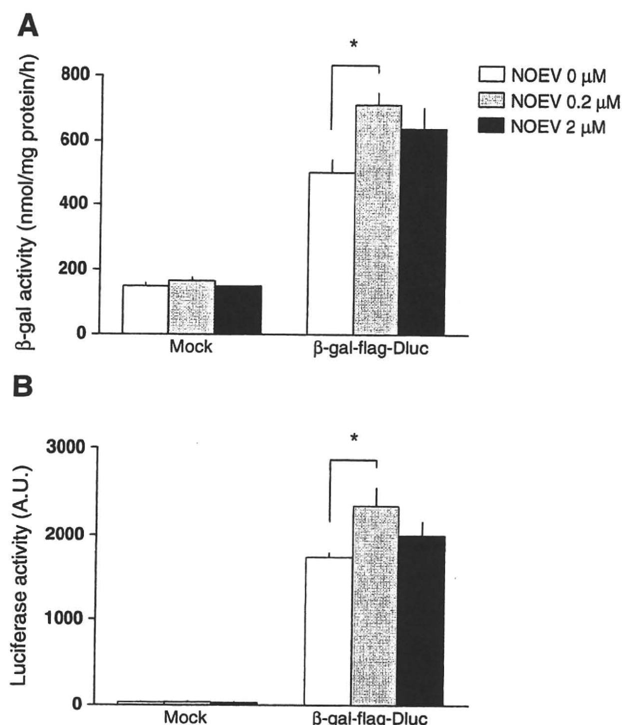

Fig. 3. NOEV effect on β-gal-flag-Dluc protein. COS7 cells were transiently transfected with β-gal-flag-Dluc. (A) β-Gal activity in the cell lysate. (B) Luciferase activity in the medium after NH_4Cl treatment. Each bar represents the mean ± SEM of three determinations each done in triplicate. *$p<0.05$, statistically different from the values in the absence of NOEV.

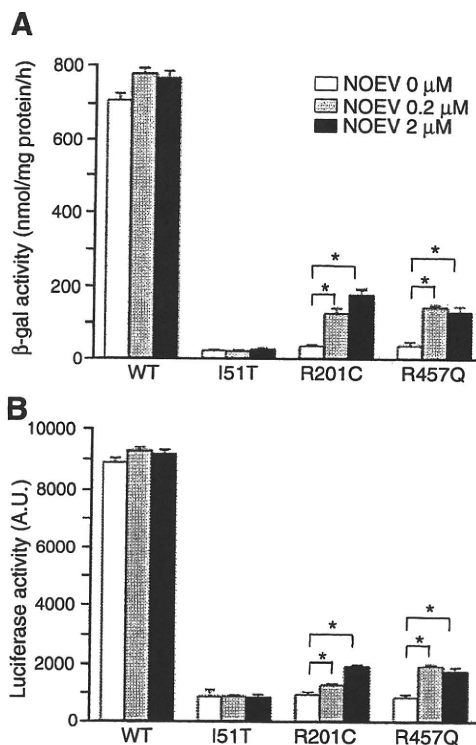

Fig. 4. NOEV effect on mutant β-gal-flag-Dluc proteins. COS7 cells were transiently transfected with mutant β-gal-flag-Dluc constructs. (A) β-Gal activity in the cell lysate. (B) Luciferase activity in the medium after NH_4Cl treatment. The endogenous activities were subtracted from each activities. Each bar represents the mean ± SEM of three determinations each done in triplicate. *$p<0.05$, statistically different from the values in the absence of NOEV.

target enzymes *in vitro*. Then they were evaluated for chaperone effects using cultured fibroblasts from patients. In most of these studies, drug screening was carried out using standard 4-MU-conjugated artificial substrate [11,24–27]. In addition, novel chemical approaches to ameliorate protein folding were explored in Gaucher disease and Tay–Sachs disease, which showed proteostasis regulators further increased enzyme activity by co-administering a chaperone compound in patients' fibroblasts [21]. Further studies will likely to be directed for the identification of new chaperones for additional LSDs.

In an attempt to develop an alternative assay method, we constructed luciferase-tagged β-gal mutants. In the current study, we obtained WT, I51T, R201C and R457Q-β-gal-flag-Dluc recombinant plasmids and validated their enzymatic activities by transient transfection. As expected, their responses to NOEV were essentially the same as observed in patients' fibroblasts. Our data proved that the level of enzymatic activity in cell lysates was well represented by the luciferase activity in the medium. We also observed that mutant β-gal activities in the medium were at undetectable levels measured by 4-MU assay, probably because of the neutral pH (data not shown). Our luciferase assay is advantageous over the 4-MU assay in that it can measure the exact amount of the enzyme protein and that it can be applicable to live cell screening. We have already established lines derived from β-gal KO mouse fibroblasts that stably express β-gal-flag-Dluc. NOEV effects were reproduced in these cells (data not showed) and will be applicable for finding novel chaperone [28] However, to make expression constructs and cell lines with each novel mutations is laborious. Moreover, our current protocol cannot examine the processes of the enzyme protein maturation, particularly the integration into multiprotein structures with neuraminidase and protective protein/cathepsin A (PPCA), and intralysosomal protein stability. However most mutations detected in GM1-gangliosidosis were shown to affect precursor stability and intracellular transport [11,24–27]. Particularly R201C and R457Q were shown to result in misfolded precursor proteins rapidly degraded by ER-associated degradation machinery [11,19]. And the chaperone effect is based on the concept that small molecular enzyme inhibitors may assist the folding of mutated enzyme proteins and prevent their recognition by the quality control systems. Therefore, as we showed in Table 1, proteostasis regulators have synergetic effect of NOEV on R201C mutant enzyme and such combination therapy should be developed to improve therapeutic effects not only LSDs but other loss-of-function diseases [21].

In summary, we have established the luciferase-based assay and this assay will be a convenient and reliable system for screening and

Table 1
Synergetic effects of NOEV together with celasterol or MG132 on R201C expressing COS7 cells.

	NOEV		
	0 μM	0.2 μM	2 μM
(A) β-gal activity in the cell lysates			
Blank	167.90 ± 4.85	219.62 ± 3.08*	254.45 ± 6.74*
MG132	188.61 ± 7.38#	256.00 ± 5.61*#	258.97 ± 9.83*
Celasterol	191.40 ± 6.25#	236.74 ± 6.51*#	274.24 ± 5.60*#
(B) Luciferase activity in the medium			
Blank	1435.78 ± 56.73	2047.42 ± 69.76*	2737.77 ± 102.29*
MG132	1570.84 ± 34.99#	2630.70 ± 104.54*#	2655.63 ± 63.81*
Celasterol	1603.48 ± 35.39#	2238.50 ± 45.57*#	2969.26 ± 48.09*#

COS7 cells expressing R201C mutant β-gal were treated with 0, 0.2 or 2 μM NOEV in absence or presence of 2 μM celasterol or 0.1 μM MG132. Growth medium containing celasterol was changed every 24 h. Cell lysates and mediums were collected after 72 h treatment for β-gal and luciferase assay. The concentrations used here had no influence on cell viability. Values in the table are Mean ± SD of three determinations each done in triplicate. *$p<0.05$, compare with "NOEV 0 μM" in the same row. #$p<0.05$, compare with "Blank" in the same column.

evaluation of chemical chaperones in live cells. It will be a useful tool for a survey of novel chemical chaperones on future studies.

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Increased globotriaosylceramide levels in a transgenic mouse expressing human α1,4-galactosyltransferase and a mouse model for treating Fabry disease

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Chikara Shiozuka[1], Atsumi Taguchi[1,2], Junichiro Matsuda[3], Yoko Noguchi[3], Takanori Kunieda[3], Kozue Uchio-Yamada[3], Hidekatsu Yoshioka[2], Ryoji Hamanaka[4], Shinji Yano[5], Shigeo Yokoyama[5], Kazuaki Mannen[6], Ashok B. Kulkarni[7], Koichi Furukawa[8] and Satoshi Ishii[1,2,9,*]

[1]Department of Agricultural and Life Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555; [2]Department of Matrix Medicine, Faculty of Medicine, Oita University, Yufu, Oita 879-5593; [3]Laboratory of Animal Models for Human Diseases, National Institute of Biomedical Innovation, Ibaraki, Osaka 567-0085; [4]Department of Cell Biology, Faculty of Medicine, Oita University, Yufu, Oita 879-5593; [5]Department of Diagnostic Pathology, Faculty of Medicine, Oita University, Yufu, Oita 879-5593; [6]Division of Laboratory Animal Sciences, Research Promotion Project, Oita University, Yufu, Oita 879-5593, Japan; [7]National Institute of Dental and Craniofacial Research, Bethesda, MD 20892, USA; [8]Department of Biochemistry II, Nagoya University School of Medicine, Nagoya, Aichi 466-0065; and [9]Biochemical Laboratory, GlycoPharma Corporation, Oita 870-0822, Japan

*Satoshi Ishii, Ph.D., Department of Matrix Medicine, Faculty of Medicine, Oita University, Hasama-cho Idaigaoka 1-1, Yufu-shi, Oita 879-5593, Japan, Tel: +81 97 586 5661, Fax: +81 97 546 2534, email: ishiis01@oita-u.ac.jp

Fabry disease is a lysosomal storage disorder caused by an α-galactosidase A (α-Gal A) deficiency and resulting in the accumulation of glycosphingolipids, predominantly globotriaosylceramide (Gb3). A transgenic mouse expressing the human α-Gal A R301Q mutant in an α-Gal A-knockout background (TgM/KO) should be useful for studying active-site-specific chaperone (ASSC) therapy for Fabry disease. However, the Gb3 content in the heart tissue of this mouse was too low to detect an ASSC-induced effect. To increase the Gb3 levels in mouse organs, we created transgenic mice (TgG3S) expressing human α1,4-galactosyltransferase (Gb3 synthase). High levels of Gb3 were observed in all major organs of the TgG3S mouse. A TgG3S(+/−)M(+/−)/KO mouse was prepared by cross-breeding the TgG3S and TgM/KO mice and the Gb3 content in the heart of the TgG3S(+/−)M(+/−)/KO mouse was 1.4 μg/mg protein, higher than in the TgM(+/−)/KO (<0.1 μg/mg protein). Treatment with an ASSC, 1-deoxygalactonojirimycin, caused a marked induction of α-Gal A activity and a concomitant reduction of the Gb3 content in the TgG3S(+/−)M(+/−)/KO mouse organs. These data indicated that the TgG3S(+/−) M(+/−)/KO mouse was suitable for studying ASSC therapy for Fabry disease, and that the TgG3S mouse would be useful for studying the effect of high Gb3 levels in mouse organs.

Keywords: active-site-specific chaperone therapy/Fabry disease/globotriaosylceramide/mouse model.

Abbreviations: A4GalT, α1,4-galactosyltransferase; ASSC, active-site-specific chaperone; α-Gal A, α-galactosidase A; DGJ, 1-deoxygalactonojirimycin; Gb3, globotriaosylceramide (Galα1,4Galβ1,4Glc-Ceramide); GLAko mouse, α-Gal A knockout mouse; Gb4, globotetraosylceramide; G3Stg/GLAko mouse, transgenic mouse expressing human Gb3 synthase in α-Gal A knockout background; G3S/COS-7 cells, COS-7 cells expressing human Gb3 synthase; HPTLC, high-performance thin-layer chromatography; LacCer, lactosylceramide; PBS, phosphate-buffered saline; Stx1B, Shiga toxin 1 B-subunit; TgM/KO mouse, transgenic mouse expressing human mutant R301Qα-Gal A in α-Gal A knockout background; TgG3S mouse, transgenic mouse expressing human Gb3 synthase; TgG3S(+/−)M(+/−)/KO mouse, transgenic mouse expressing human Gb3 synthase and mutant R301Qα-Gal A in α-Gal A knockout background.

Fabry disease is an X-linked recessive disease caused by insufficient activity of lysosomal α-galactosidase A (α-Gal A, EC 3.2.1.22), an enzyme responsible for the catabolism of glycosphingolipids, predominantly globotriaosylceramide (Gb3) (*1*). Patients with Fabry disease show diverse clinical manifestations caused by generalized vasculopathy—pain in the extremities, hypohidrosis, angiokeratoma, corneal opacity, ischaemic heart disease, progressive nephropathy and cerebrovascular disease (*2*). Approximately 60% of the mutations reported in patients with Fabry disease are missense mutations in the α-Gal A gene. Although many products encoded by these missense mutations have normal catalytic properties, they also have low thermostability and degrade rapidly after their synthesis in the endoplasmic reticulum (ER) (*3–5*). A novel therapeutic strategy for the treatment of Fabry disease, which uses competitive inhibitors of α-Gal A as active-site-specific chaperones (ASSCs), has been suggested (*6–8*). The normal folding of those missense mutant enzymes can be restored by ASSC treatment, thus preventing excessive ER-associated degradation and improving the transport of the mutant enzyme to the lysosomes (*4*). Cultivation of patients' cells with an ASSC of α-Gal A [*e.g.* 1-deoxygalactonojirimycin (DGJ)] at low concentrations resulted in a substantial

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increase in both residual enzyme activity and the amount of intracellular enzyme protein (4, 6, 9).

A mouse line deficient in α-Gal A (GLAko mice) was established in 1997 by disruption of the murine α-Gal A gene (10). Despite the accumulation of Gb3 in Fabry disease-relevant organs of the GLAko mice, the mice were clinically normal and had a typical life span. These mice have served as an excellent model for studies on enzyme replacement therapy (11), gene therapy (12–14) and substrate reduction therapy (15), wherein the increased α-Gal A activity and reduced Gb3 accumulation were primary objectives. However, this mouse model is not suitable for studying ASSC therapy, which requires the expression of a human mutant enzyme. In a previous report (16), we established a homozygous transgenic mouse in a murine α-Gal A knockout background (TgM/KO mouse) by crossbreeding a TgM mouse expressing the human α-Gal A R301Q mutant (17) in GLAko mice. Like the GLAko mouse, the TgM/KO mouse does not exhibit a disease phenotype, however these mice do express human mutant α-Gal A and serve as an excellent biochemical model for studying ASSC (18). Heterozygous TgM/KO mice show a lower α-Gal A activity than the homozygotes, and Gb3 accumulates in the kidney of the heterozygous mouse, but not in the heart, which is affected in Fabry disease (16).

The purpose of this study was to establish a transgenic mouse that expressed high levels of Gb3 as well as the human mutant α-Gal A enzyme in heart tissue, which would permit us to assess the effect of ASSC (enhancement of α-Gal A activity and decrease in Gb3 accumulation) in the heart. Here, in order to increase cardiac Gb3 production, we simply overexpressed human α1,4-galactosyltransferase [*A4GalT*, Gb3 synthase (G3S, EC 2.4.1.228)] to obtain the TgG3S mouse line. We confirmed that these mice expressed high levels of Gb3 in their major organs, and then bred them with the TgM/KO mice to obtain TgG3S(+/−)M(+/−)/KO mice. Here we report the characterization and potential usefulness of this new mouse line.

Materials and Methods

Establishment of a stable transformant expressing A4GalT in COS-7 cells and A4GalT-overexpressing transgenic mice (TgG3S)

The human Gb3 synthase (G3S) cDNA containing the full-length coding sequence and an *Eco*RI site at both ends was prepared by polymerase chain reaction (PCR) using a Phusion™ High-Fidelity PCR Kit [New England BioLabs (NEB), Ipswich, MA, USA]. The cDNA for α1,4Gal-TpVTR1 (19) was the template, and the primer sequences were 5′-TGGGAATTCCATGTCCAAGCC-3′ and 5′-GGGGAATTCACAAGTACATTTTCATGGC-3′. The 1.1-kb PCR product was purified with a PCR Purification Kit (QIAGEN K.K., Japan) and digested with *Eco*RI (NEB). The digested cDNA was subcloned into the *Eco*RI site of expression vector pCXN2 (20), and the product was designated as pCXN2-G3S. For the preparation of the stably transformed G3S/COS-7 cells, pCXN2-G3S was linearized with *Hin*dIII (NEB) and then transfected into COS-7 cells with the FuGENE™6 transfection reagent (Roche Molecular Biochemicals, Basel, Switzerland), according to the manufacturer's protocol. Stably transformed G3S/COS-7 cells were selected by growing them in culture medium containing 400 µg/ml of G418 (Sigma, St Louis, MO, USA) for 4 weeks.

A DNA fragment comprising a mammalian expression unit and the human Gb3 synthase cDNA was prepared by digesting the pCXN2-G3S with *Sal*I and *Bam*HI (NEB). A fragment that was ~3.5 kb was isolated by agarose gel electrophoresis and purified by a Gel Extraction Kit (QIAGEN). A transgenic mouse (TgG3S) expressing human Gb3 synthase was generated by injecting the DNA fragments into the pronuclei of fertilized eggs taken from superovulated C57BL/6J Jms female mice, and the embryos were implanted into pseudopregnant Jcl:MCH mice, as described previously (21). Transgenic founder mice were identified by PCR with a primer set (5′-ATTGTTCTCAAGAACCTGCG-3′ and 5′-ATTTGTGAGCC AGGGCATTG-3′). A transgene fragment was confirmed as a single 548-bp band on agarose gel electrophoresis. To generate an animal model exclusively expressing both human G3S and human mutant α-Gal A in an endogenous *GLA* knockout background [TgG3S(+/−)M(+/−)/KO mice], we first prepared a mouse line overexpressing the human G3S transgene in an α-Gal A knockout background (G3Stg/GLAko) by crossbreeding male TgG3S mice and homozygous female GLAko mice (since *GLA* is located on the X chromosome). After the G3Stg/GLAko mouse line was established, TgM/KO and G3Stg/GLAko mice were crossbred to obtain TgG3S(+/−)M(+/−)/KO mice.

Determination of A4GalT mRNA expression in mouse tissues

The *A4GalT* mRNA expression of the transgene (human *A4GalT*) was determined by a reverse transcriptase (RT)-PCR using Takara RNA PCR Kit (AMV) Ver.3.0 (Takara Bio Inc., Shiga, Japan). The total RNA samples from tissues of wild-type and transgenic mice were prepared with RNAiso (Takara Bio Inc.), and the RNA concentrations were determined by absorbance at 260 nm. The RT reaction was performed at 30°C for 10 min, and then at 42°C for 15 min, followed by incubation at 99°C for 5 min. PCR amplification was performed using the following conditions: initial denaturation (94°C, 5 min) followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, elongation at 72°C for 30 s and further elongation at 72°C for 2 min. The DNA fragment (409 bp), which contained a sequence that is highly conserved between the mouse and human *A4GalT* genes, was amplified with primers 5′-GGCATC TC(A/T)CTTCTGAGCTG-3′ and 5′-GGATGGAACACCACTTC TTG-3′. PCR products were run on a 2% agarose gel, stained with ethidium bromide and photographed. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an expression control gene as described previously (22).

Administration of DGJ to TgG3S(+/−)M(+/−)/KO mice

TgG3S(+/−)M(+/−)/KO mice were supplied with fresh tap water *ad libitum* and rodent pellets. DGJ (Toronto Research Chemicals, Toronto, Canada) was administrated to one group of female TgG3S(+/−)M(+/−)/KO mice in tap water as a DGJ aqueous solution without any other substances. After 4 weeks, the animals were sacrificed and the organs were quickly removed and rinsed with phosphate-buffered saline (PBS). Tissue homogenates were subjected to enzyme assays and lipid extraction.

Assay of enzyme activity and protein content

All samples were kept on ice and processed as rapidly as possible. Approximately 10% (w/v) tissue homogenates were prepared in water using a micro-homogenizer (Physcotron, Niti-on Inc., Chiba, Japan). The supernatant obtained from the homogenate after centrifugation at 10,000*g* for 5 min was used in the enzyme assays. The α-Gal A activity was assayed with 4-methylumbelliferyl α-D-galactoside (Sigma) as the substrate and N-acetyl-D-galactosamine (75 mM) as the inhibitor for α-N-acetylgalactosaminidase in 0.1 M sodium citrate buffer (pH 4.6) as described previously (16). The protein concentration was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard.

Detection of neutral and acidic glycosphingolipids by thin-layer chromatography analysis

Glycosphingolipids were extracted from cultured cells and mouse tissues and were analysed as described previously (14), with some modifications. Crude lipids were extracted from cultured cells (eq. to 1 mg of protein) and tissue homogenates (eq. to 5 mg of protein), using a mixture of chloroform : methanol (2 : 1, v/v). Glycosphingolipids were dried under a stream of nitrogen.

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The dried samples were dissolved in chloroform : methanol : water (30 : 60 : 8, v/v) and applied to a TOYOPEARL DEAE-650 column (Tosoh Corporation, Tokyo, Japan). The column was washed with chloroform : methanol : water (30 : 60 : 8, v/v). The pass-through fraction was pooled for the preparation of neutral glycosphingolipids. The acidic glycosphingolipids including gangliosides were eluted with chloroform : methanol : 0.8 M sodium acetate (30 : 60 : 8, v/v), and they were desalted with Sep-Pak C18 reverse-phase cartridge (Waters, Milford, MA, USA) and then analysed by thin-layer chromatography (TLC). The neutral glycosphingolipids fraction was dried, and then subjected to mild alkaline treatment with 1 ml of 0.1 N NaOH in methanol at 40°C for 2 h. After neutralizing the solution with glacial acetic acid, glycosphingolipids were further subjected to the Folch's partition (choloroform : methanol : H_2O, 8 : 4 : 3 in v/v/v), recovered in the lower phase, and then quantitatively applied to TLC plates. TLC analyses were performed with HPTLC-Silica gel 60 plates (Merck & Co., Inc., Whitehouse Station, NJ, USA) using a solvent system of chloroform : methanol : water (60 : 35 : 8, v/v/v) and chloroform : methanol : 0.2% $CaCl_2$ (60 : 35 : 8, v/v/v) for the separation of neutral and acidic glycosphingolipids, respectively. Glycosphingolipids were detected by spraying the plate with orcinol-sulphuric acid reagent, and heating the plate at 110°C. The Gb3 from porcine erythrocytes was purchased from Nakalai Tesque (Kyoto, Japan), and other glycosphingolipid standards were purchased from Wako Pure Chemicals (Osaka, Japan). The ganglioside standards from bovine brain (GM1, GM2 and GM3) were purchased from Wako.

Binding assay

The Stx1B binding assay was performed as described previously (*23*), with some modifications. After glycosphingolipids were separated by TLC as described above, a TLC plate was sunk in a 0.4% polyisobutylmethacrylate (GlycoTech, Rockville, MD, USA) solution (2.5% polyisobutylmethacrylate in chloroform was diluted to 0.4% with hexane) and then blocked with 1% bovine serum albumin in PBS (BSA-PBS). The plate was incubated with 2.5 μg/ml of Stx1B in BSA-PBS at room temperature for 20 min and washed with PBS. After incubation with 7.0 μg/ml of anti-Stx1B polyclonal antibody (produced in rabbits with purified Stx1B) for 20 min the plate was washed with PBS. After further incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Pierce Chemical, Rockford, IL, USA) at room temperature for 20 min and the following final washing, Stx1B-binding was visualized with an enhanced chemiluminescent substrate (Pierce).

Immunoelectron microscopy

The G3S/COS-7 cells were cultured with or without 10 mM DGJ for 4 days, then fixed in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) for 3 h; they were then further incubated in 1% osmium tetraoxide for 2 h, dehydrated in ethanol and embedded in Epok 812. Immunoelectron microscopy was performed as described previously (*24*). In summary, each thin section was briefly microwaved in Target Retrieval Solution, pH 10 (DAKO, Carpinteria, CA, USA) and then incubated for 30 min at room temperature with Stx1B (2.5 μg/ml). After being washed with Wash Buffer [50 mM Tris–HCl (pH 7.6) containing 0.8% NaCl and 0.1% BSA], the ultrathin section was incubated with anti-Stx1B antibody (7 μg/ml). After another wash, the section was incubated with gold-conjugated goat anti-rabbit IgG. The section was then washed again, stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (JEM-1200EXII, JEOL, Akishima, Tokyo, Japan).

Results

Establishment of TgG3S mice

First, we prepared a stable transformant COS-7 cell line expressing Gb3 synthase (G3S/COS-7 cells) to confirm that the expression construct prepared in our present study could increase intracellular Gb3 content. We extracted neutral glycosphingolipids from the cells and quantitated them by high performance TLC (HPTLC) (Fig. 1). The Gb3 and globotetraosylceramide (Gb4) levels in the G3S/COS-7 cells were markedly higher than those in the parental COS-7 cells or in a control line transformed with only the empty vector. The content of lactosylceramide (LacCer) in the G3S/COS-7 cells was lower than it was in the COS-7 cells, indicating that the expression product from the *A4GalT* gene in our construct catalysed the construction of Gb3 from LacCer.

A mouse line expressing the *A4GalT* gene (TgG3S) was generated by injecting the DNA fragment into the pronuclei of fertilized mouse ova taken from C57BL/6J female mice. A marked increase in the expression of the *A4GalT* gene in heart, kidney, liver, spleen, small intestine, brain, lungs and muscle of the TgG3S mice compared with wild-type C57BL/6J mice was observed by RT-PCR (Fig. 2A). High levels of Gb3 synthase expression resulted in an increase in Gb3 levels of all organs examined in this study (Fig. 2B). A glycosphingolipid band (possibly galabiosylceramide) with an Rf value similar to that of LacCer by HPTLC was also increased in the kidney and brain of the TgG3S mouse. No change was observed in the content of acidic glycosphingolipids including gangliosides in organs of TgG3S mice (data not shown). The TgG3S mouse line did not show any clinical phenotype and had a typical life span.

Preparation of TgG3S(+/−)M(+/−)/KO mouse line as a model mouse for ASSC therapy

To study the effect of ASSC on Gb3 content in the heart, we tried to increase the cardiac Gb3 content of the TgM/KO mouse by introducing the G3S-overexpressing transgene. The TgG3S(+/−)M(+/−)/KO mouse was created by crossbreeding the G3Stg/GLAko mouse and the TgM/KO mouse. The Gb3 content in the TgG3S(+/−)M(+/−)/KO heart and kidney was determined and compared with that in

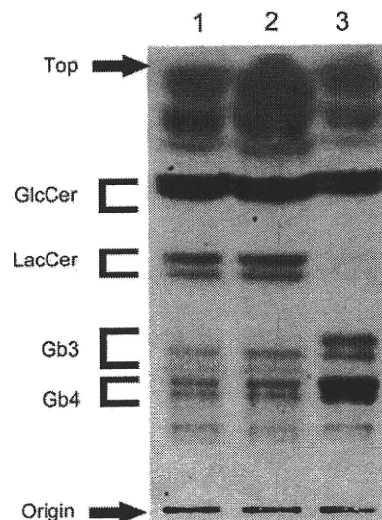

Fig. 1 Neutral glycosphingolipids in stably transformed G3S/COS-7 cells. Neutral glycosphingolipids were extracted from cell homogenates (containing 1 mg protein), as described in 'Materials and Methods' section. Glycosphingolipids were visualized with orcinol-sulphuric acid reagent. Lane 1, intact COS-7 cells; lane 2, mock transfection; lane 3, stable transformant G3S/COS-7 cells.

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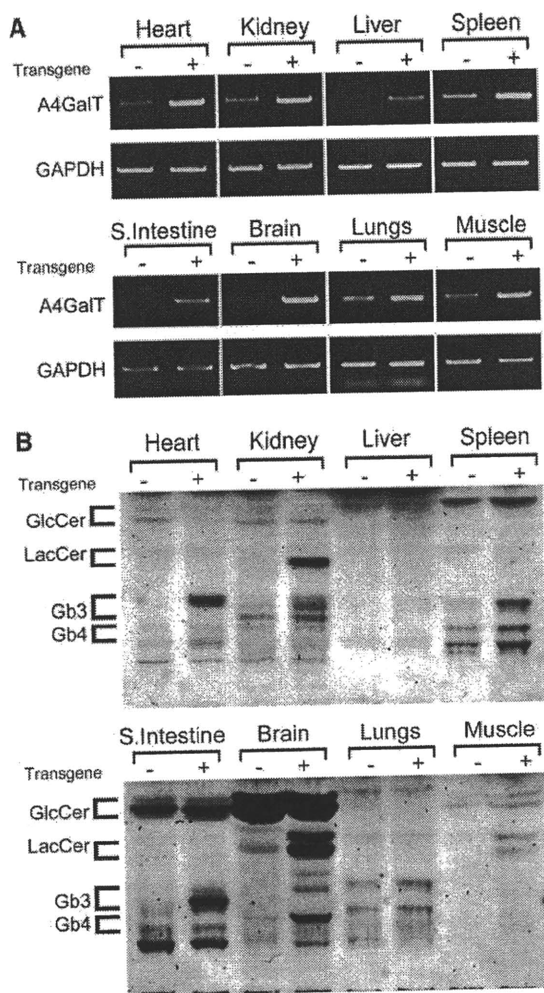

Fig. 2 Expression of *A4GalT* gene and glycosphingolipids in tissues of TgG3S mice. A human G3S-overexpressing mouse line was prepared as described in 'Materials and Methods' section. (A) The expression of *A4GalT* mRNA was determined by RT–PCR analysis with a primer set that can amplify both mouse and human genes. The glyceraldehyde phosphate dehydrogenase (GAPDH) gene was amplified as an internal control. (B) Neutral glycosphingolipids were extracted from tissue homogenates (containing 5 mg protein) prepared from age-matched TgG3S and wild-type mice and applied to HPTLC, as described in the legend to Fig. 1.

wild-type C57BL/6J, TgG3S(+/−) and TgM(+/−)/KO mice (Fig. 3). A Gb3 level as high as that of the TgG3S mice (1.3 ± 0.3 μg/mg protein) was observed in the heart of the TgG3S(+/−)M(+/−)/KO mice (1.1 ± 0.3 μg/mg protein) (Fig. 3A). Furthermore, when we probed the HPTLC plate with Shiga toxin 1 B-subunit (Stx1B), which selectively binds Gb3, we did not detect Gb3 in the heart tissue of mouse lines that did not express the *A4GalT* transgene (*i.e.* wild-type and TgM(+/−)/KO mice) (Fig. 3C). In contrast, Gb3 was detectable in kidney tissue of all the mouse lines, and a slight increase was observed in the lines expressing the *A4GalT* transgene (Fig. 3B, D and F). The *A4GalT* transgene did not change the α-Gal A activity in either the heart or kidney (Fig. 3G and H). Marked increase in Gb3 content was detected in the heart and kidney by the expression of Gb3 synthase, however no abnormality was observed in the histological examination of both organs from TgG3S(+/−)M(+/−)/KO mouse (data not shown).

Age-related increase in the kidney Gb3 content in TgG3S(+/−)M(+/−)/KO mice

The Gb3 content in the kidney of both the TgM(+/−)/KO and the TgG3S(+/−)M(+/−)/KO mice increased ~3-fold between 5 and 10 weeks of age (Fig. 4A and B). In contrast, neither the Gb3 content in the heart nor the α-Gal A activity in the heart or kidney changed during this time (Fig. 4B–D). These data showed that we needed to use age-matched TgG3S(+/−)M(+/−)/KO mice to determine the effect of DGJ on Gb3 content in the kidney. Because the change in the Gb3 content in the kidney changed relatively little between 10 and 15 weeks of age, we used mice in this age range to study the effects of DGJ treatment.

Effect of 0.05 mM DGJ treatment on Gb3 content in heart and kidney of TgG3S(+/−)M(+/−)/KO mice

DGJ was administrated to TgG3S(+/−)M(+/−)/KO mice in their water as a 0.05 mM solution, available *ad libitum*, for 4 weeks. Based on the daily water consumption, the DGJ dosage was calculated to be ~3 mg/kg body weight/day. In the heart tissues of TgG3S(+/−)M(+/−)/KO mice after treatment with DGJ, a 0.69-fold decrease in Gb3 content (1.44 ± 0.38 and 0.99 ± 0.40 μg/mg protein in control and DGJ-treated mice, respectively) was observed, along with a 5.4-fold increase in α-Gal A activity (24.7 ± 7.6 and 133.0 ± 24.9 unit/mg protein in control and DGJ-treated mice, respectively) (Fig. 5). Likewise, the Gb3 content of the kidney tissue decreased 0.61-fold (6.56 ± 1.33 and 3.97 ± 0.57 μg/mg protein in control and DGJ-treated mice, respectively), and the α-Gal A activity in the kidney increased 4.4-fold (6.4 ± 3.1 and 28.0 ± 2.8 unit/mg protein in control and DGJ-treated mice, respectively) after treatment. These data indicate that the enhancement of the mutant α-Gal A activity by treatment with DGJ reduced the Gb3 content of the heart as well as the kidney, and that the TgG3S(+/−)M(+/−)/KO mouse line is a useful mouse model for the study of ASSC treatment in Fabry disease-relevant organs.

Inhibitory effect of high-concentration DGJ on α-Gal A activity causes Gb3 accumulation in G3S/COS-7 cells

To determine the critical concentration of DGJ required to reduce the Gb3 accumulation of mammalian cells, G3S/COS-7 cells were cultured in DGJ (0–10 mM)-supplemented medium for 4 days. Significant Gb3 accumulation was observed in cases where DGJ dosage was >1 mM (Fig. 6A and B). As an explanation, we found that DGJ decreased the intracellular α-Gal A activity at these higher concentrations, although it slightly increased the α-Gal A activity at 10 μM DGJ, by the ASSC effect (Fig. 6C).

To confirm that the Gb3 accumulated in the lysosomes of the G3S/COS-7 cells, an immunoelectron microscopic study was conducted using the Stx1B and anti-Stx1B polyclonal antibodies to label the

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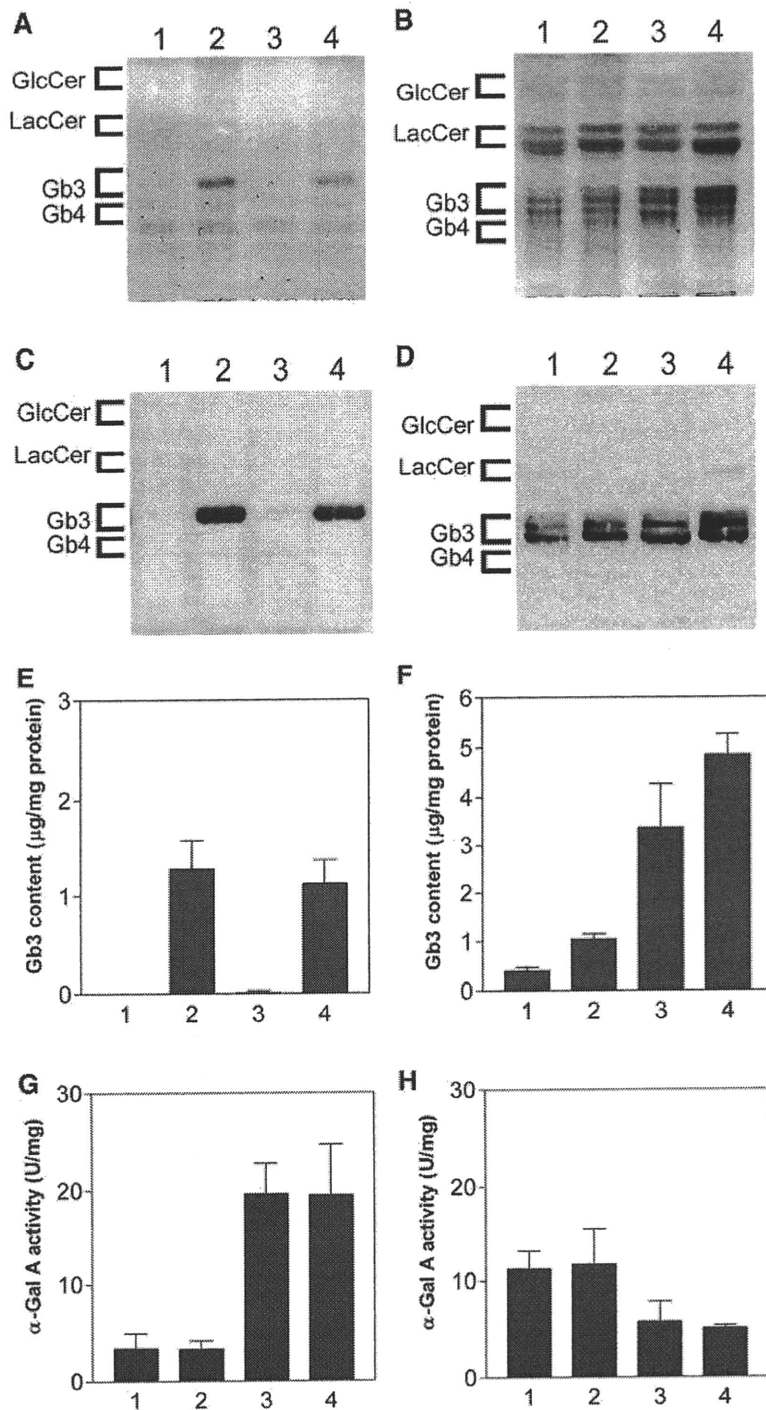

Fig. 3 Characterization of TgG3S(+/−)M(+/−)/KO mouse line. Gb3 content and α-Gal A activity in heart (A, C, E, and G) and kidney (B, D, F, and H) tissue from 10-week-old TgG3S(+/−)M(+/−)/KO mice were compared to those from age-matched mice from other lines. In (A–H), the lanes and columns show results from the following genotypes: 1, wild-type; 2, TgG3S(+/−); 3, TgM(+/−)/KO; 4, TgG3S(+/−)M(+/−)/KO. The neutral glycosphingolipids were extracted and visualized with orcinol-sulphuric acid reagent (A and B) or by Stx1B-binding (C and D). The Gb3 content (E and F) and α-Gal A activity (G and H) were determined as described in 'Materials and Methods' section.

Gb3 (Fig. 6D and E). The number of gold particles in each lysosome was markedly increased in the samples treated with 10-mM DGJ, indicating that the Gb3 had accumulated in them.

Administration of DGJ at high concentrations to TgG3S(+/−)M(+/−)/KO mice

To elucidate whether high concentrations of DGJ would also cause Gb3 accumulation in mouse tissues,

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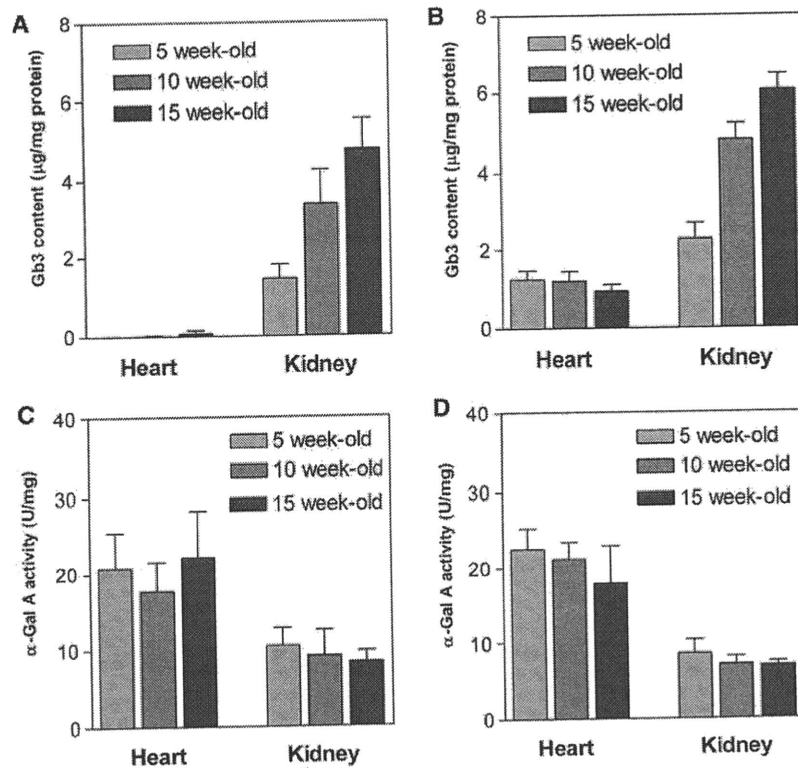

Fig. 4 Developmental changes in Gb3 content and α-Gal A activity in mouse heart and kidney. The Gb3 content and α-Gal A activity in the heart and kidney of TgM(+/−)/KO (A and C) and TgG3S(+/−)M(+/−)/KO mice (B and D) were determined at the indicated ages, as described in 'Materials and Methods' section. Each bar represents the means ± SD of data from three or four mice.

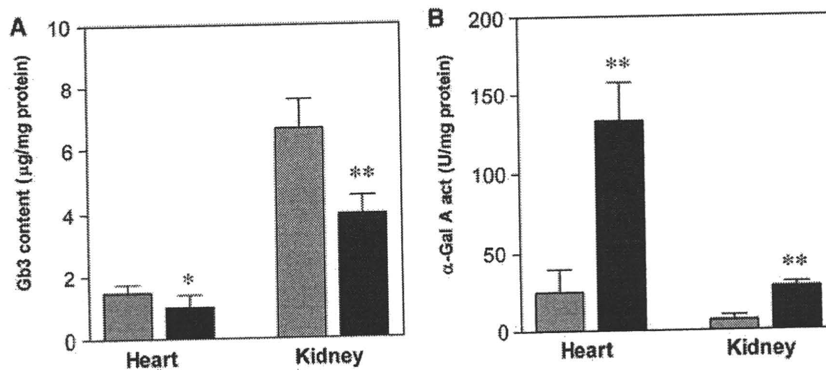

Fig. 5 Effect of DGJ treatment on Gb3 content and α-Gal A activity in TgG3S(+/−)M(+/−)/KO mice. DGJ (0.05 mM) was administrated to 10-week-old TgG3S(+/−)M(+/−)/KO mice in their drinking water for 4 weeks. The dosage of DGJ was estimated to be 3 mg/kg/day. Six female mice each were used in the DGJ-treated (dark-coloured bar) and control (light-coloured bar) groups. The Gb3 content (A) and α-Gal A activity (B) in the heart and kidney were determined as described in 'Materials and Methods' section. The statistical significance of the difference was determined by Student's t test. $^{*}P<0.05$, $^{**}P<0.01$.

TgG3S(+/−)M(+/−)/KO mice (three 10-week-old female mice for each group) were treated with DGJ at 5-fold (0.25 mM) and 25-fold (1.25 mM) higher concentrations than in the original experiment, for 4 weeks. No significant increase in Gb3 content either in heart or kidney tissue was observed after treatment with DGJ at high concentrations, and the low level of Gb3 in the TgG3S(+/−)M(+/−)/KO mouse tissues (~1.0 and 3.7 µg/mg protein in heart and kidney, respectively) was maintained (Fig. 7A and B), compared to that of age-matched G3Stg/GLAko mice (14 and 38 µg/mg protein in heart and kidney, respectively), which have no α-Gal A activity in their organs. Likewise, DGJ increased α-Gal A activity dose-dependently in the heart and kidney of the TgG3S(+/−)M(+/−)/KO mice (Fig. 7C and D). These data indicated that at least a 25-fold higher DGJ concentration (75 mg/kg body weight/day) may be safe for the treatment of patients with Fabry disease.

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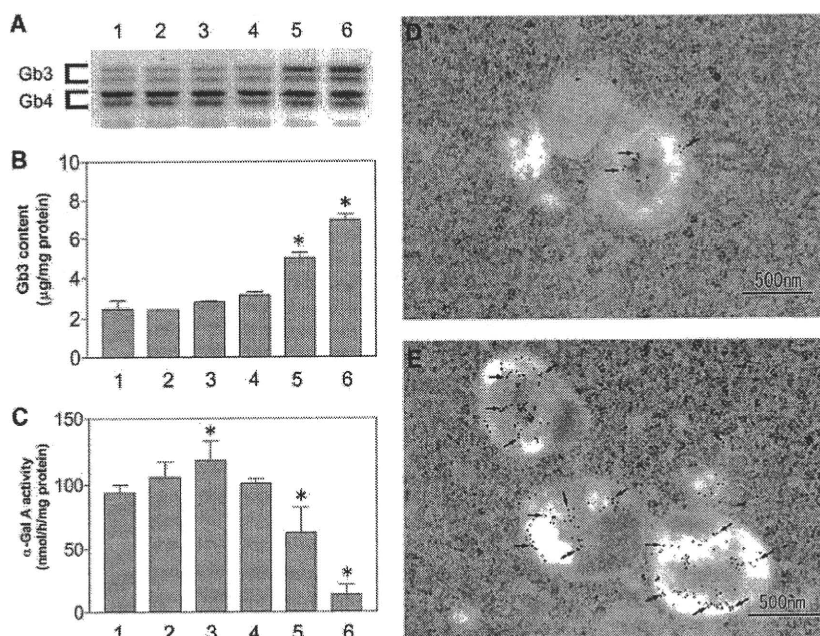

Fig. 6 Treatment of G3S/COS-7 cells with high-concentration DGJ. G3S/COS-7 cells were cultured for 4 days in high-glucose DMEM medium containing 10% foetal bovine serum with different concentrations of DGJ. After cells were rinsed with PBS, cells were harvested with PBS by a plastic scraper, and collected by centrifugation (3000*g*, 5 min). The cell pellet was homogenized with water and used for the determination of Gb3 content and α-Gal A activity. In (A), HPTLC analysis of Gb3 and Gb4 was visualized by orcinol-sulphuric acid reagent. The values of Gb3 content (B) and α-Gal A activity (C) are the means ± SD from three cultures. The statistical significance of the difference was determined by Student's *t* test; $*P < 0.05$ versus DGJ-free culture. In (A, B and C), lanes and columns are the same, and 1, DGJ-free control culture; 2, 1 μM DGJ; 3, 10 μM DGJ; 4, 100 μM DGJ; 5, 1 mM DGJ; 6, 10 mM DGJ. The accumulation of Gb3 in lysosomes was determined by Stx1B-binding immunoelectron microscopic study in DGJ-free (D) and 10 mM DGJ-treated G3S/COS-7 cells (E). The ultrathin sections were incubated with Stx1B, then with anti-Stx1B polyclonal antibody, followed by immunogold labeling. Typical gold particles are pointed out by arrows.

Discussion

To increase the Gb3 content in mouse tissues, we tried overexpressing human Gb3 synthase in them. Since high Gb3 content was observed in tissues of the TgG3S mouse compared with wild-type mouse organs (Fig. 2), Gb3 synthase may be a critical enzyme for the synthesis of Gb3 in mouse organs, particularly in the heart and small intestine, where the increase was greatest. The increase in the tissue Gb3 level was determined by the binding of Stx1B (Fig. 3), which selectively binds Gb3 (*25*, *26*). The effect of G3S-overexpression on Gb3 content was greater in the heart than in the kidney, indicating that Gb3 synthase is expressed at low levels in wild-type mouse heart. The absence of Gb3 in the TgM/KO mouse heart was caused not only by its low expression of the Gb3 synthase gene, but also by the high activity of the mutant α-Gal A. However, the G3S-stimulated Gb3 production outstripped Gb3's decomposition by α-Gal A activity in the heart of the TgG3S (+/−)M(+/−)/KO mouse, leading to the accumulation of Gb3 (even though the Gb3 content was still lower than it was in the kidney).

We did not identify the neutral glycolipid, which has a similar Rf value to that of LacCer on HPTLC and which increased in the kidney and brain tissues in response to G3S-overexpression. However, it may be galabiosylceramide (Galα1,4Gal-Cer), since this glycolipid can be made by Gb3 synthase (*19*), and galactosylceramide (Gal-Cer) is produced in the kidney as well as in the brain (*27*).

The kidney Gb3 level increased with age in both TgM(+/−)/KO and TgG3S(+/−)M(+/−)/KO mice (Fig. 4). However, the α-Gal A activity was not changed in kidney tissue by the additional expression of the Gb3 synthase gene, and an increase in the kidney Gb3 level with age was also reported in GLAko mice (*28*), indicating that the age-related Gb3 accumulation in the kidney is not caused by a decrease in the catabolism of Gb3. Although the mechanism by which kidney Gb3 levels increase with age is unknown, these findings meant that we had to use age-matched mice to determine the ASSC effect on the kidney Gb3 level.

After successfully increasing the heart Gb3 level in mice, we were able to examine the ASSC effect of DGJ on Gb3 content in the heart tissue of TgG3S(+/−)M(+/−)/KO mice. A significant reduction of Gb3 in the heart was observed following treatment with 0.05 mM DGJ for 4 weeks, indicating that TgG3S(+/−)M(+/−)/KO mice are a useful animal model for determining the effect of ASSC therapy. A recent report on ASSC by Khanna *et al.* (*29*) using model mice also demonstrated that treatment with DGJ decreases the Gb3 level in the mouse heart. Although they used the same human mutant α-Gal A (R301Q) cDNA for the preparation of a model mouse, their strategy was very different from ours. Their mouse model, which shows Gb3 accumulation in heart

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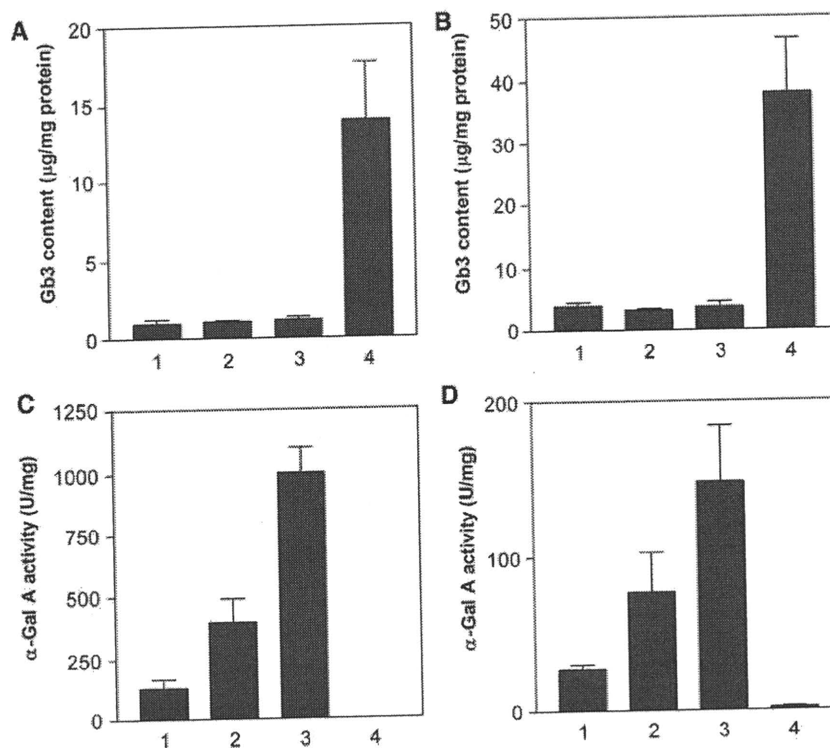

Fig. 7 Administration of high concentrations of DGJ to TgG3S(+/−)M(+/−)/KO mice. High concentrations of DGJ were administered to TgG3S(+/−)M(+/−)/KO mice (three or four 11-week-old female mice in each group) for 4 weeks. The Gb3 content and α-Gal A activity in the heart (A and C, respectively) and in the kidney (B and D, respectively) were determined as described in 'Materials and Methods' section. Lanes in A–D were the same, and lane 1, 0.05 mM DGJ treated; lane 2, 0.25 mM DGJ treated; and lane 3, 1.25 mM DGJ treated TgG3S(+/−)M(+/−)/KO mice, and lane 4, age-matched TgG3S(+/−)/KO mice.

tissues, was prepared by reducing the expression level of the mutant α-Gal A gene by using the human GLA promoter—which is a very weak promoter— instead of the stronger CAG (cytomegalovirus immediate-early enhancer/chicken β-actin hybrid) promoter. In contrast, we prepared a mouse model overexpressing both Gb3 synthase and the mutant α-Gal A genes. Our study confirmed the work of Khanna *et al.* (*29*) by reproducing their findings on the effect of DGJ in the heart, in a different mouse model. Although the effect of ASSC on Gb3 content can be determined in our mice and mice reported by Khanna *et al.* (*29*), the benefit of our mice is to be able to determine the localization and content of mutant enzyme in mice tissue with its antibody (*16*, *18*) as well as the localization of Gb3. Our mice will be useful for the elucidation of the mechanism of ASSC effect in detail by the detection of both mutant enzyme and Gb3 at the same time.

Although treatment with DGJ at a low concentration (<100 μM) can increase mutant α-Gal A activity, at high concentrations (>1 mM) DGJ significantly inhibits α-Gal A (*30*), leading to a corresponding increase in Gb3 levels in G3S/COS-7 cells (Fig. 6). Here, the increase in Gb3 content correlated with a decrease in the intracellular α-Gal A activity, and we used immunoelectron microscopy to show that Gb3 accumulated in the lysosomes of G3S/COS-7 cells treated with 10 mM DGJ. These data demonstrate the importance of determining a safe and effective concentration of DGJ for use in clinical therapy. In our present study, we used a 25-fold higher concentration (~75 mg/kg/day treatment) of DGJ than the usual dose (3 mg/kg/day treatment), which did not inhibit α-Gal A activity nor increase the tissue Gb3 level (Fig. 7), indicating that the tissue concentration of DGJ is below the inhibitory level at oral dosage. From no further reduction of Gb3 content either in heart or kidney tissue was observed by the treatment with DGJ at higher concentrations, the usual dose (3 mg/kg/day treatment) may be a suitable dose for the ASSC effect in our mouse model.

In conclusion, the Gb3-synthase-overexpressing mouse (TgG3S) shows an increased Gb3 level in its organs, and was useful in generating a mouse line (TgG3S(+/−)M(+/−)/KO) that can be used as an animal model for studying the efficacy of ASSC therapy for Fabry disease.

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Conflict of interest

None declared.

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Review article

Molecular basis of neurogenetic diseases

Yoshiyuki Suzuki

International University of Health and Welfare Graduate School, 2600-1 Kita Kanemaru, Otawara, Tochigi 324-8501, Japan

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Abstract

Molecular background of neurogenetic disease is briefly reviewed. Importance and usefulness of genetic testing are emphasized. Molecular genetics is a powerful tool for investigation of epileptic syndromes. Diagnosis based on gene analysis will give a new insight for pathophysiology and clinical outcome of the patient, and there is a hope to develop a new therapeutic approach in the near future. Among them a new molecular therapeutic trial for lysosomal diseases is being developed: chemical chaperone therapy. It will become a new approach to brain damage causing epilepsy and other phenotypic expressions of a large number of genetic diseases in the near future.
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Keywords: Epilepsy; Neurogenetic disease; Neurometabolic disease; Genetic analysis; Gene mutation; Genetic engineering

1. Introduction

Genetics is a scientific research field of biology, and has given the most important impact on clinical medicine during the past 30 years for investigation of pathophysiology, diagnosis, and possible treatment of intractable diseases, particularly of those affecting the central nervous system. Currently we cannot talk about diseases in general without consideration of molecular events in individual cells and tissues affected by diseases. The major targets of clinical genetics are genes and proteins in genetic and non-genetic diseases. Molecular analysis is important and useful for clinical practice related to infectious diseases, allergic diseases, hypoxic and vascular diseases, and many other common childhood diseases.

In this article I briefly summarize the historical background of genetics research, and review currently available information about molecular pathophysiology of diseases, mainly focusing on the aberrant genetic information in genetic metabolic diseases affecting the central nervous system. Then gene mutations causing epilepsy and related diseases in the pediatric age group will be summarized in order to emphasize the importance of molecular diagnosis and possible molecular therapy in the new future.

2. History of genetics research (Table 1)

Research in genetics started when Gregor Mendel published experimental data on plant hybridization in 1866 [1]. At present this achievement is well known as Mendel's law. However, this important breakthrough in the field of biology remained unnoticed for the subsequent more than 30 years. At the turn of the century, three biologists from Europe, Hugo de Vries (the Netherlands) [2], Carl Correns (Germany) [3] and Erich von Tschermak (Austria) [4], independently did the same experiments, and, after survey of the literature, came to the same conclusion (re-discovery of Mendel's law), and consequently active search for genetic mechanisms started for human diseases. However, some reservations have been made for the re-discovery by these three biologists [5–7].

E-mail address: suzukiy@iuhw.ac.jp.

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Table 1
Research milestones in genetics*.

Researcher	Year	Achievement	References
Gregor Mendel (Austria)	1866	Experiments on plant hybridization	[1]
Hugo de Vries (Netherands)	1900	Re-discovery of Mendel's law	[2]
Carl Correns (Germany)			[3]
Erich von Tschermak (Austria)			[4]
Archibald Garrod (UK)	1909	Inborn errors of metabolism	[8]
George Beadle (USA)	1941	One gene-one enzyme hypothesis	[9,10]
Edward Tatum (USA)			

* The names of main investigators are listed.

In parallel with basic research in biology, an important observation was made by a British physician Archibald Garrod on four rare diseases: albinism, homocystinuria, cystinuria, and pentosuria. He stated that the liability to develop diabetes or gout is often inherited, but the diseases themselves are not inherited for they are never congenital. Although this statement is nowadays not completely correct, he then proposed the concept of inborn errors of metabolism, first delivered as the Croonian Lecture in 1908, and then published as a monograph in 1909 [8]. A chemical error pursues an even course and shows no tendency to become aggravated as time goes on in the diseases of this type. It was pointed out that the epithets inborn ("genetic" or "hereditary" at present) and congenital are by no means synonymous.

Garrod proposed the view that alkaptonuria is what may be described as a "freak" of metabolism, a chemical abnormality more or less analogous to structural malformations. However, the substantial background was not known causing metabolic derangements in these disorders.

During the subsequent 30 years, Garrod's book had little influence on genetics. It was widely known and cited by biochemists, but only few authors mentioned or even gave reference to it in the standard genetic books written in the twenties and thirties. Most geneticists were not yet inclined to think of hereditary traits in chemical terms.

George Beadle and his colleagues were the first to demonstrate evidence indicating specific substance involved in the development of wild type eye color [9], and then found that mutant strains of a fungus species Neurospora was unable to carry out specific biochemical processes [10]. They proposed one gene-one enzyme theory and opened a new era of genetics based on chemical background.

3. Molecular basis of genetic control

Deoxynucleic acid (DNA) remained a mysterious substance in the cell nucleus after discovery in the middle of the 19th century by Friedrich Miescher [11], followed by extensive molecular characterization by organic chemists, particularly by Albrecht Kossel [12] (Table 2).

Molecular basis of genetic control on biochemical events was finally confirmed by Oswald Avery and his colleagues by demonstrating that minute amounts of DNA could transform the unencapsulated R variant of pneumococci to the fully encapsulated variant [13]. The chemically induced alterations in cellular structure and function were predictable, type-specific, and transmissible in series. This is the first report confirming that DNA is in fact the molecule controlling genetic phenomena in living cells.

In the same period another important studies were made on the composition of DNA molecules. Erwin Chargaff did thorough chemical analysis of DNA molecules, and found that double-stranded DNA from any cell of all organisms have a 1:1 ratio of pyrimidine and purine bases and more specifically that the amount of

Table 2
History of gene research*.

Researcher	Year	Achievement	References
Friedrich Miescher (Germany)	1869	Discovery of nucleic acid ("nuclein")	[11]
Albrecht Kossel (Germany)	1885–94	Characterization of nucleic acids	[12]
Oswald Theodore Avery (USA)	1944	*Pneumococcus* transforming factor (DNA)	[13]
Erwin Chargaff (Austria)	1950	Chargaff's rules	[14]
James Watson (USA)	1953	Double helix	[15]
Francis Crick (UK)			

* The names of main investigators are listed.

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guanine is equal to cytosine and the amount of adenine is equal to thymine (Chargaff's rules) [14].

These contributions to basic information of the gene structure were followed by theoretical calculation of the three-dimensional structure of DNA, double helix [15], and a new era of molecular biology started, stimulating interest in human diseases caused by gene mutations.

After confirmation of DNA structure by subsequent laboratory experiments, genetic engineering techniques were developed for application of basic research achievements (Table 3). Among them DNA cutting [16–18] and new DNA synthesis using reverse transcriptase were most important for analysis of human DNA samples [19,20]. In addition DNA pasting using DNA ligase was developed [21,22], and a new recombinant technology was established [23]. Two different methods of DNA sequencing were developed and applied for mutant DNA analysis in human patients [24,25]. Finally amplification of minute amount of DNA by polymerase chain reaction [26] enabled us to handle clinically available human samples for identifying a human subject and identifying various gene mutations.

4. Neurogenetic disease: hereditary metabolic diseases affecting the brain

Two reports appeared in late 1880s describing a new genetic neurological disease in children. A British ophthalmologist Warren Tay described the red spot on the retina of the eye of a patient [27], and an American neurologist Bernard Sachs described the cellular changes of the disease and noted its higher occurrence in Ashkenazi Jews from Eastern Europe [28]. Currently this disease is known as Tay-Sachs disease, the most famous prototype of neurogenetic diseases in children. In the first half of the 20th century a large number of genetic diseases, particularly those involving the central nervous system, were reported mainly based on clinical and pathological observations.

In the middle of the 20th century electron microscope was introduced to clinical medicine and revealed a completely new morphologic aspect of neurogenetic diseases, such as membranous cytoplasmic body (known as MCB) in the brain of Tay-Sachs disease patients [29]. Simultaneously techniques of molecular biology were introduced to clinical analysis, and understanding of human diseases became deeper at the level of gene and protein molecules. At present the techniques summarized in Table 3 are indispensable tools for clinical application of basic scientific research in molecular biology.

At present the term genetic disease is used almost synonymously as metabolic disease, because mutant functional proteins are under the control of mutant genes, causing metabolic dysregulations as enzymes, receptors, structural materials and others, in somatic cells of our body. Information about genetic diseases is regularly updated on the website of Online Mendelian Inheritance in Man (OMIM). The number of genetic diseases in this list is 6921 as of July 16, 2010 [30]. Biological markers have been identified in 35–40% of them, and responsible genes in 5–10%. In clinical child neurology the central nervous system is involved in 60–70% of genetic diseases, counting roughly up to 4000–5000 as neurogenetic diseases (Suzuki, Personal estimation). We have to keep in mind the possibility of some neurogenetic diseases for differential diagnosis whenever there is a neurological patient without solid clinical diagnosis.

5. Gene diagnosis of neurogenetic diseases

When we come across a child presenting with neurological signs and symptoms of unknown etiology, any of

Table 4
Gene analysis of neurogenetic diseases (proband or affected family).

1	Gene diagnosis of the proband
2	Prediction of phenotypic expression (clinical course)
3	Prenatal diagnosis
4	Carrier detection
5	Possibility of therapy and prevention

Table 3
Development of Genetic Engineering Technology*.

Researcher	Year	Achievement	References
Werner Arber (Switzerland)	1962	DNA cutting: restriction enzyme	[16]
Daniel Nathans (USA)	1968		[17]
Hamilton O. Smith (USA)	1970		[18]
David Baltimore (USA)	1970	Reverse transcriptase	[19]
Howard M. Temin (USA)	1970		[20]
Stanley Cohen (USA)	1971	DNA pasting: polymerase (ligase)	[21]
Paul Berg (USA)	1972		[22]
	1973		[23]
Walter Gilbert (USA)	1977	DNA sequencing	[24]
Frederick Sanger (UK)	1977		[25]
Kary B. Mullis (USA)	1986	Polymerase chain reaction	[26]

* The names of main investigators are listed.

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the genetic diseases listed on the OMIM website can be screened on the basis of phenotypic expressions (clinical signs and symptoms). In general the incidence is low for one single disease (1 out of 100,000 births or even lower). However, the total number of patients is not small when all diseases are counted up. OMIM is a powerful tool for survey of this type personally for me; one or more keywords will give hundreds of diseases compatible with clinical manifestations of the patient in question.

When a genetic disease is suspected clinically and/or pathologically, a wide metabolic screening should be made for identification of a single disease or a small group of diseases. Final diagnosis of a genetic disease is made by confirming a specific mutation of a single gene responsible for the pathogenesis of the disease. There are several steps for further investigation of the family members after establishment of the diagnosis in the proband (Table 4). Among them carrier diagnosis is accurate and not ambiguous as compared to metabolic diagnosis if the mutant gene of the patient and the genotype of the parents have been established [31,32].

In general development of a disease is influenced by both genetic and environmental factors. Single gene diseases are not exceptions. Phenylketonuria is a typical genetic metabolic disease affecting the central nervous system. The clinical disease will not develop unless dietary intake of phenylalanine surpasses the limit of tolerance to handle the amino acid in the liver. This is an example of significant environmental influence to clinical onset of a single gene disease. The outline of mutual interactions between genetic and environmental factors is illustrated in Fig. 1.

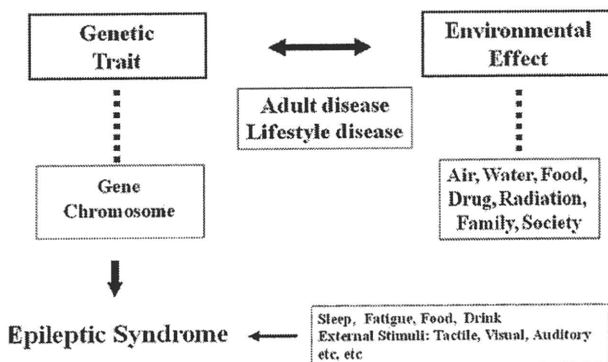

Fig. 1. Interactions between gene and environment. Genetic disease results mainly from the genetic background of the patient. However, various environmental factors influence clinical onset and course of the disease. In addition epileptic seizures are triggered by physical, chemical and social environments. These factors should be counted for evaluation and treatment of each patient.

Table 5
Hereditary Epilepsies.

Trait	Number
Dominant	211
Recessive	199
X-linked	88
Y-linked	2
Total	500

OMIM, July 16, 2010.

6. Molecular genetics of epileptic syndromes

Identification of genes that influence risk for epilepsies is important for basic research and clinical practice. As of July 16, 2010, 500 hereditary epilepsies are listed in the OMIM database, with 40% each of autosomal dominant and recessive traits (Table 5). These numbers indicate that we should keep in mind the possibility of genetic trait in each epileptic patient, although the incidence of hereditary epilepsy is not high in the general population affected by epilepsy.

Genetic testing is important for clinical practice for elucidation of pathogenesis and for possible development of new therapeutic approaches [33]. The molecular genetic information is useful not only for diagnosis of the patient in question but also for prediction of developing epilepsy in the family members as shown in Table 4. Currently available molecular methods for genetic testing are well described in the report by Ottman et al. (2010) [33].

Some well-known and validated diseases and genes are listed in Table 6. They are single gene disorders mainly manifesting epileptic symptomatology. Genes causing cerebral cortical malformations or progressive myoclonus epilepsies such as Unverricht Lundborg disease, Lafora disease and neuronal ceroid lipofuscinosis are not included in this list. However, symptomatic epilepsies in generalized brain disorders are important for genetic testing in clinical practice.

It is noteworthy that gene mutation analysis gives an accurate diagnosis when the analytical result is positive. However, a specific gene mutation is not always identified in the exon sequence of the gene responsible for an epileptic syndrome as a genetic disorder (Table 6). In addition we should keep in mind that other genes unknown at present may be involved in an epileptic syndrome. Accuracy of diagnosis depends on our understanding of the genetic spectrum of a symptomatic disease group. It is almost impossible to identify specific gene mutations in all patients affected by a clinically recognized epileptic syndrome as well as many other single gene disorders.

Usefulness of molecular genetic information in clinical practice is summarized in Fig. 2. When mutation of a gene is identified in a patient with epilepsy, the negative effects on neurophysiology and neurodevelopment

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Table 6
Genes Identified in Idiopathic Epilepsy Syndromes.

Syndrome	Disease	Locus	Gene	Product	Proportion of mutations	Accuracy of diagnosis
Syndromes beginning in the first year of life	Benign familial neonatal seizures	20q13.3	KCNQ2	$K_v7.2$ (K^+ channel)	>50% of families	Accurate
		8q24	KCNQ3	$K_v7.3$ (K^+ channel)	~7% families	Accurate
	Benign familial neonatal-infantile seizures	2q23-q24.3	SCN2A	$Na_v1.2$ (Na^+ channel)	Unknown	Accurate
	Ohtahara syndrome	9q34.1	STXBP1	Syntaxin binding protein 1	~35% patients	Accurate
		Xp22.13	ARX	Aristaless-related homeobox protein	Unknown	Accurate
	Early onset spasms	Xp22	STK9/CDKL5	Cyclin-dependent kinase-like 5	10–17% patients	Accurate
	X-linked infantile spasms	Xp22.13	ARX	Aristaless-related homeobox protein	<5% male patients	Accurate
Syndromes with prominent febrile seizures	Dravet syndrome (severe myoclonic epilepsy in infancy)	2q24	SCN1A	$Na_v1.1$ (Na^+ channel)	70–80% patients	Truncation: accurate Missense: less clear
	Genetic epilepsy with febrile seizures plus (GEFS+)	2q24	SCN1A	$Na_v1.1$ (Na^+ channel)	5–10% families	Missense: accurate
		19q13.1	SCN1B	b_1 Subunit (Na^+ channel)	<5% families	
		5q24	GABRG2	g2 Subunit (Na^+ channel)	<1% families	
	Childhood absence epilepsy with febrile seizures	5q34	GABRG2	g2 Subunit (Na^+ channel)		
	Epilepsy and mental retardation limited to females	Xq22	PCDH19	Protocadherin	Unknown	Accurate
Idiopathic generalized epilepsies	Early-onset absence epilepsy	1p35-p31.1	SLC2A1	GLUT1 (glucose transporter type 1)	~10% patients	Accurate
	Juvenile myoclonic epilepsy	5q34-q13.3	GABRA1	a_1 Subunit ($GABA_A$ receptor)		
		6p12-p11	EFHC1	EF hand motif protein		
Focal epilepsies	Autosomal dominant nocturnal frontal lobe epilepsy	20q13.2-q13.3	CHRNA4	a_4 Subunit (nACh receptor)	<10% families	Accurate
		1q21	CHRNB2	b_2 Subunit (nACh receptor)	<5% families	
		8p21	CHRNA2	a_2 Subunit (nACh receptor)	Unknown (rare)	
	Autosomal dominant partial epilepsy with auditory features (Autosomal dominant lateral temporal epilepsy)	10q24	LGI1	Leucine-rich repeat protein	~50% patients	
Epilepsies associated with other paroxysmal disorders	Generalized epilepsy and paroxysmal dyskinesia	10q22	KCNMA1	$K_{Ca}1.1$ (K^+ channel)		
	Epilepsy with paroxysmal exercise-induced dyskinesia	1p35-p31.3	SLC2A1	GLUT1 (glucose transporter type 1)	Unknown	Accurate
	Absence epilepsy and episodic ataxia	19p13	CACNA1A	$Ca_v2.1$ (Ca^+ channel)		
	Focal epilepsy and episodic ataxia	12p13	KCNA1	$K_v1.1$ (K^+ channel)		
	Familial hemiplegic migraine and epilepsy	1q21–23	ATP1A2	Sodium–potassium ATPase		

Modified from Ref. [33].

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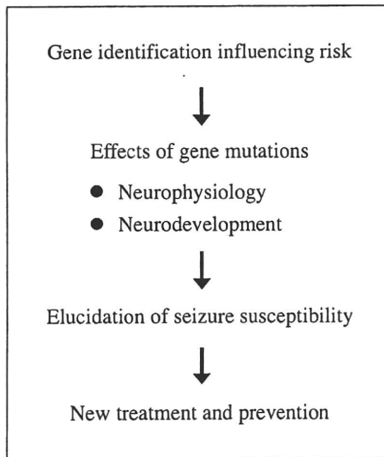

Fig. 2. Clinical usefulness of genetics in epilepsy practice. Identification of a mutant gene closely related to epilepsy will provide information about negative effects on neurophysiology and neurodevelopment. The risk of seizure susceptibility will be logically calculated. Furthermore we may be able to devise an approach to new treatment and prevention of the disease in question.

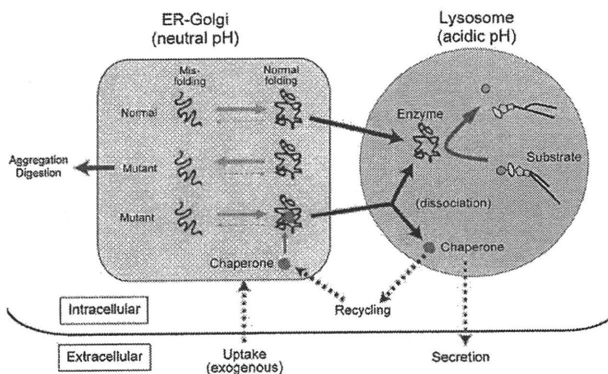

Fig. 3. Molecular interactions between mutant enzyme and chaperone compound. Mutant enzyme protein is unstable in the endoplasmic reticulum (ER)-Golgi compartment at neutral pH, and rapidly degraded or aggregated possibly to cause ER stress. An appropriate substrate analogue inhibitor binds to misfolded mutant protein as chemical chaperone at the ER-Golgi compartment, resulting in normal folding and formation of a stable complex at neutral pH. The protein-chaperone complex is safely transported to the lysosome. The complex is dissociated under the acidic condition and in the presence of excessive storage of the substrate. The mutant enzyme remains stabilized, and express catalytic function. The released chaperone is either secreted from the cell or recycled to interact with another mutant protein. These molecular events have been partially clarified by analytical and morphological analyses, and computer-assisted prediction of molecular interactions. Modified from ref [38].

will be anticipated and the risk of seizure susceptibility will be logically calculated. Furthermore we may be able to devise an approach to new treatment and prevention of the disease in question.

Epileptic seizures are one of the major manifestations in lysosomal disease. Enzyme replacement therapy has been widely conducted to improve non-neurological manifestations in some diseases, such as Gaucher disease, Fabry disease, mucopolysaccharidoses, and Pompe disease. However, the clinical effect on neurological manifestations has not been confirmed.

I have been trying to develop a molecular therapeutic technology based on a new concept, toward a few lysosomal diseases as first examples [34–37]. An exogenous competitive inhibitor of a lysosomal enzyme binds to the mutant enzyme protein to form a stable complex at neutral pH of the rough endoplasmic reticulum-Golgi compartment. The complex is safely transported to the lysosome, and dissociated under the acidic condition and in the presence of excessive storage of the substrate. The mutant enzyme remains stabilized, and express catalytic function [38] (Fig. 3). This approach has been shown to be clinically effective in a G_{M1}-gangliosidosis model mouse strain expressing R201C mutant β-galactosidase [39], and the clinical course is improved with a prolonged life span (Suzuki, unpublished data). I hope human trial will be started in the near future. This is one of the few medical approaches to the possibility of clinical treatment. I hope epilepsy also will become the target of this new therapeutic trial.

7. Conclusion

The genetic background of a clinically established disease should always be kept in mind whenever we see a patient with a neurological disease without known etiological information. There may be many genetic diseases not known to us at present. Gene analysis will give us solid diagnosis and insight into the pathogenesis of a disease, when specific information has been provided for a patient. Further, a new therapeutic aspect may be expected on the basis of the pathophysiology of epileptic phenomenon.

Our understanding of genetic diseases started from careful clinical and pathological observations in the first half of the 20th century, and then the stage of molecular genetic analysis appeared in the second half of the 20th century. Clinical analysis of patients was followed by molecular analysis for diagnosis and possible treatment ("from patient to molecule"). Information about molecular pathology was accumulated, and we had an illusion that every detail of the disease in question could be elucidated by analysis of gene and protein molecules. However, almost nothing was revealed about the pathological sequence of events in cells and tissues of the patient with a single disorder. Cellular and tissue pathophysiology should be investigated on the basis of molecular pathology in genetic diseases, including epileptic syndromes ("from molecule to patient"). There is a huge black box (the cell) with complicated metabolic systems.

Without clarification of these details we may not be able to reach our final destination of clinical medicine; treatment and prevention of genetic diseases.

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