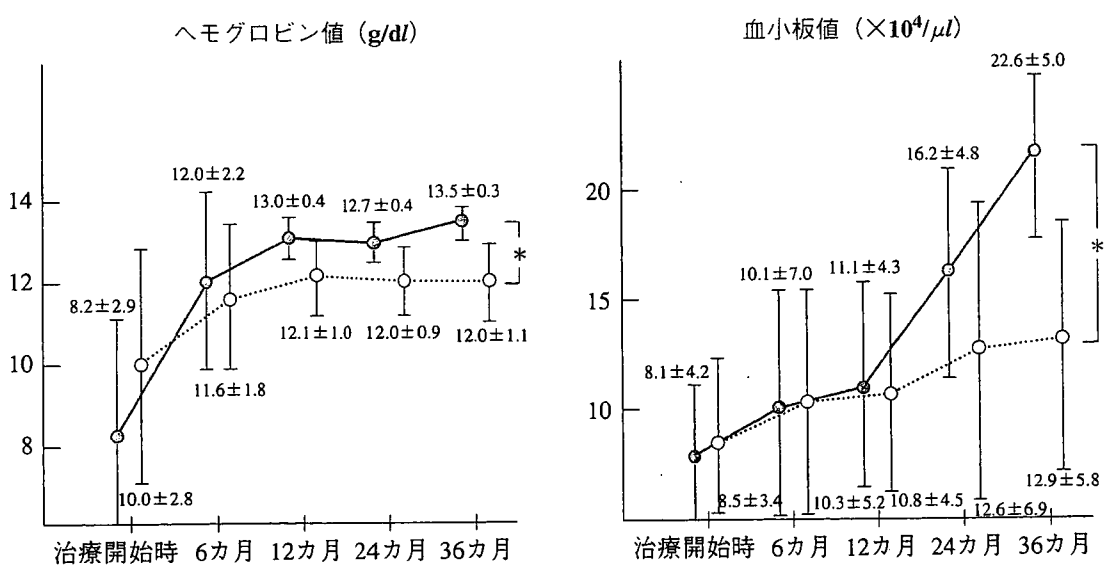
報告	患者数	治療開始時期	投与量（投与期間）	転　帰
1	1例	16カ月	60単位/kg/月，隔週投与	眼球運動失行のみ
2	2例	20カ月，15歳	50 ～ 60単位/kg/月	神経学的評価不完全
3	5例	2 ～ 7歳	30単位/kg/月，隔週投与 （1 ～ 4年間）	眼球運動失行変化なし 1例で神経学的退行 1例で眼球運動失行出現
4	3例	3歳，6歳6カ月，24歳	60単位/kg/月，隔週投与（1 ～ 4年間）	全例で神経学的退行
5	5例	3歳6カ月～8歳6カ月	120 ～ 480単位/kg/月 （1年6カ月～5年間）	全例で神経学的退行
6	8例	4 ～ 42歳	60 ～ 120単位/kg/月（26 ～ 42カ月）	神経学的退行なし
7	1例	7歳	120単位/kg/月（24カ月）	神経学的徴候の改善なし

症度の把握が重要である．日本人Gaucher病の遺伝子変異分布は欧米人のそれとは異なり，軽症型にリンクするN370S変異がまったく存在しない．それゆえ日本人においては重症型の頻度が高い．

図3に小児期発症の日本人Gaucher病におけ

表2　神経型Gaucher病に対する勧告治療ガイドライン（文献4）より引用）

1. 最近，酵素補充療法はひとつの治療選択である
2. 以下のグループは治療を受けるべきである
   (1) 慢性神経型Gaucher病患者
   (2) 神経型Gaucher病の同胞をもつ患者
   (3) L444P/L444P, D409H/D409H, L444P/D409Hの遺伝子型のGaucher病患者
3. 同定されたら，あるいは診断されたら可及的速やかに酵素補充療法を開始すべきである（120単位/kg/回，2週間ごと投与）そして，
   (1) 患者が成人に達し，そして明らかに軽微なGaucher病を呈し，神経症状が安定している
   この時点で継続投与量の減量を考慮したほうがよいかもしれない
   (2) 神経症状が進行している．この時点で短期間（6カ月を超えない），投与量を2倍，
   すなわち240単位/kg/回，2週間ごと投与を考慮すべきである
4. もし，投与量を増加させたのにもかかわらず，神経学的徴候がQOLを受け入れられないように変化する程進行する場合は酵素量はGaucher病の全身症候をコントロールするレベルまで減少させるべきでる
5. 神経症状はないが，神経型Gaucher病のリスクを持つすべての患者は少なくとも2週間ごと，最少60単位/kg/回の酵素補充療法を受けるべきである．そして，注意深いモニターを続けるべきである
6. 2型Gaucher病タイプA（認識能障害，不機嫌，錐体路症状が軽微なもの）の治療を許可しない十分な根拠はない．このような患者は特別なセンターで1カ月ごとのフォローアップを受けながら120単位/kg，2週間ごとの6カ月間の試験投与をなされるべきである．しかしながら，2型Gaucher病タイプB（認識能障害，不機嫌，錐体路症状が著明なもの）に対して現在のところ酵素補充療法は推奨できない
7. 腹部臓器症状をコントロールするためには，推奨投与量よりも高単位投与が必要かもしれない
8. 脾摘は緊急時を除いては禁忌である．そのような場合，全脾摘よりむしろ部分脾摘が考慮されるべきである．このような患者は全脾摘された患者のように治療されるべきである



○…○：60単位/kg/回，隔週投与を6カ月間，30単位/kg/回，隔週投与を6カ月間そして15単位/kg回，隔週投与を維持療法として行った群（n＝5）

○—○：60単位/kg/回，隔週投与を維持した群（n＝5）　　＊：P<0.05

図3　日本人Gaucher病の投与酵素量が効果に与える影響

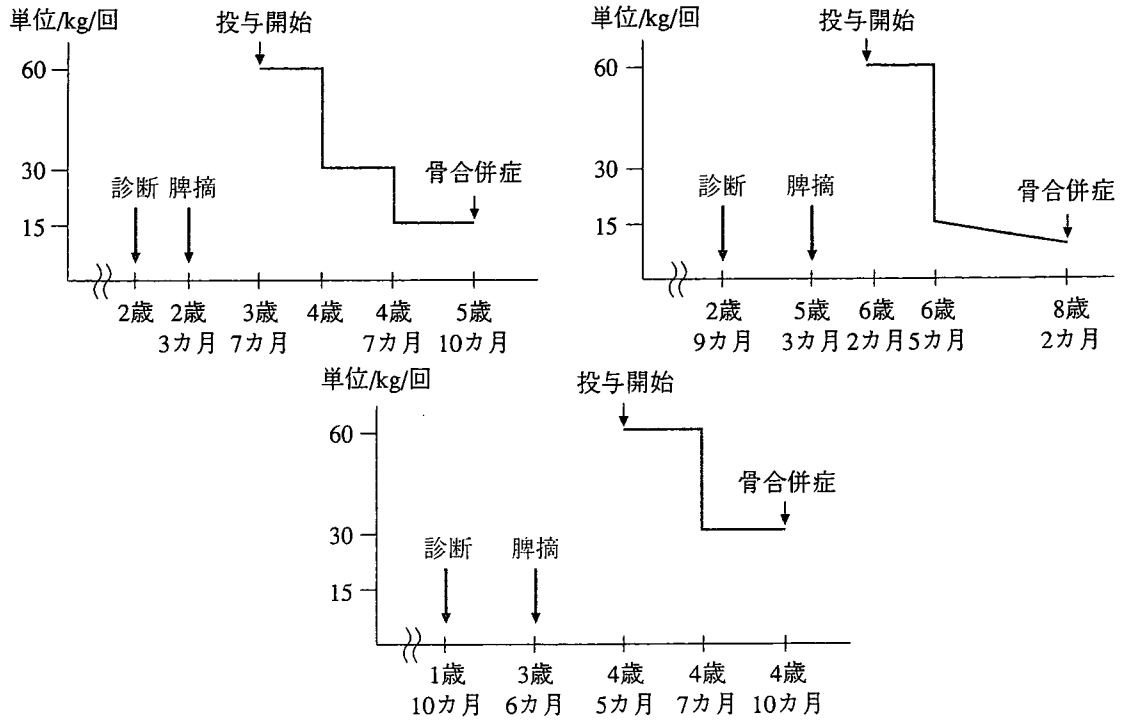


図4　酵素補充療法中に骨合併症を発症した日本人 Gaucher 病の3例のまとめ

る投与量による効果の相違を示す[5]．60単位/kg/回を2週間ごとに3年間投与した群と60単位/kg/回，2週間ごと投与を6カ月継続し，その後，投与量を漸減した群のヘモグロビン値と血小板値を示した．漸減した群においては3年間の治療にもかかわらず，十分なヘモグロビン値，および血小板値の改善は認められず，3年後の値は統計学的有意差を呈していた．このデータは小児期発症の日本人 Gaucher 病においては，高単位（60単位/kg/回）の酵素量を長期間継続投与する必要性を示している．

また前述したように，酵素の取り込みは各臓器によって異なり，とくに骨への取り込み不良は臨床上，問題をおこすことがあるので，今後の課題と考えられる．日本人 Gaucher 病は重症型の頻度が高く，重篤な骨合併症は57％に認められ，しかも酵素補充療法中にもかかわらず，骨合併症を認めた症例も報告されている[6]．

図4は，これら3例の臨床経過を要約したものである．いずれも幼小児期発症で，脾摘を施行されており，貧血，血小板減少が著明な症例であった．さらに初期投与量である60単位/kg/回，2週間ごとの投与を6カ月以内に減量している．遺伝子型は日本人 Gaucher 病に頻度の高い重症型遺伝子変異である L444P/F213I の compound heterozygote である．このデータは血液学的所見が改善したとしても，安易な酵素量の減量は骨合併症を発症することを示している．とくに重症型の遺伝子変異をもち，幼小児期発症で，脾摘されている症例では注意しなければならない．

まとめ

Gaucher 病に対する酵素補充療法はきわめて安全で，血液学的異常，肝脾腫への効果は著明である．日本でも現在までに，約100名の Gaucher 病の患者さんが酵素補充療法の恩恵を受けている．しかしながら，上記のような課題，問題もあり，酵素製剤の更なる改善が期待される．

●文　献

1) Weineb NJ et al.: Effectiveness of enzyme replacement therapy in 1028 patients with type 1 Gaucher disease after 2 to 5 years of treatment. Am J Med 113:112-119, 2002
2) Elstein D et al.: Ethical considerations for enzyme replacement therapy in neuronopathic Gaucher disease. Clin Genet 54:179-184, 1998
3) Altarescu G et al.: The efficacy of enzyme replacement therapy in patients with chronic neuronopathic Gaucher's disease. J Pediatr 138:539-547, 2001
4) Vellodi A et al.: Management neuronopathic Gaucher disease: A European consensus. J Inhert Metab Dis 24:319-327, 2001
5) Ida H et al: Effect of enzyme replacement therapy in thirteen Japanese pediatric patients with Gaucher disease. Eur J Pediatr 160:21-25, 2001
6) Ida H et al: Severe skeletal complication in Japanese patients with type 1 Gaucher disease. J Inhert Metab Dis 22:67-73, 1999

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第5回日本乳・幼児側弯症研究会のお知らせ

会　　期　平成18年11月23日（木曜）9時より
会　　場　ぱ・る・るプラザ京都（TEL 075-352-7444）
JR京都駅中央改札口出て右手すぐ（3分）
参加申込　事前登録をお願いしております．11月9日（木曜）までにご氏名，〒，住所，勤務先，所属，電話・FAX番号，参加希望人数（氏名）を名記のうえ，郵便またはFAXで事務局宛にお申し込みください
参 加 費　1,000円（当日お支払い下さい）

事 務 局　〒108-0073　東京都港区三田1-4-17
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Characterization of the sensitivity to various stress agents in Roberts syndrome lymphoblastoid cell lines

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ABSTRACT

Hypersensitivity to DNA damaging agents has been useful in elucidating the role that genes mutated in chromosomal instability syndromes have in DNA damage sensing or repair pathways. Roberts syndrome (RBS) is a developmental disorder characterized by tetraphocomelia growth retardation and craniofacial abnormalities. Cells from RBS affected individuals exhibit premature separation of heterochromatic regions and cell cycle defects. We recently found that RBS is caused by mutations in ESCO2 gene, a human homolog of yeast ECO1/CTF7 that is essential for sister chromatid cohesion. It has been proposed that sister chromatid cohesion is involved not only in accurate segregation of chromosomes but also in other cellular processes such as DNA damage repair. To gain insight into the pathophysiology of RBS and ESCO2 function, we investigated the sensitivity of RBS lymphoblastoid cell lines to a diverse set of stress agents including DNA damaging agents. We found that RBS cells show increased cell death in response to mitomycin C (MMC) but not to camptothecin treatment, indicating selective sensitivity to the mechanism of DNA damage. Cell cycle analysis of RBS cells treated with a low dose of MMC (0.3μM) showed accumulation of cells in S phase at 18 to 24h and later a G2/M arrest with subsequent apoptosis. Treatment with higher dosages (1.4 μM) resulted in S-phase arrest and induced a similar high level of apoptosis in RBS and normal cells. RBS cells were also more sensitive to G418 and sodium orthovanadate, but not to staurosporine or okadaic acid. Despite the apparent absence of cell cycle disturbance after G418 or orthovanadate treatment we found that, 24 and 48 hr after orthovanadate treatment, p53 induction was greater in RBS cells, implicating a p53 triggered cell death. These observations will help define the key defect in Roberts Syndrome.

Key words: sister chromatid cohesion, Roberts syndrome, ESCO2, mitomycin C, sodium orthovanadate, cell death

INTRODUCTION

Well-characterized cell mutants have been crucial to delineating the role of genes mutated in disorders associated with either defects in response to DNA damage or repair. There are at least 15 known human genetic disorders associated with such defects representing deficiencies in approximately 35 genes (1). Cells from each of these diseases are specifically sensitive to different classes of DNA-damaging or stress inducing agents (2). Roberts Syndrome (RBS) patients and cells share some of these characteristics.

RBS (MIM 268300) is an autosomal recessive developmental disorder characterized at the clinical level by pre- and post-natal growth retardation, craniofacial anomalies and tetraphocomelia. Craniofacial anomalies include bilateral cleft lip and palate, microbrachycephaly, hypertelorism, downslanting palpebral fissures, hypoplastic alae nasal, dysplastic and low set ears, malar hypoplasia, and micrognathia (3, 4).

At the cellular level, RBS is associated with abnormalities of different aspects of the cell cycle with the most remarkable phenotype resulting from the loss of cohesion at heterochromatic regions of centromeres and the long arm of the Y chromosome (5, 6). Aneuploidy, a 30 fold reduced plating efficiency for fibroblast cultures, micronucleation and lobulated nuclei have also been described (7, 8). A further characteristic of Roberts syndrome cells is an increased sensitivity to cell killing by DNA damaging agents such as UV irradiation, methylnitrosourea, mitomycin C (MMC), cisplatin and γ irradiation (9, 10, 11).

We recently identified mutations in *ESCO2* in individuals with RBS (12). *ESCO2* encodes a human homolog of *Saccharomyces cerevisiae* Eco1/Ctf7, an essential protein for the establishment of sister chromatid cohesion (13, 14). As in the case of RBS cells, mutants of this gene family present sister chromatid defects across different species (13, 14, 15, 16).

Sister chromatid cohesion plays a fundamental role preventing missegregation of chromosomes that may result in birth defects, and is frequently associated with cancer (17). Sister chromatids are held together by a protein complex known as cohesin that consists of at least four subunits: Smc1, Smc3, the kleisin subunit Scc1 (also called Rad21 or Mcd1), and Scc3 (18). To establish cohesion, cohesin must be loaded by Scc2 and Scc4, and then actual establishment of cohesion is performed in a process in which Eco1 plays a central role (13, 14). Mutants of smc1, scc1, scc2 and eco1 are all hypersensitive to γ-irradiation and are deficient in postreplicative double-strand break (DSB) repair pointing to a link between cohesion and DNA damage repair (19, 20, 21).

In this report we explore the cause underlying the hypersensitivity of RBS cells to DNA damaging and other stress agents. As RBS cells are known to exhibit impaired cell growth capabilities we exploited flow cytometry and fluorescein diacetate (FDA) to overcome the need for large cell numbers. We found that growth inhibition, previously reported for MMC and G418, is instigated by cell death. We also report that RBS cells are sensitive to sodium orthovanadate (OV), an inhibitor of tyrosine phosphatases, and show that the response to OV involves p53 deregulation. We show that apoptosis induction is common to the three drugs but cell cycle perturbations are induced exclusively by MMC.

MATERIALS AND METHODS

Cell lines and Growth Conditions

EBV-inmortalized LCLs were established by infecting peripheral blood lymphocytes from either normal donors or RS patients with the EBV strain B95-8 (22). Blood samples were taken after informed consent of the donors. Established LCLs lines were always cultured in RPMI 1640 medium supplemented with penicillin, streptomycin and amphotericin B (Gibco) plus 10 % fetal bovine serum (Hyclone) and maintained at 37°C in a 5% CO_2 incubator. The R19 cell line has an E251fsX30 mutation on both *ESCO2* alleles. The R12 cell line has an R169X mutation also in both *ESCO2* alleles (12).

Drug sensitivity assays

MMC (Wako) was diluted in ethanol; sodium orthovanadate (Wako) and G418 (Gibco) were diluted in water; staurosporine (Calbiochem), Okadaic acid (Calbiochem) and Camptothecin (Calbiochem) were diluted in DMSO. After complete dissolution, all the stocks were stored in aliquots at -20°C. Cells were maintained in log phase growth before setting up sensitivity assays. Excluding camptothecin, LCLs (10^5 cells/ml) were cultured continuously for up to 4 days in growth medium containing the indicated concentrations of drug. For camptothecin, cells were treated four hr and then washed and grown in medium without drug. FDA (Wako) was diluted in acetone (1 mg/ml stock solution). Stock solution was added to the cell suspensions to a final concentration of 5 µg/ml. Staining time was 5 to 10 min at 37°C (23). Cells were collected by pipetting without washes and analyzed using a FACScalibur flow cytometer (Becton-Dickinson). FDA fluorescence signal was collected in log mode. For each measurement, 10^4 cells were analyzed for fluorescence; debris was excluded on the basis of forward and side light-scattering properties. Viability index was established as the percentage of viable cells grown in drug divided by the percentage of cells grown in media alone. To ascertain equivalent dosages (10) the respective viability curve of each dose was replotted to identify the doses at which the curves for all the cell lines were similar. The dose identified for the normal control divided by the dose found for the RBS cell lines was determined to establish the degree of sensitivity.

Apoptosis assays and cell cycle analysis.

Apoptotic cells and cell cycle distribution were assayed using propidium iodide (PI) staining followed by flow cytometry analysis. At each time point after drug exposure, 10^6 cells were washed once with PBS, fixed in 70% ethanol and stored at 4°C for up to 1 week. They were centrifuged at 1,400 rpm for 10 min and washed twice with PBS containing 0.5% bovine serum albumin ([BSA], Sigma) stained with propidium iodide solution (30 µg/ml of PI and 0.1 mg/ml of DNase free ribonuclease; stock reagents from Wako) for 30 min at 37° and the cells were analyzed on a FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Propidium iodide fluorescence was acquired using linear (FL2-585/20BP) and log (FL3-630LP) amplification. Threshold was set on log PI (FL3-630LP) in order to acquire all DNA-positive populations and a proportion of debris. Low scatter debris was excluded postacquisition. Percent apoptotic cells was calculated using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA), using both side and forward scatter to differentiate apoptotic from nonapoptotic cells, as well as linear and log red fluorescence. Debris (low forward scatter, low side scatter) were excluded from analysis. For DNA content, data were analyzed by the ModFit LT cell-cycle analysis software (Verity Software House, Topsham, ME).

To assess apoptosis after OV treatment, cytospin slides were prepared and stained with May-Grünwald/Giemsa for morphological evaluation. Apoptotic cells were microscopically identified by chromatin condensation and nuclear fragmentation (24) and quantified by counting at least five hundreds cells per slide from two separate preparations.

Immunobloting.

Cells (2 x 10^6) were washed in phosphate-buffered saline (PBS) and lysed in 50 mM tris-HCl (pH 7.5), 0.5% Nonidet P-40, 10% (v/v) glycerol, 100 mM NaCl, 5 mM ß-glycerophosphate, 50 mM NaF, 0.3 mM NA_3VO_4, and 1x Complete protease inhibitor cocktail (Roche) for 30 min at 4°C. After centrifugation at 50,000 x g for 30 min at 4 °C, supernatant was removed and protein concentration was estimated by Lowry assay and 20 to 40 ng of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to immobilon-P membrane (Millipore Corp, Bedford, MA). After blocking with *5%* dry milk in TBS (10 mmol/L Tris hydrochloride, pH 7.5, and 150 mmol/L NaCl) for 1 hour at room temperature, the membrane was incubated overnight at 4°C with the anti-human p53 FL-393 antibody (rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS containing 1% BSA. Subsequently, after washing four times in TBST (TBS supplemented with 0.05% Tween 20; Sigma), Proteins were visualized using secondary peroxidase

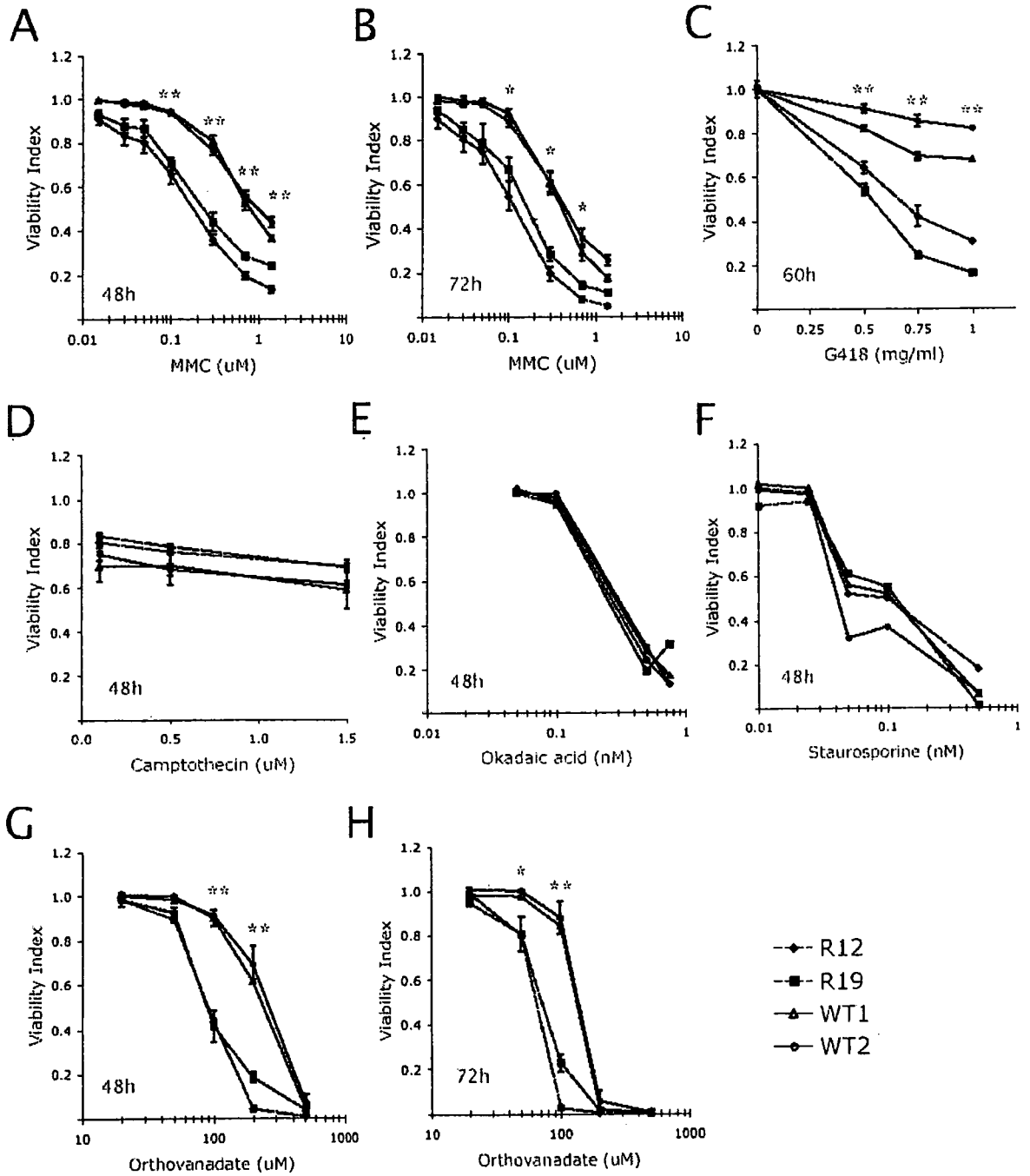

Figure 1. Cell viability response to different stress agents using FDA and flow cytometry. Roberts and control LCLs were were exposed to increasing drug concentrations of MMC for 48h (A) and 72h (B); G418 for 60h (C); camptothecin for 48h (D); okadaic acid for 48 h (E); staurosporine for 48 h (F); and sodium orthovanadate for 48h (G) and 72h (H). All data were normalized to an untreated control by determining he ratio of viable cells in exposed *vs.* unexposed cell cultures. Data point represents the mean ± sem of three to six independent assays. *$p<0.03$, **$p<0.005$

labeled antibodies and ECL plus western blotting detection reagent obtained from Amershan (Amersham Biosciences, Bukinghamshire, UK).

Statistical analysis

For sensitivity assays, statistical comparisons among groups were made using one way analysis of variance (ANOVA). Post-hoc analysis based on Scheffe test using SPSS version 12 (SPSS, Inc.) was used for comparison of individual cell lines mean values. For apoptosis, estimation of the difference between means was derived using the Student's paired t-test. A value of p less than 0.05 was considered statistically significant for all tests. All results are expressed as mean ± standard error of the mean (SEM) of at least three independent experiments unless otherwise stated. ($p < 0.05$).

RESULTS

We first evaluated the response of RBS and control LCLs to the DNA damaging agent mitomycin C and the protein synthesis inhibitor G418 that has been previously evaluated by cell counting or colony forming assays (9, 10). As shown in Fig. 1A, B and C, viability curves of RBS LCLs present a greater reduction in the proportion of viable cells after 48 and 72 hr in the presence of MMC, and after 60 hr in the presence of G418. A clear difference is evident in the response of the two RBS LCLs and the two control LCLs ($p \leq 0.003$ for 0.1 to 1.4 μM MMC at 48 hr; $p \leq 0.03$ for 0.1 to 0.7μM MMC at 72hr; $p \leq 0.005$ for 0.5 to 1mg/ml G418 at 60hr). To determine the degree of sensitivity to these drugs, we first found the equivalent dosage for MMC, i.e. the doses at which the curves for the four cell lines were very similar, and then the dose found for the control cells was divided by the dose found for the RBS cell lines (10). As shown in Fig, 2A, RBS cell lines are approximately five times more sensitive to MMC than control lines, having equivalent response curves at 0.3μM and 1.4 μM for the RBS and control cells, respectively. Although equivalent viability curves for G418 are not homogeneous for the four cell lines, they do indicate that RBS cells are at least two times more sensitive than normal LCLs with similar equivalent curves at 0.5 mg/ml and 1 mg/ml (Fig. 2B). These results are in agreement with previous reports that found that RBS cells are four to five times more sensitive to MMC and about 2.7 times more sensitive to G418 (9, 10). In addition, the similar degrees of sensitivity obtained in the present study indicate that assessment of cell viability using FDA and flow cytometry to study cell response to stress agents in RBS is a valid substitute for cell counting and colony forming assays.

In order to test the sensitivity of RBS LCLs to other DNA clastogenic agents, we evaluated the viability response to camptothecin, a DNA topoisomerase I inhibitor. In addition, as protein phosphorylation and dephosphorylation have a central role in sister chromatid cohesion (25, 26, 27, 28), we also studied the response to okadaic acid (OA), a serine/threonine phosphatases inhibitor; to staurosporine, a serine/threonine kinase inhibitor; and to sodium orthovanadate (OV), an inhibitor of tyrosine phosphatases. We found no differences between RBS and control LCLs in the viability response to camptothecin, OA or staurosporine at 48 h (Fig. 1D, E, F). Evaluation at 24 and 72 h also did not reveal differences between RBS and control cells (data not shown). On the contrary, we found that RBS cells are hypersensitive to OV as they present a larger reduction in cell viability when compared to control LCLs (Fig. 1G, H). RBS cells viability was greatly reduced after 48 and 72 hr in the presence of 100 μM OV, but also 200 μM OV for 48h and 50 μM OV for 72 hr induced a significant difference. The analysis of the response curves at 100 μM for RBS cells and 200 μM for controls indicate that RBS cells are approximately two times more sensitive to OV (Fig. 2C). However, it should be noted that the difference in cell viability decrease is more dramatic at 100 μM OV than with any dose of MMC tested (compare Fig. 1A, B and 1G, H).

To identify if apoptosis is involved in the hypersensitivity of RBS cells to cell death induced by MMC, G418 and OV we determined the number of cells entering into apoptosis after exposure to each drug. Apoptosis was determined for MMC and G418 by flow cytometry of PI-stained cells. In this assay, apoptotic cells appear in a sub-G1 peak, since chromatin condensation and DNA cleavage, which is characteristic of