EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 41, No. 1, 1-7, January 2009

Effects of a chemical chaperone on genetic mutations in α-galactosidase A in Korean patients with Fabry disease

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DOI 10.3858/emm.2009.41.1.001

Accepted 22 September 2008

Abbreviations: DGJ, 1-deoxygalatonojirimycin; ERAD, ER-associated degradation; ERP, enzyme replacement therapy; Gb3, globotriaosylceramide; GLA, α-galactosidase A; LSD, lysosomal storage disorder

Abstract

Fabry disease is an X-linked inborn error of glycosphingolipid catabolism that results from mutations in the gene encoding the α-galactosidase A (GLA) enzyme. We have identified 15 distinct mutations in the *GLA* gene in 13 unrelated patients with classic Fabry disease and 2 unrelated patients with atypical Fabry disease. Two of the identified mutations were novel (i.e., the D231G missense mutation and the L268delfsX1 deletion mutation). This study evaluated the effects of the chemical chaperones 1-deoxygalactonojirimycin (DGJ) on the function of GLA *in vitro*, in cells containing missense mutations in the *GLA* gene. Nine missense and a nonsense mutations, including one novel mutation were cloned into mammalian expression vectors. After transient expression in COS-7 cells, GLA enzyme activity and protein expression were analyzed using fluorescence spectrophotometry and Western blot analysis, respectively. DGJ enhanced GLA enzyme activity in the M42V, I91T, R112C and F113L mutants. Interestingly, the I91T and F113L mutations are associated with the atypical form of Fabry disease. However, DGJ treatment did not have any significant effect on the GLA enzyme activity and protein expression of other mutants, including C142W, D231G, D266N, and S297F. Of note, GLA enzyme activity was not detected in the novel mutant (i.e., D231G), although protein expression was similar to the wild type. In the absence of DGJ, the E66Q mutant had wild-type levels of GLA protein expression and approximately 40% GLA activity, indicating that E66Q is either a mild mutation or a functional single nucleotide polymorphism (SNP). Thus, the results of this study suggest that the chemical chaperone DGJ enhances GLA enzyme activity and protein expression in milder mutations associated with the atypical form of Fabry disease.

Keywords: 1-deoxygalactonojirimycin; Fabry disease; globotriaosylceramide; lysosomal storage diseases; α-galactosidase

Introduction

Fabry disease (OMIM #301500) is an X-linked recessive lysosomal storage disorder (LSD) caused by deficiency of the α-galactosidase A (GLA) enzyme (EC 3.2.1.22). This deficiency results in the systemic accumulation of globotriaosylceramide (Gb3) and related glycosphingolipids in the lysosomes of several different types of cells, including vascular endothelial, smooth muscle, epithelial, perithelial, reticuloendothelial, myocardial, ganglion and perineural cells (Desnick *et al*., 2001). Early symptoms of Fabry disease include acroparesthesias, angiokeratoma, and corneal opacities (Peter *et al*., 1997). In elderly patients, glycolipid accumulation can lead to chronic renal failure and stroke. Before the advent of dialysis, transplantation, and enzyme replacement therapy, males afflicted with this disease often died in their fifth decade (Branton *et al*., 2002).

Mutations in *GLA* gene cause misfolding, misassembly and aggregation of the protein in the endoplasmic reticulum. Hence, lysosomal trafficking is impaired because the enzyme is retained or degraded by the ubiqutin-proteosome pathway (Frustaci *et al*., 2001; Yam *et al*., 2006). However, enzyme activity of the mutant protein can be restored by chemical chaperones, such as galactose and its structural analog, 1-deoxygalactonojirimycin (DGJ) (Okumiya *et al*., 1995; Fan *et al*., 1999).

Table 1. Distribution of mutations in the human *GLA* gene in Korean patients with Fabry disease.

Mutations		Location	Phenotype	Mutagenesis construct
Nucleotide	Amino acids			
Missense				
c.124A > G	p.Met42Val	Exon1	Classical	+
c.196G > C	p.Glu66Gln	Exon2	Classical	+
c.272T > C	p.Ile91Thr	Exon2	Atypical	+
c.334C > T	p.Arg112Cys	Exon2	Classical	+
c.337T > C	p.phe113Leu	Exon2	Atypical	+
c.426C > G	p.Cys142Trp	Exon3	Classical	+
c.692A > G	***p.Asp231Gly***	Exon5	Classical	+
c.796G > A	p.Asp266Asn	Exon5	Classical	+
c.890C > T	p.Ser297Phe	Exon6	Classical	+
Nonsense				
c.258T > G	p.Tyr86X	Exon2	Classical	+
c.1024C > T	p.Arg342X	Exon7	Classical	N/A
Deletion				
c.296_297del	p.Gln98fsX23	Exon2	Classical	N/A
c.803_806del	***p.Leu268delfsX1***	Exon6	Classical	N/A
c.1077del	p.Ile359MetfsX32	Exon7	Classical	N/A
c.1235_1236del	p.Thr412SerfsX38	Exon7	Classical	N/A

Italicized, bolded text refers to the novel mutant identified in the study. The mutations were described according to established mutation nomenclature (http://www.hgvs.org/mutnomen). NM_000169.1 (GenBank) was used as a reference sequence. The +1 notation corresponds to the A of the ATG translation initiation codon. N/A, not applicable.

These chemical chaperones bind to the active site of GLA to promote proper folding and stabilization of the protein (Frustaci *et al*., 2001). However, the efficacy of chemical chaperones is limited in patients with atypical Fabry disease, and these chaperones are ineffective for most patients with the classic form of the condition (Okumiya *et al*., 1995; Matsuzawa *et al*., 2005). The GLA protein is encoded by the *GLA* gene, which consists of 7 exons on chromosome Xq22.1 (Kornerich *et al*., 1989). In humans, GLA is synthesized as a 50 kDa precursor, which is further processed to a 46 kDa mature form of the protein (Lenmansky *et al*., 1987; Froissart *et al*., 2003). To date, more than 350 mutations have been identified in the human *GLA* gene. We screened the *GLA* genes in Korean patients with Fabry disease and identified 15 different mutations, two of which were novel. Also, this study was undertaken to evaluate the effects of the chemical chaperones DGJ on the function of GLA *in vitro*, in nine groups of cells containing distinct missense mutations in the *GLA* gene.

Results

Identification of mutations in the human *GLA* gene

We identified 15 mutations in the unrelated Korean probands with Fabry disease. Of these, 9 were missense mutations and 6 nonsense or deletion mutations (Table 1). Two (i.e., D231G and L268delfsX1) were novel mutations, which were not observed in any of the 50 healthy Korean individuals.

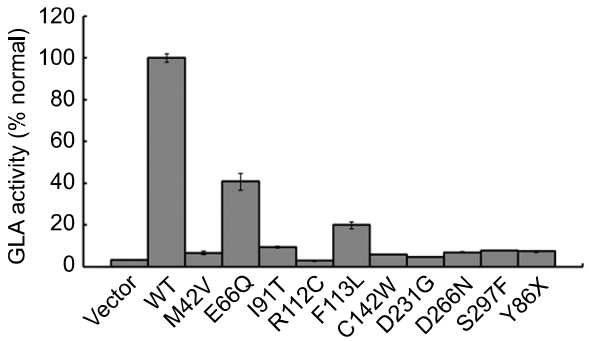

Figure 1. GLA enzyme activity analysis. Mutants were cultured in the absence of chemical chaperone for 2 days before enzyme assays for basal GLA activity. The mean of each standard deviation was calculated from five independent experiments.

Enzyme activity of the *GLA* mutants

Constructs that each contained one of 9 distinct missense and a nonsense mutations of *GLA* were over-expressed in COS-7 cells to verify that the mutants disrupted enzyme activity. The nonsense mutation was used as a negative control. After the

mutant constructs were transiently over-expressed in the absence of a chemical chaperone, GLA enzyme activity was measured (Figure 1). Enzyme activities of the E66Q and F113L mutants were approximately 40% and 20% lower than that of the wild type, respectively. No residual enzyme activity was detected in the other mutants.

Effects of DGJ on enzyme activity

To evaluate the effects of the chemical chaperone DGJ, we analyzed GLA enzyme activity and protein expression in mutants cultured in medium containing DGJ for 48 h (Figure 2). In the presence of 20 μM DGJ, the GLA activity of the mutants was similar to that observed in cells cultured without DGJ. As shown in Figure 2 and Table 2, GLA activity was enhanced about 2-3 fold in the M42V ($P < 0.0001$), I91T ($P < 0.0001$), R112C ($P < 0.0001$) and F113L ($P < 0.0001$) mutants, but enzyme activity of the other mutants was not affected by the addition of DGJ.

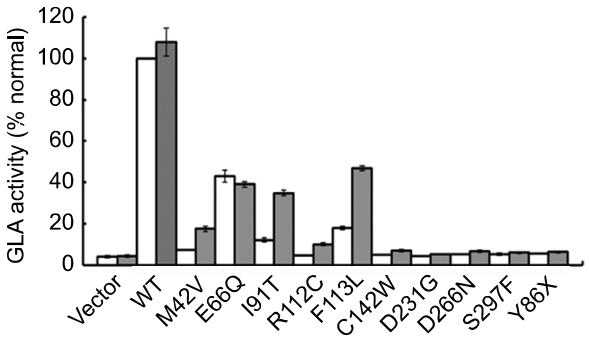

Figure 2. Effect of DGJ on activity of GLA. Mutants were cultured in the presence or absence of 20 μM DGJ for 2 days prior to GLA activity assay. The mean of each standard deviation was calculated from five independent experiments. Open bars indicate the absence of DGJ; solid bars indicate the presence of DGJ.

Protein expression in the GLA mutants

Western analyses revealed that GLA was expressed as a 46 kDa protein in COS-7 cells expressing either wild type GLA or the E66Q mutant, indicating that the mature form of GLA was present. In contrast, a 50 kDa protein corresponding to the immature form of GLA was identified in several other mutants. In particular, the D231G mutant yielded both the 50 kDa and 46 kDa forms of the GLA protein, although catalytic activity of GLA was absent (Figure 3A). Also, although the D266N was missense mutation, neither form of the GLA protein was identified (Figure 3A). To certify this result, pEGFP-C1 vector tagged GFP were co-transfected with the D266N mutant in the COS-7 cells. As shown in Figure 3B, GFP protein was detected in both WT and D266N, but GLA protein was not detected in the D266N mutant. Furthermore, to evaluate the effects of the chemical chaperone DGJ, we analyzed GLA protein expression in mutants cultured in medium containing DGJ for 48 h (Figure 3A). In Western analyses, expression of the mature GLA protein increased in the presence of 20 μM DGJ in the I91T and F113L mutants. A marked increase was observed in the F113L mutant, while a much smaller increase was observed in the I91T mutant.

Table 2. Enhancement of GLA activity in COS7 cells cultured with DGJ.

Mutation	Residual enzyme activity (% normal)		*P* value	Enhancement ratio
	DGJ (-)	DGJ (+)		
M42V	7.2 ± 0.22	17.2 ± 1.31	0.0001	2.5
E66Q	43.8 ± 3.03	39.3 ± 1.26	NE	NE
I91T	12.0 ± 0.69	35.7 ± 1.48	0.0001	2.9
R112C	4.7 ± 0.13	9.3 ± 0.60	0.0001	2
F113L	17.8 ± 0.88	47.1 ± 1.07	0.0001	2.6
C142W	5.2 ± 0.39	7.0 ± 0.59	NE	NE
D231G	4.4 ± 0.32	5.1 ± 0.20	NE	NE
D266N	5.2 ± 0.23	6.4 ± 0.67	NE	NE
S297F	5.2 ± 0.41	5.9 ± 0.45	NE	NE
Y86X	5.4 ± 0.27	5.7 ± 0.82	NE	NE

Enhancement of GLA activity by DGJ was compared in cultured COS-7 cells with a variety of mutations. The mean of each standard deviation was calculated from five independent experiments. Student T-test was used for *P* value calculation. *P* values below 0.05 were considered statistically significant. NE, non-enhanceable.

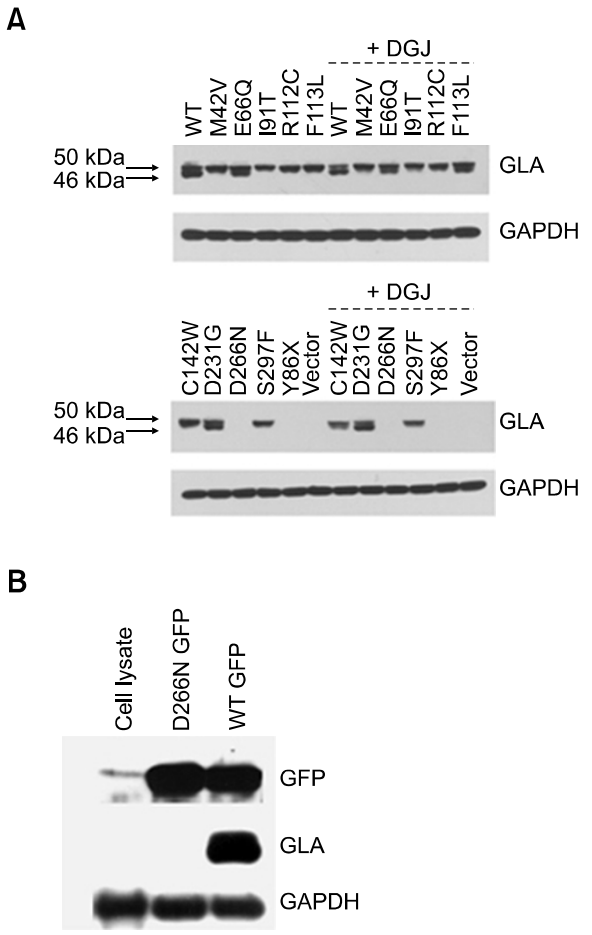

Figure 3. Western blot analysis. (A) Effect of DGJ on expression of GLA. Mutants were cultured in the presence or absence of 20 μM DGJ for 2 days prior to GLA assay. (B) Protein expression of D266N mutant. GAPDH was used as an internal control.

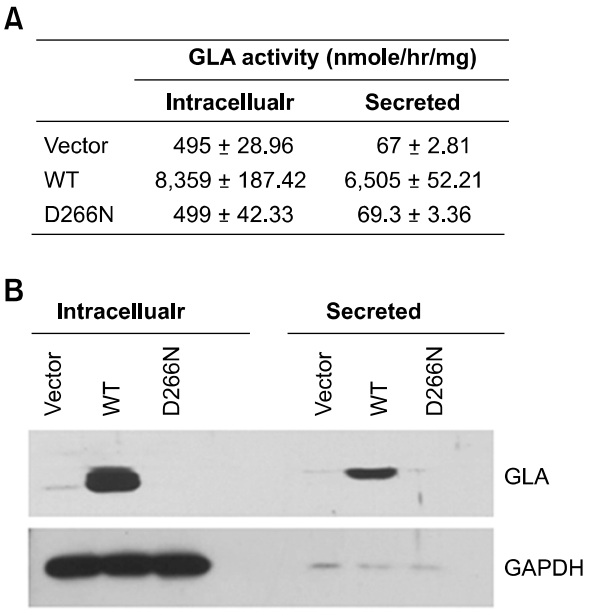

Figure 4. Enzyme activity (A) and protein expression (B) of the D266N mutant during expression in COS-7 cells. The D266N mutant was cultured in the absence of chemical chaperone for 2 days prior to enzyme assays and Western analyses. GAPDH was used as an internal control. The mean of each standard deviation was calculated from three independent experiments.

However, the mature GLA protein was not observed in the M42V and R112C mutants.

Discussion

Enzyme replacement therapy (ERT) has proved to be an effective treatment modality for Fabry disease. Unfortunately, ERT is expensive and patients often find the frequent intravenous infusions and adverse immunological events highly inconvenient. Furthermore, the enzyme is not efficiently delivered into the central nervous system, as in other lysosomal storage diseases (LSD) involving the nervous system. One plausible treatment modality for LSD is small molecule therapy, which consists of two main strategies. The first strategy involves substrate reduction by a specific enzyme inhibitor. The other strategy, known as pharmacological chaperone therapy, involves stabilizing the enzyme using small chemical chaperones that bind the active site of the target enzyme. In fact, pharmacological chaperone therapy has been clinically tested in the treatment of other LSDs (Matsuda *et al*., 2003; Tropak *et al*., 2004).

Galactose and its structural analog DGJ are well known chemical chaperones that bind the active site of GLA and act as competitive inhibitors (Garman and Garboczi, 2004; Fan *et al*., 2007). Binding promotes proper folding, dimerization, and processing of the GLA protein, thereby enhancing residual GLA activity in patients with GLA deficiencies (Frustaci *et al*., 2001; Matsuzawa *et al*., 2005; Ishii *et al*., 2007; Shin *et al*., 2007).

The active site of GLA contains side chains with residues W47, D92, D93, Y134, C142, K168, D170, E203, L206, Y207, R227, D231, D266 and M267. The C172 residue is connected to C142 via a disulfide bond (Garman and Garboczi, 2002, 2004). The active site of this enzyme is extremely sensitive to mutation: Even conservative substitutions around the active site can result in the complete loss of enzymatic activity. Furthermore, mutations can lead to folding defects in the GLA polypeptide, wherein the hydrophobic core of the protein is disrupted and the enzyme cannot fold or remain folded in the acidic lysozyme environment (Garman and Garboczi, 2002, 2004). Of the nine missense mutations explored in the present study,

C142W, D231G and D266N are located in the enzyme's active site, while the M42V, E66Q, I91T, R112C, F113L and S297 mutations are predicted to cause folding defects in the GLA polypeptide.

Addition of DGJ to the culture medium increased GLA enzyme activity in the M42V, I91T, R112C and F113L mutants in mutation-expressing COS-7 cells. Furthermore, addition of DGJ boosted production of the mature protein in two mutations involved in the atypical form of the disease (i.e., I91T and F113L), while production of the mature protein was not detected in two mutations involved in the classical form of the disease (i.e., M42V and R112C). These results indicated that the amount of M42V and R112C protein was too low to be detected comparing with wild type. Also, the amount of protein does not always reflect its functional activity. The D266N mutation yielded particularly interesting results, considering that *in vitro* transient expression data detected no residual enzyme activity and CRIM findings were negative on Western blot analysis. Specific aspartate or glutamate residues function as nucleophiles in the active site of the enzyme. We hypothesize that the D266N mutation affects posttranslational glycosylation by introducing a novel site for N-glycosylation. However, as shown in Figure 4, enzyme activity and protein were not detected in cells or supernatants, suggesting that the enzyme was rapidly degraded via ERAD (ER-associated degradation) without appropriate molecular folding (Sitia and Braakman, 2003; Helenius and Aebi, 2004). Residual GLA activity was not observed in the D231G and C142W mutants, although D231G was determined to be CRIM-positive on Western blot analysis. Enzyme activity in the D231G and C142W mutants was not enhanced by DGJ, indicating that the mutation occurred in an extremely sensitive active site. Furthermore, the C142 residue in the disulfide bond of the enzyme's active site is strictly conserved.

No residual enzyme activities were observed in the S297F mutant and CRIM findings were positive on Western blot analysis. The GLA activity of this mutant was not enhanced in the presence of DGJ, indicating that the mutation caused a significant conformational change in the enzyme. The other novel mutation, L268delfsX1, results in premature termination and thus was not expected to yield detectable GLA activity or protein expression. Enzyme activity in the E66Q mutant was approximately 40% of the activity of wild-type GLA and the protein was expressed normally, indicating that E66Q is either a mild mutation or a functional SNP. A previous study found that the double mutation of E66Q and R112C eliminated enzyme activity in a transient expression assay (Ishii *et al*., 1992).

We screened effect of a chemical chaperone both on enzyme activity and on protein expression in various missense mutations which identified in Korean Patients with Fabry Disease. The chemical chaperone DGJ enhanced more effectively in milder mutations associated with the atypical form of Fabry disease.

Methods

Patients

To date, 13 male patients with classic Fabry disease and 2 male patients with the atypical form of the disease have been enrolled in the Korean Fabry Registry. The patients are between 13 and 48 years of age (mean 28.6 $\pm$ 11.89 years) and are unrelated. Diagnosis was confirmed via the GLA assay and DNA analysis of peripheral blood leukocytes. Mutations in the *GLA* gene were identified in all patients. This study was approved by the institutional review boards (# 2007-9006) of Asan Medical Center, Seoul, and all patients or their parents provided written informed consent.

Genomic DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes or EBV immortalized cell lines using the Puregene DNA isolation kit (Gentra). The GLA coding regions were amplified via PCR. The PCR products were directly sequenced using the BigDye termination kit, version 3.0 (Applied Biosystems), with the same primers used in the amplification reactions.

Plasmid construction and mutagenesis

The *GLA* cDNA used in this study was kindly provided by Genzyme. The pcDNA3 mammalian expression vector (Invitrogen) was used for the cloning of non-tagged normal GLA. Mutant *GLA* constructs were generated using the PCR-based *Dpn*I-treatment site-directed mutagenesis method as a series of base substitutions in full-length GLA cDNA of pcDNA3 (Li and Wilkinson, 1997). Mutation sites were confirmed via sequence analysis with the BigDye termination kit, version 3.0 (Applied Biosystems).

Cell culture and transfection

COS-7 cells were maintained in DMEM, supplemented with 10% FBS, 100 mg/ml streptomycin and 100 U/ml of penicillin. Transient trasfection was performed using Lipofectamine 2000 reagent (Invitrogen) with 2 μg of plasmid construct, according to the manufacturer's instructions. 1 μg of pSV-B-galactosidase plasmid (Promega) was used to monitor the transfection efficiency. Cells were further incubated until harvest at 37^{o}C in an atmosphere containing 5% CO_2.

DGJ treatment and GLA assays

COS-7 cells were cultured for two days in DMEM medium, with and without 20 μM DGJ (Sigma Inc., St. Louis. MO) (Fan and Ishii, 2003). Assays of GLA enzyme activity were performed using a standard fluorometric method (Desnick *et al*., 1973) with the following modification: Reactions containing 0.01-0.02 mg of total protein, 10 μl of 10 mM 4-methylumbelliferyl-α-D-galactopyranoside (i.e., substrate) and 2 μl of 1 M N-acetyl-D-galactosamine (i.e., an inhibitor of N-acetylgalactosaminidase) were incubated at 37°C for 1 h. All chemicals were obtained from Sigma. The reaction was stopped by adding 130 μl of 0.17 M glycine-carbonate buffer, pH 9.8. Fluorescence was detected using a fluorescence spectrophotometer (Molecular devices) with excitation and emission wavelengths of 360 nm and 415 nm.

Cell extracts and Western blot analyses

Cells were homogenized in a RIPA buffer containing 1 mM PMSF (Sigma) and Protease Inhibitor Cocktail (Roche). After centrifugation, the supernatant was used in GLA enzyme assays and Western blot analyses. Proteins were denatured using the Lowry method. For Western blot analyses, 20 μg of cell extract was separated on a 10% SDS-PAGE gels and immunoblotted onto a nitrocellulose membrane (Amersham Bioscience). Membranes were blocked in TBST containing 5% skim milk for 1 h at room temperature and then incubated with a 1:1,000 dilution of the GLA polyclonal antibody and the GFP monoclonal antibody (Santa Cruz, CA). Protein bands were visualized using the ECL detection system (Amersham-Pharmacia Biotech). GAPDH was used as an internal control in each Western blot analysis.

Acknowledgements

We thank the patients and their families for participating in this study, which was supported by grant No A010384 and A080588 from the Korean Ministry for Health, Welfare and Family Affairs.

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