Shield) as described (Lin et al., 2004). Briefly, cells were homogenized with a potter homogenizer in ice-cold buffer (Hepes 10 mM pH 7.0, 1 mM EDTA, 1 mM EGTA supplemented with a protease inhibitor cocktail). After centrifugation at 100,000 *g* for 1 hour at 4°C, the supernatant was discarded and the pellet was resuspended in the same buffer, overlaid onto an Opti-prep gradient and centrifuged at 100,000 *g* for 16 hours at 4°C. The top 12 fractions of the gradient were recovered and numbered accordingly.

Immunofluorescence

All procedures were carried out at room temperature. Cells were fixed for 2 minutes with acetone:methanol (1:1 v/v) and incubated for 30 minutes in PBS + 1% bovine serum albumin (BSA). They were then incubated for 1 hour with anti-Flag antibody or anti-His_6 antibody in PBS + 1% BSA. Bound antibodies were visualized with an Alexa Fluor 546-conjugated secondary antibody and images were obtained using a BioRad MRC1024 confocal laser-scanning microscope.

Bacterial expression of GST-SKD1 and in vitro binding assays

The *EcoRI*/*XhoI* fragment of pME/SKD1 was transferred to pGEX-6P (Amersham Pharmacia). DH5α competent cells were transformed with the plasmid and protein expression was induced with 1 mM IPTG at 37°C for 3 hours. GST-SKD1 was recovered from bacterial pellets in 8 M urea, dialyzed against TBS and immobilized on glutathione sepharose. For in vitro binding assays, GST-SKD1 glutathione sepharose was incubated with 0.5% CHAPS extracts from Flag-NPC1-expressing cells at 4°C for 2 hours, in the absence or presence of ADP, ATP or ATPγs (all at 0.5 mM). The sepharose resin was washed with TBS + 0.5% CHAPS and bound proteins were eluted with glutathione and analyzed by SDS-PAGE followed by immunoblotting with anti-Flag antibody.

We thank J. F. Strauss III for mutant NPC1 cDNAs and R. Kopito for ubiquitin cDNA. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Babst, M., Sato, T. K., Banta, L. M. and Emr, S. D. (1997). Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p. *EMBO J.* **16**, 1820-1831.

Babst, M., Wendland, B., Estepa, E. J. and Emr, S. D. (1998). The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function. *EMBO J.* **17**, 2982-2993.

Babst, M., Katzmann, D. J., Estepa-Sabal, E. J., Meerloo, T. and Emr, S. D. (2002a). ESCRT-III: an endosome-associated heterooligomeric protein complex required for MVB sorting. *Dev. Cell* **3**, 271-282.

Babst, M., Katzmann, D. J., Snyder, W. B., Wendland, B. and Emr, S. D. (2002b). Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body. *Dev. Cell* **3**, 283-289.

Berger, A. C., Hanson, P. K., Wylie Nichols, J. and Corbett, A. H. (2005). A yeast model system for functional analysis of the niemann-pick type C protein 1 homolog, ncr1p. *Traffic* **6**, 907-917.

Bishop, N. and Woodman, P. (2000). ATPase-defective mammalian VPS4 localizes to aberrant endosomes and impairs cholesterol trafficking. *Mol. Biol. Cell* **11**, 227-239.

Bishop, N. and Woodman, P. (2001). TSG101/mammalian VPS23 and mammalian VPS28 interact directly and are recruited to VPS4-induced endosomes. *J. Biol. Chem.* **276**, 11735-11742.

Bishop, N., Horman, A. and Woodman, P. (2002). Mammalian class E vps proteins recognize ubiquitin and act in the removal of endosomal protein-ubiquitin conjugates. *J. Cell Biol.* **157**, 91-101.

Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M., Pavan, W. J., Krizman, D. B. et al. (1997). Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science* **277**, 228-231.

Davies, J. P. and Ioannou, Y. A. (2000). Topological analysis of Niemann-Pick C1 protein reveals that the membrane orientation of the putative sterol-sensing domain is identical to those of 3-hydroxy-3-methylglutaryl-CoA reductase and sterol regulatory element binding protein cleavage-activating protein. *J. Biol. Chem.* **275**, 24367-24374.

Davies, J. P., Chen, F. W. and Ioannou, Y. A. (2000). Transmembrane molecular pump activity of Niemann-Pick C1 protein. *Science* **290**, 2295-2298.

Fujita, H., Yamanaka, M., Imamura, K., Tanaka, Y., Nara, A., Yoshimori, T., Yokota, S. and Himeno, M. (2003). A dominant negative form of the AAA ATPase SKD1/VPS4 impairs membrane trafficking out of endosomal/lysosomal compartments: class E vps phenotype in mammalian cells. *J. Cell Sci.* **116**, 401-414.

Fujita, H., Umezuki, Y., Imamura, K., Ishikawa, D., Uchimura, S., Nara, A., Yoshimori, T., Hayashizaki, Y., Kawai, J., Ishidoh, K. et al. (2004). Mammalian class E Vps proteins, SBP1 and mVps2/CHMP2A, interact with and regulate the function of an AAA-ATPase SKD1/Vps4B. *J. Cell Sci.* **117**, 2997-3009.

Garver, W. S., Heidenreich, R. A., Erickson, R. P., Thomas, M. A. and Wilson, J. M. (2000). Localization of the murine Niemann-Pick C1 protein to two distinct intracellular compartments. *J. Lipid Res.* **41**, 673-687.

Hicke, L. (2001). A new ticket for entry into budding vesicles-ubiquitin. *Cell* **106**, 527-530.

Higaki, K., Ninomiya, H., Sugimoto, Y., Suzuki, T., Taniguchi, M., Niwa, H., Pentchev, P. G., Vanier, M. T. and Ohno, K. (2001). Isolation of NPC1-deficient Chinese hamster ovary cell mutants by gene trap mutagenesis. *J. Biochem.* **129**, 875-880.

Higgins, M. E., Davies, J. P., Chen, F. W. and Ioannou, Y. A. (1999). Niemann-Pick C1 is a late endosome-resident protein that transiently associates with lysosomes and the trans-Golgi network. *Mol. Genet. Metab.* **68**, 1-13.

Inohara, N. and Nunez, G. (2002). ML – a conserved domain involved in innate immunity and lipid metabolism. *Trends Biochem. Sci.* **27**, 219-221.

Katzmann, D. J., Babst, M. and Emr, S. D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell* **106**, 145-155.

Ko, D. C., Gordon, M. D., Jin, J. Y. and Scott, M. P. (2001). Dynamic movements of organelles containing Niemann-Pick C1 protein: NPC1 involvement in late endocytic events. *Mol. Biol. Cell* **12**, 601-614.

Lin, H., Sugimoto, Y., Ohsaki, Y., Ninomiya, H., Oka, A., Taniguchi, M., Ida, H., Eto, Y., Ogawa, S., Matsuzaki, Y. et al. (2004). N-octyl-beta-valienamine up-regulates activity of F213I mutant beta-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease. *Biochim. Biophys. Acta* **1689**, 219-228.

Millard, E. E., Gale, S. E., Dudley, N., Zhang, J., Schaffer, J. E. and Ory, D. S. (2005). The sterol-sensing domain of the Niemann-Pick C1 (NPC1) protein regulates trafficking of low density lipoprotein cholesterol. *J. Biol. Chem.* **280**, 28581-28590.

Millat, G., Marcais, C., Tomasetto, C., Chikh, K., Fensom, A. H., Harzer, K., Wenger, D. A., Ohno, K. and Vanier, M. T. (2001a). Niemann-Pick C1 disease: correlations between NPC1 mutations, levels of NPC1 protein, and phenotypes emphasize the functional significance of the putative sterol-sensing domain and of the cysteine-rich luminal loop. *Am. J. Hum. Genet.* **68**, 1373-1385.

Millat, G., Chikh, K., Naureckiene, S., Sleat, D. E., Fensom, A. H., Higaki, K., Elleder, M., Lobel, P. and Vanier, M. T. (2001b). Niemann-Pick disease type C: spectrum of HE1 mutations and genotype/phenotype correlations in the NPC2 group. *Am. J. Hum. Genet.* **69**, 1013-1021.

Naureckiene, S., Sleat, D. E., Lackland, H., Fensom, A., Vanier, M. T., Wattiaux, R., Jadot, M. and Lobel, P. (2000). Identification of HE1 as the second gene of Niemann-Pick C disease. *Science* **290**, 2298-2301.

Patterson, M. C., Vanier, M. T., Suzuki, K., Morris, J. A., Carstea, E. D., Neufeld, E. B., Blanchette-Mackie, E. J. and Pentchev, P. G. (2001). Niemann-Pick disease type C: a lipid trafficking disorder. In *The Metabolic and Molecular Bases of Inherited Disease* (ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle, B. Childs, K. W. Kinzler and B. Vogelstein), pp. 3611-3634. New York: McGraw Hill.

Peck, J. W., Bowden, E. T. and Burbelo, P. D. (2004). Structure and function of human Vps20 and Snf7 proteins. *Biochem. J.* **377**, 693-700.

Ravid, T., Doolman, R., Avner, R., Harats, D. and Roitelman, J. (2000). The ubiquitin-proteasome pathway mediates the regulated degradation of mammalian 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J. Biol. Chem.* **275**, 35840-35847.

Scheuring, S., Rohricht, R. A., Schoning-Burkhardt, B., Beyer, A., Muller, S., Abts, H. F. and Kohrer, K. (2001). Mammalian cells express two VPS4 proteins both of which are involved in intracellular protein trafficking. *J. Mol. Biol.* **312**, 469-480.

Scott, C., Higgins, M. E., Davies, J. P. and Ioannou, Y. A. (2004). Targeting of NPC1 to late endosomes involves multiple signals, including one residing within the putative sterol-sensing domain. *J. Biol. Chem.* **279**, 48214-48223.

Sever, N., Song, B. L., Yabe, D., Goldstein, J. L., Brown, M. S. and DeBose-Boyd, R. A. (2003a). Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol. *J. Biol. Chem.* **278**, 52479-52490.

Sever, N., Yang, T., Brown, M. S., Goldstein, J. L. and DeBose-Boyd, R. A. (2003b). Accelerated degradation of HMG CoA reductase mediated by binding of insig-1 to its sterol-sensing domain. *Mol. Cell* **11**, 25-33.

Sharma, M., Pampinella, F., Nemes, C., Benharouga, M., So, J., Du, K., Bache, K. G., Papsin, B., Zerangue, N., Stenmark, H. et al. (2004). Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. *J. Cell Biol.* **164**, 923-933.

Shenoy, S. K., McDonald, P. H., Kohout, T. A. and Lefkowitz, R. J. (2001). Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science* **294**, 1307-1313.

Simons, K. and Ikonen, E. (1997). Functional rafts in cell membranes. *Nature* **387**, 569-572.

Sleat, D. E., Wiseman, J. A., El-Banna, M., Price, S. M., Verot, L., Shen, M. M., Tint, G. S., Vanier, M. T., Walkley, S. U. and Lobel, P. (2004). Genetic evidence for nonredundant functional cooperativity between NPC1 and NPC2 in lipid transport. *Proc. Natl. Acad. Sci. USA* **101**, 5886-5891.

Sugimoto, Y., Ninomiya, H., Ohsaki, Y., Higaki, K., Davies, J. P., Ioannou, Y. A. and Ohno, K. (2001). Accumulation of cholera toxin and GM1 ganglioside in the early endosome of Niemann-Pick C1-deficient cells. *Proc. Natl. Acad. Sci. USA* **98**, 12391-12396.

Vanier, M. T. and Millat, G. (2004). Structure and function of the NPC2 protein. *Biochim. Biophys. Acta* **1685**, 14-21.

Watari, H., Blanchette-Mackie, E. J., Dwyer, N. K., Watari, M., Neufeld, E. B., Patel, S., Pentchev, P. G. and Strauss, J. F., III (1999a). Mutations in the leucine zipper motif and sterol-sensing domain inactivate the Niemann-Pick C1 glycoprotein. *J. Biol. Chem.* **274**, 21861-21866.

Watari, H., Blanchette-Mackie, E. J., Dwyer, N. K., Glick, J. M., Patel, S., Neufeld, E. B., Brady, R. O., Pentchev, P. G. and Strauss, J. F., III (1999b). Niemann-Pick

C1 protein: obligatory roles for N-terminal domains and lysosomal targeting in cholesterol mobilization. *Proc. Natl. Acad. Sci. USA* **96**, 805-810.

Yabe, D., Brown, M. S. and Goldstein, J. L. (2002). Insig-2, a second endoplasmic reticulum protein that binds SCAP and blocks export of sterol regulatory element-binding proteins. *Proc. Natl. Acad. Sci. USA* **99**, 12753-12758.

Yamamoto, T., Ninomiya, H., Matsumoto, M., Ohta, Y., Nanba, E., Tsutsumi, Y., Yamakawa, K., Millat, G., Vanier, M. T., Pentchev, P. G. et al. (2000). Genotype-phenotype relationship of Niemann-Pick disease type C: a possible correlation between clinical onset and levels of NPC1 protein in isolated skin fibroblasts. *J. Med. Genet.* **37**, 707-712.

Yan, Q., Hunt, P. R., Frelin, L., Vida, T. A., Pevsner, J. and Bean, A. J. (2005). mVps24p functions in EGF receptor sorting/trafficking from the early endosome. *Exp. Cell Res.* **304**, 265-273.

Yang, T., Espenshade, P. J., Wright, M. E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J. L. and Brown, M. S. (2002). Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. *Cell* **110**, 489-500.

Yoshimori, T., Yamagata, F., Yamamoto, A., Mizushima, N., Kabeya, Y., Nara, A., Miwako, I., Ohashi, M., Ohsumi, M. and Ohsumi, Y. (2000). The mouse SKD1, a homologue of yeast Vps4p, is required for normal endosomal trafficking and morphology in mammalian cells. *Mol. Biol. Cell* **11**, 747-763.

Zhang, M., Dwyer, N. K., Neufeld, E. B., Love, D. C., Cooney, A., Comly, M., Patel, S., Watari, H., Strauss, J. F., III, Pentchev, P. G. et al. (2001). Sterol-modulated glycolipid sorting occurs in niemann-pick C1 late endosomes. *J. Biol. Chem.* **276**, 3417-3425.

Zhang, S., Ren, J., Li, H., Zhang, Q., Armstrong, J. S., Munn, A. L. and Yang, H. (2004). Ncr1p, the yeast ortholog of mammalian Niemann Pick C1 protein, is dispensable for endocytic transport. *Traffic* **5**, 1017-1030.

Histidine-Tagged Shiga Toxin B Subunit Binding Assay: Simple and Specific Determination of Gb3 Content in Mammalian Cells

In-Sun SHIN,[a,b] Kiyotaka NISHIKAWA,[c,d] Hiroki MARUYAMA,[e] and Satoshi ISHII*,[a]

[a] *Department of Agricultural and Life Sciences, Obihiro University of Agriculture and Veterinary Medicine; Obihiro 080–8555, Japan:* [b] *Course of Science of Bioresources, The United Graduate School of Agricultural Sciences, Iwate University; Morioka 020–8550, Japan:* [c] *Department of Clinical Pharmacology, Research Institute, International Medical Center of Japan; Tokyo 162–8655, Japan:* [d] *PRESTO, Japan Science and Technology Agency; Saitama 332–0012, Japan: and* [e] *Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences; Niigata 951–8510, Japan.*

Received November 22, 2005; accepted January 11, 2006; published online January 18, 2006

A two-step binding assay for globotriaosylceramide (Gb3) content was developed by histidine-tagging strategy, which is a well-established method for the purification of recombinant proteins. The complete binding of the recombinant His-tagged Shiga toxin 1B subunit (1B-His) (1 μg/ml) to the standard Gb3 adsorbed on a multi-well H type plate was observed within 30 min at 37 °C; and its binding could be visualized by the following applications of HisProbe-HRP (8 μg/ml) and tetramethylbenzidine (TMB) peroxidase substrate. The 1B-His binding assay was linear over the range of 1 to 100 ng of Gb3 per well. The binding of 1B-His was specific to Gb3 separated from HeLa cells, and no major cross-reactivity of other glycolipids in Folch's lower fractions extracted from HeLa cells was detected. The glycolipids in Folch's lower fractions from HeLa cells, human fibroblasts and mouse heart were suitable for this assay, but the further purification was needed for glycolipids from human plasma, thus sample preparation is critical factor for the reliable determination of Gb3 content. The 1B-His binding to Gb3 was inhibited by the addition of galactose, but not mannose. This 1B-His binding assay will be useful not only for the determination of Gb3 content, but also for screening for the compounds which inhibit the toxin-binding to Gb3. The strategy of our present method may be applicable for other binding assay, such as Cholera toxin B-subunit for ganglioside GM1.

Key words globotriaosylceramide; Shiga toxin; binding assay method; His-tagged protein

Globotriaosylceramide (Galα1-4Galβ1-4Glcβ1-1Cer; Gb3) is the major neutral glycosphingolipid accumulating in patients with Fabry disease, which is an inherited disease caused by a deficiency of α-galactosidase A,[1,2] and is also known as the receptor for the Shiga toxin (Stx, also called verotoxin), which is a class of protein toxin produced by Stx-producing strains of *Escherichia coli* (STEC) associated with diarrhea, hemorrhagic colitis,[3] and hemolytic uremic syndrome (HUS).[4,5] Stx is an approximately 70-kDa complex comprised of one A-subunit (32 kDa) and five B-subunits (7.5 kDa each).[6] The holotoxin binds *via* B-subunit pentamer with high affinity to the terminal digalactose of Gb3.[7,8] Although the cytotoxicity of Stx was mainly caused by the A-subunit, which has an RNA-*N*-glycosidase activity, resulting in inhibition of protein synthesis,[7] Jacewicz *et al.*[9] suggested that the basal levels of Gb3 in human cells may relate to their sensitivity toward Stx. The assay of Gb3 content in mammalian cells is important for the study on the mechanism of Stx-cytotoxicity and on the pathogenesis of Fabry disease.

Various assay methods for Gb3 content have been previously reported, such as HPLC analysis of benzoylated glycolipids,[10] TLC-orcinol staining,[11] liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis,[12] and matrix-assisted laser desorption ionisation with time-of-flight (MALDI-TOF) mass spectrometry.[13] High cost equipment is sometimes required for these methods. In contrast, the simple assay method has been reported as an enzyme-linked immunosorbent assay (ELISA) method using verotoxin B subunit.[14] This method has many benefits including no special equipment is required, many samples can be assayed at once, and a specific Gb3 assay can be accomplished because of the high affinity of the verotoxin B subunit toward Gb3.

The purpose of this study was to simplify the ELISA method using the verotoxin B subunit and to determine the specificity of Gb3 assay in various sources. In this study, we used the recombinant histidine-tagged B subunit of Stx 1 (1B-His), because 1B-His has a higher affinity toward Gb3 in comparison with His-tagged Stx 2B subunit (2B-His).[15] The two-step binding assay was established by using 1B-His and HisProbe-HRP, which is a nickel (Ni^{2+}) activated derivative of horseradish peroxidase (HRP) and is specifically bound to the poly-histidine residue; and the sample preparation of glycosphingolipid suitable for this Gb3 assay was studied in different sources. Other applications of this binding assay were also suggested in this study.

Experimental

Preparation of Recombinant 1B-His The recombinant 1B-His, in which 6 histidine residues were added at the carboxy termini of the B subunits, was prepared as described previously.[15] The BL21DE cells expressing 1B-His were cultured in 300 ml of LB broth (Difco laboratories, Detroit, MI, U.S.A.) supplemented with 50 μg/ml kanamycin (Nacalai Tesque, Inc., Kyoto, Japan) at 37 °C for 2 h. The cells were subsequently treated with 1.0 mmol/l isopropyl β-D(−)thiogalactopyranoside (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 37 °C for 4 h. Cells were pooled by centrifugation at 6000 rpm for 15 min at 4 °C. The 1B-His was extracted from cell pellets with the Bugbuster protein extraction reagent (Novagen, Madison, WI, U.S.A.) and purified by using the His-bind purification kit (Novagen) according to manufacturer's recommendations. Purified 1B-His fractions were applied to an NAP10 column (Amersham Biosciences, Uppsala, Sweden) equilibrated with phosphate-buffered saline (PBS). The purified 1B-His was revealed as a single band on SDS-PAGE (data not shown) and its aliquots were stored at −20 °C.

Materials HisProbe-HRP was purchased from Pierce Biotechnology,

* To whom correspondence should be addressed. e-mail: ishiis@obihiro.ac.jp.

Inc. (Rockford, IL, U.S.A.). Purified glycosphingolipids, Gb3 from porcine erythrocytes and globotetraosylceramide (Gb4) from porcine erythrocytes were purchased from Nacalai Tesque, Inc. and Wako Pure Chemical Industries, Ltd., respectively.

Cell Lines and Specimen HeLa cell line (JCRB9004) was purchased from Human Science Research Resources Bank (Osaka, Japan) and human fibroblast cell line was established from a heterozygous patient with Fabry disease. Cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.) supplemented with 10% fatal calf serum (Biological Industries, Haemek, Israel) under a humidified 5% CO_2 atmosphere. Cultured cells were washed with PBS and then harvested by a plastic scraper. Cells were precipitated by centrifugation at 2000 rpm for 5 min. Human plasma was prepared from the heparinized blood of a heterozygous patient with Fabry disease by centrifugation at 3000×*g* for 5 min. All samples were stored at −20 °C.

Glycosphingolipid Extraction Cell pellets and minced heart from an α-galactosidase A-knock out (KO) mouse[16] were homogenized using a handy micro homogenizer (Physcotron, Niti-on, Inc., Chiba, Japan) in 0.2-ml H_2O. After the determination of protein content with a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.), cellular lipids were extracted from homogenates containing 5—10 mg protein with 20 volumes of chloroform–methanol (2 : 1, v/v). The lipid from human plasma was also extracted with 20-fold excess volume of chloroform–methanol (2 : 1, v/v). Following filtration by 90-mm 5A filter papers (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), crude extracts were dried and stored as a crude extract fraction. Crude extracts were further treated with 1 ml of methanolic NaOH (0.2 M 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 (chloroform–methanol–H_2O, 8 : 4 : 3 in v/v/v).[17] Glycosphingolipids recovered in the lower phase were pooled and designated as a Folch's lower fraction. The glycosphingolipids in a Folch's lower fraction were further purified by the Iatrobeads (Iatron Laboratories, Tokyo, Japan) column chromatography. Glycosphingolipids suspended in 1 ml of chloroform–methanol (2 : 1, v/v) were applied to an Iatrobeads column (0.5×2 cm) equilibrated with isopropanol–hexane (55 : 45, v/v). The bound glycolipids were eluted with isopropanol–hexane–water (55 : 30 : 15, v/v/v). Eluates were pooled and used as an Iatrobeads-binding fraction.

Separation of Glycosphingolipids by HPLC Glycosphingolipids in Folch's lower fraction extracted from HeLa cells were separated by HPLC (LC-10AD, Shimadzu Corporation, Kyoto, Japan) according to the method of Kannagi *et al.*[18] Folch's lower fraction from HeLa cells was dissolved in 2 ml of chloroform–methanol (2 : 1, v/v), and applied to a packed column of Iatrobeads (6RS-8010, Iatron Laboratories; 1×50 cm) equilibrated with isopropanol–hexane (55 : 45, v/v). Glycosphingolipids were eluted with a gradient of isopropanol–hexane–water from 55 : 45 : 0 to 55 : 30 : 15 (v/v/v). The flow rate was set at 2 ml/min by applying approximately 60—70 kgf/cm^2. Fractions were collected by a fraction collector (FC 203B, Gilson Inc., Middleton, WI, U.S.A.) at 2-min intervals and the total volume of eluate was 400 ml. Each fraction was dried and resuspended in chloroform–methanol (2 : 1, v/v). A small aliquot of each fraction was applied to TLC analysis, and 7 major lipid components were pooled in 7 fractions.

1B-His Binding Assay The 1B-His binding assay for the determination of Gb3 content was performed as follows. Samples containing glycosphingolipids in 10 μl of 100% methanol were plated onto a multi-well H type plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The plates were placed to evaporate methanol at room temperature for 1 h, and then 200 μl of 1% bovine serum albumin solution in PBS (BSA-PBS) was added to each well and incubated at 37 °C for 1 h. Plates were washed once with 400 μl of 0.2% BSA-PBS, and then incubated with purified 1B-His (1 μg/ml) in 0.2% BSA-PBS at 37 °C for 30 min. Following washing three times with 200 μl of PBS, the plates were incubated with the HisProbe-HRP (8 μg/ml) in 0.2% BSA-PBS at 37 °C for 30 min. The plates were washed with 200 μl of PBS and then visualized with tetramethylbenzidine (TMB) peroxidase substrate system (100 μl/well) (KPL, Gaithersburg, MD, U.S.A.) at room temperature for 10—15 min. Reactions were terminated by the addition of 100 μl of 1 M phosphoric acid and gentle shaking. The absorbance of each well at 415 nm was determined using a micro plate reader (MPR-A4, Tosoh Co., Tokyo, Japan). For the determination of Gb3 content, 10—100 ng of standard Gb3 was consistently applied on the same plate, and absorbances were determined at each assay.

TLC and TLC-Blotting TLC analysis was quantitatively performed with high-performance TLC (HPTLC)-Silica gel 60 plates (Merck & Co., Inc., Whitehouse Station, NJ, U.S.A.) using a solvent system of chloroform–methanol–water (60 : 35 : 8, v/v/v). Glycosphingolipids were visualized by spraying orcinol-sulfuric acid reagent; and their band intensities were determined by the image processing software Scion Image (Scion Corporation, Frederick, MD, U.S.A.). TLC-blotting with 1B-His was performed by the method of Taki *et al.*[19] Glycosphingolipids were separated on a TLC plate as described above. The plate was sunk in 0.4% polyisobutylmethacrylate (PIM) solution (2.5% PIM in chloroform was diluted to 0.4% with hexane) and followed by blocking with 1% BSA-PBS. After incubation with 1B-His at 1 μg/ml at room temperature for 30 min, the plate was washed with PBS. After further incubation with HisPorbe-HRP at 8 μg/ml at room temperature for 1 h and the following final washing, 1B-His binding was visualized with an enhanced chemiluminescent substrate (Pierce Biotechnology, Inc.).

Results

1B-His Binding Assay with Standard Glycosphingolipids To establish the 1B-His binding assay, we first used standard glycosphingolipids (Gb3 and Gb4) in the amount of 1—100 ng/well (Fig. 1). Absorbance (abs.) at 415 nm and amount of Gb3 were good corresponding in the range of 1 to 100 ng of Gb3. 1B-His binding was partially observed when a higher amount of Gb4 was used. One hundred nanograms of Gb4 showed 0.062 abs. units; and 7.5% of 0.823 abs. units was observed with 100 ng of Gb3. The assay was highly reproducible, had a very low background (<0.05 abs. units), and there was minimal variation between three independent determinations for each Gb3 concentration. The effect of incubation time on 1B-His binding to standard Gb3 was analyzed. The 1B-His solution (1 μg/ml) in 0.2% BSA-PBS was applied to multi-well H type plates, coated with standard Gb3 (100 ng), and incubated for 5 different lengths (10, 20, 30, 60, 120 min) at 37 °C. The 1B-His binding was low for the shortest incubation, and reached a plateau at 30 min; and the effect of incubation time on the 1B-His binding was similar for lower Gb3 content (data not shown). This data suggested that at least 30-min incubation at 37 °C was necessary for the completion of 1B-His binding to Gb3. In Fig. 2, the optimum concentration of 1B-His and HisProbe-HRP was determined. The Gb3-dependent binding of 1B-His increased, according to the concentration of 1B-His, and reached a plateau at 1 μg/ml (Fig. 2A). 1B-His binding activity was also dependent on the addition of HisProbe-HRP, and reached a plateau at 8 μg/ml (Fig. 2B). These data indicated that the optimum concentrations of 1B-His and HisProbe-HRP for the detection of Gb3 content were 1 μg/ml and 8 μg/ml, respectively.

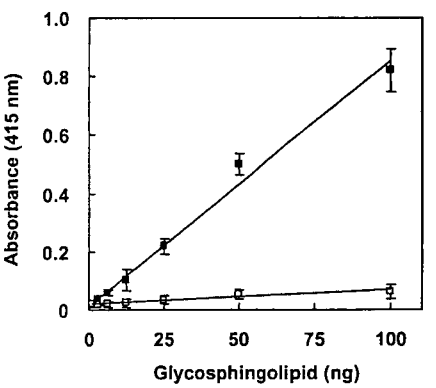

Fig. 1. 1B-His Binding Assay with Standard Glycosphingolipids

Doubled dilutions of standard Gb3 (■) and Gb4 (□) were applied to a multi-well H type plate and assayed with 1 μg/ml 1B-His and 8 μg/ml HisProbe-HRP. Results are the mean±S.D. of three independent assays.

Specificity of the 1B-His Binding Assay In order to determine the specificity of the 1B-His binding assay, Folch's lower fraction from HeLa cells was used as the source of various lipids. As shown in Fig. 3A, more than 6 different glycolipids and a group of cholesterol were present in this fraction. All major lipid components were separated by an HPLC attached Iatrobeads column, and pooled into 7 fractions (Fig. 3A). Fraction 1 was the pass through fraction using Iatrobeads column chromatography, and Fractions 2 to 7 were once bound and then eluted with different solvent conditions. The highest abs. units (1.238) in 1B-His binding was observed in Fraction 5, which contained Gb3. A partial 1B-His binding (0.104 abs. units) was detected in Fraction 6, which contained Gb4, but other fractions were less than background level (<0.05 abs. units) (Fig. 3B). To clarify whether the presence of non-Gb3 lipids negatively or positively affect the 1B-His binding assay, all 6 fractions other than Fraction 5 were combined, and a different concentration of standard Gb3 (Fig. 4A) was added to the pooled lipids (Fig. 4B). The abs. unit in the 1B-His binding simply corresponded to the content of standard Gb3, and no significant difference was observed between samples with or without combined lipids (Fig. 4C). These data indicated that no marked effect of other lipids on the 1B-His binding assay and low Gb3 content in HeLa cells can be correctly determined. The specificity of the 1B-His binding was further determined by TLC-blotting (Fig. 5). Iatrobeads-binding fractions from human cells, human plasma and KO-mouse heart were applied to TLC plate. More than 5 kinds of neutral glycolipids were detected in all Iatrobeads-binding fractions by the orcinol-staining

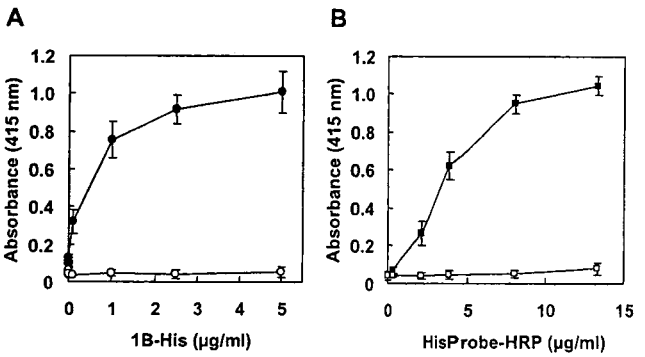

Fig. 2. Optimum Concentration of 1B-His (A) and HisProbe-HRP (B)

In A, wells with (●) or without (○) 100 ng of standard Gb3 were treated with indicated concentrations of 1B-His for 30 min. Bound 1B-His was detected by 8 μg/ml HisProbe-HRP. In B, wells coated with 100 ng of Gb3 were treated with (■) or without (□) 1 μg/ml of 1B-His. Bound 1B-His was determined by indicated concentrations of HisProbe-HRP. The error bars represent S.D. (n=3).

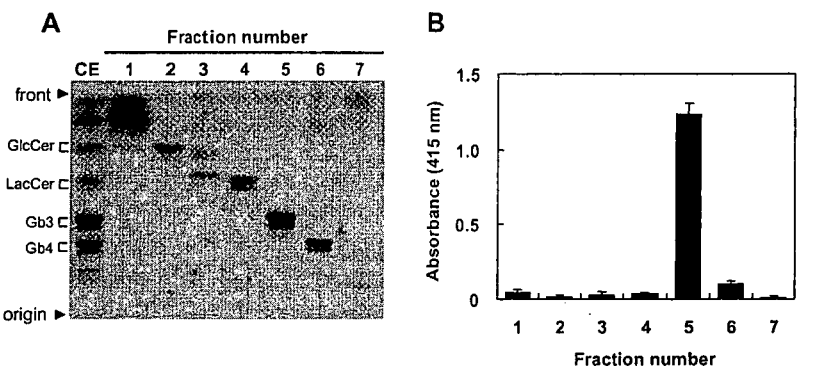

Fig. 3. 1B-His Binding to Glycosphingolipids Fractionated by HPLC from HeLa Cells

In A, glycosphingolipids in Folch's lower fraction extracted from HeLa cells were separated through low-pressure HPLC on an Iatrobeads 6RS-8010 column. TLC analysis was quantitatively performed with a solvent system of chloroform–methanol–water (60 : 35 : 8), and glycosphingolipids were visualized by orcinol reagent as described in Experimental. CE is crude extract in Folch's lower fraction extracted from HeLa cells. In B, the 1B-His binding assay of each glycosphingolipid fraction was performed. 1/10 aliquot of each fraction suspended in 100 μl of methanol was applied on a multi-well H type plate, and assayed as described in the legend to Fig. 1. Experiments were performed in triplicate, and the means±S.D. of the values are indicated.

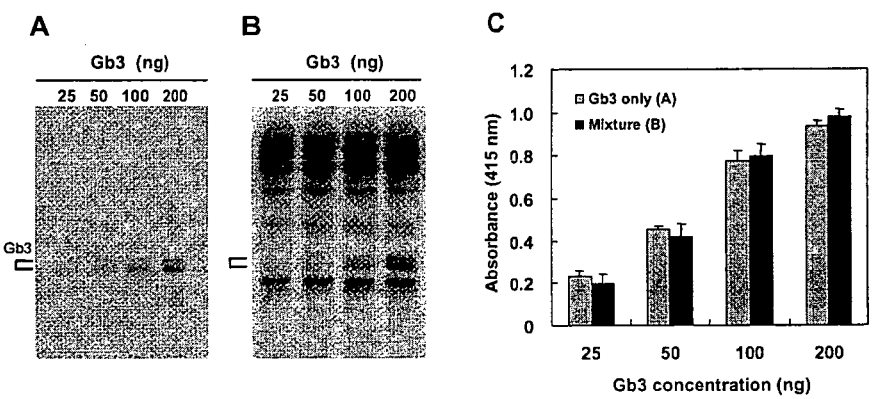

Fig. 4. Effect of All Components Other Than Gb3 in Folch's Lower Fraction from HeLa Cells on the 1B-His Binding Assay

Standard Gb3 at indicated concentrations with (B) or without (A) all components other than Gb3 in Folch's lower fraction from HeLa cells were applied onto TLC, and after its development with chloroform–methanol–water (60 : 35 : 8) TLC plates were stained with orcinol reagent. In C, the 1B-His binding assay of the same samples used in A and B was performed as described in the legend to Fig. 1. Experiments were performed in triplicate and the means±S.D. of the values indicated.

(Fig. 5A). 1B-His strongly bound to Gb3 in all samples, and weak binding to Gb4 was also observed in HeLa and human fibroblasts; but no other binding was observed in the glycolipids from human and mouse sources (Fig. 5B).

Effect of Sample Preparation on the 1B-His Binding Assay For a practical application of the 1B-His binding assay, the effect of a sample preparation on the assay condition was studied (Fig. 6). The glycolipids were sequentially prepared from human cells and plasma, and mouse tissue, and pooled as a crude extract fraction, a Folch's lower fraction and an Iatrobeads-binding fraction. All Iatrobeads-binding fractions showed good correlation between sample concentration and the 1B-His binding, but no correlation was observed in all crude extract fractions. Although Folch's lower fraction from human plasma did not show linear correlation, Folch's lower fractions from human cells and mouse tissue corresponded well with Iatrobeads-binding fractions from respective sources. This data indicated that a Folch's lower fraction may be suitable preparation for the 1B-His binding assay, but not for human plasma.

Comparison with Another Assay Method Gb3 content was determined in 4 different sources by the 1B-His binding assay and the results were compared to the data from the TLC-orcinol method (Table 1). The mean of Gb3 content determined by the 1B-His binding assay in HeLa cells, human fibroblasts and human plasma from a heterozygous patient with Fabry disease, and a heart from a KO mouse (27.3 μg/mg protein, 37.4 μg/mg protein, 11.1 μg/ml and 7.5 μg/mg protein, respectively), corresponded well with the values measured by the TLC-orcinol method (25.9 μg/mg protein, 34.7 μg/mg protein, 13.9 μg/ml and 6.3 μg/mg protein, respectively). Although the TLC-orcinol method was reproducible, a high amount of Gb3 (100—500 ng) was necessary for the assay; while, 10—50 ng Gb3 was enough for the 1B-His binding assay.

Other Applications of the 1B-His Binding Assay Blocking of 1B-His binding to Gb3 occurred with the addition of a high concentration of galactose but not mannose (Fig. 7). The abs. unit was decreased to 40% of the control by the addition of 300 mM galactose. These inhibition data indicated that 1B-His binding to Gb3 was specific to galactose.

The possibility that the 1B-His binding assay will be applicable to the measurement of Gb3 synthase (α1,4-galactosyltransferase) activity was studied. HeLa-cell homogenate was incubated with 0.5 mM UDP-Gal and 0.2 mM lactosylceramide (LacCer) at 37 °C for the indicated period. Folch's lower fraction was then prepared from each reaction solution and Gb3 content determined by the 1B-His binding assay (Fig. 8). The increase in Gb3 content was 3.2, 6.8, and 9.4 (μg/mg protein), after incubation for 1, 2 and 4 h, respec-

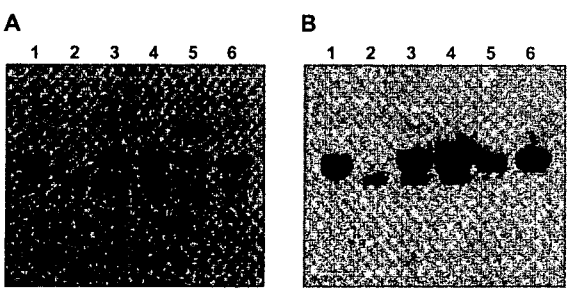

Fig. 5. 1B-His Binding on Glycosphingolipids Separated by TLC of Iatrobeads-Binding Fractions from Human Cells and Plasma, and Mouse Heart

Glycosphingolipid samples were applied to a HPTLC plate, and separated by a solvent system of chloroform : methanol : water (60 : 35 : 8). After the fixation of the plate with 0.4% PIM solution, TLC-blotting with 1B-His (B) was performed as described in Experimental. After the determination of 1B-His binding with chemiluminescent substrate, the same TLC plate was stained with orcinol reagent (A). Lanes 1 and 2, 500 ng of standard Gb3 and Gb4, respectively; lanes 3, 4, 5 and 6, Iatrobeads-binding fractions from HeLa cells, human fibroblasts, human plasma and the heart from KO mouse, respectively.

Table 1. Comparison with Gb3 Contents Determined by the Assay Methods of TLC-Orcinol and the 1B-His Binding

Sample	TLC-orcinol	1B-His binding
HeLa cells	25.89±2.94	27.28±0.88
Human fibroblasts	34.68±3.08	37.36±0.64
Human plasma	13.91±2.66	11.13±1.70
Mouse heart	6.28±1.58	7.52±0.57

Folch's lower fractions of human cells and mouse heart, and the Iatrobeads-binding fraction of human plasma were used as sample sources for both assays. The determination of Gb3 content by the TLC-orcinol and 1B-His binding assays was performed as described in Experimental. Values are expressed as μg/mg of protein±S.D. for human cells and mouse heart, and as μg/ml of plasma±S.D. for human plasma.

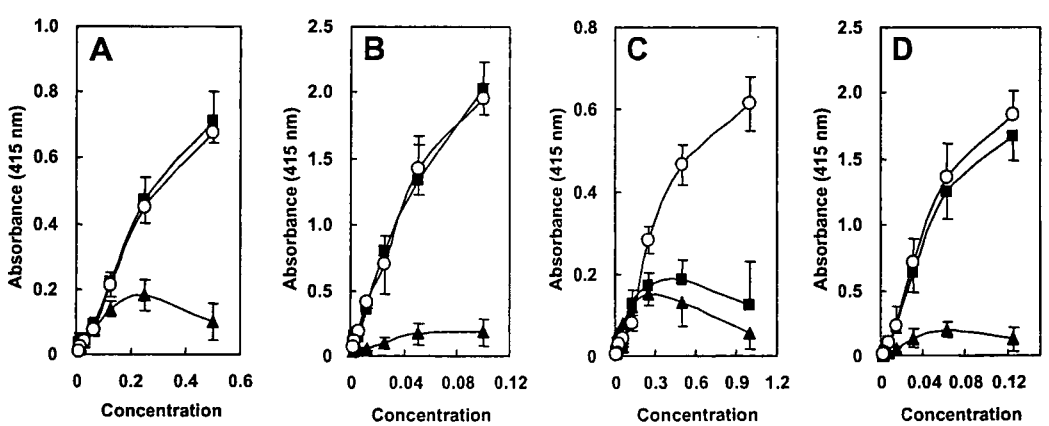

Fig. 6. Effect of Preparation of Lipid Samples on 1B-His Binding Assay

Lipids were extracted from HeLa cells (A), human fibroblasts (B), human plasma (C) and heart from KO mouse (D). Preparation of crude extract fraction (▲), Folch's lower fraction (■) and Iatrobeads-binding fraction (○) was performed as described in Experimental. Doubled dilutions of lipid samples were dried on the plate and then assayed by 1B-His binding. Concentration 1 was corresponding to 5 ng protein content of HeLa cells, fibroblasts, mouse heart and 5 μl of human plasma. Results are mean±S.D.

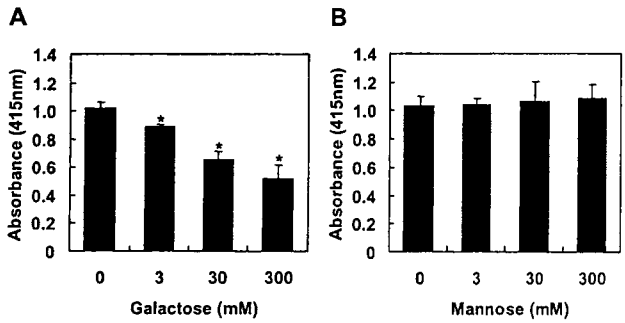

Fig. 7. Effect of Galactose and Mannose Addition in 1B-His Binding

1B-His in 0.2% BSA-PBS solutions (1 μg/ml) containing galactose (A) or mannose (B) at indicated concentrations was applied to a plate coated with 100 ng of standard Gb3. After incubation at 37 °C for 30 min, bound 1B-His was assayed as described in the legend to Fig. 1. Data represent mean±S.D. of three different experiments per point. *$p<0.05$ (compared with concentration=0).

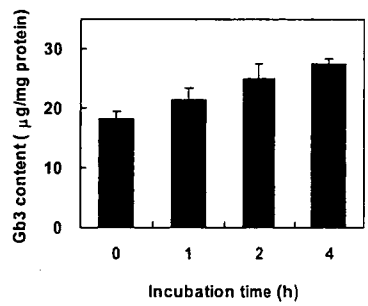

Fig. 8. The Application for Gb3 Synthase Assay

100 μl of homogenate from HeLa cells (5×10^6) was incubated at 37 °C in assay mixture containing 25 mM citrate/phosphate (pH 6.0), 10 mM $MgCl_2$, 0.2 mM LacCer, 0.3% Triton X-100, 3 mM phosphatidylglycerol, 0.5 mM UDP-Gal. Folch's lower fractions extracted from incubated samples were applied to a multi-well H type plate, and the Gb3 content was standardized by the protein amount. The error bars represent the S.D. of independent assays ($n=5$).

tively. The linearity of the enzyme reaction was observed until 2 h of incubation. Gb3 synthase activity in HeLa cells was calculated at 2 h incubation as 3.4 μg of Gb3 production/h/mg protein. This result indicates that the 1B-His binding assay may be applicable for the determination of Gb3 synthase activity.

Discussion

Recent studies on Stx 1B structure revealed that three sites may be involved in the recognition of Gb3[20]; and one of the 3 sites around Phe30 might play an important role for the high affinity toward Gb3 binding.[21] Although AB_5 class toxin is a complex of an A-subunit and 5 B-subunits, the stability and structure of B-subunits were not altered by the binding of the A-subunit,[22] and the intracellular trafficking of B-subunits was independent of the A-subunit.[23] From the above information, 6 histidine residues at the carboxy termini of His-tagged Stx B may not disturb its binding. We used 1B-His for the detection of Gb3 because a higher affinity of 1B-His toward Gb3 was observed in comparison with 2B-His.[15] The His-tagging strategy is useful for the purification of recombinant proteins[24] and we tried to apply for their detection in our present study. HisProbe-HRP is a unique product for the detection of His-tagged protein. Both 1B-His and HisProbe-HRP provide us simple and specific methods for the determination of Gb3 content. The strategy of this assay method may be applicable for other binding assays, such as Cholera toxin B-subunit for ganglioside GM1.[25]

Although the ELISA method using verotoxin B subunit for the determination of Gb3 content was already established,[14] the assay procedure in our method (only two-step binding procedure) was simpler than the ELISA method (three steps). We believe minimum steps in a procedure have benefits not only for the quick assay but also for the specific assay. In a paper by Zeidner *et al.*,[14] cross reactivity was determined only with some glycosphingolipids (glucosylceramide, LacCer and Gb4); however we determined the effect of all components in Folch's lower fraction and proved the specificity of our assay method for Gb3 in HeLa cells (Fig. 4), and proved its specificity. For the practical application, we also determined the effect of sample preparation on the 1B-His binding assay. Surprisingly, the sample preparation was critical for the correct determination of Gb3 content by the 1B-His binding assay (Fig. 6). Folch's lower fraction was a suitable preparation for determination in human cells and mouse heart, but further purification was needed in human plasma. Correct binding may be disturbed by lipids other than glycosphingolipids in human plasma. We also confirmed that more than 95% of Gb3 in HeLa cells was recovered in Folch's lower fraction by the TLC-orcinol method (data not shown). From this information, we concluded that a simple and specific determination of Gb3 content in HeLa cells can be performed by 1B-His binding assay with Folch's lower fraction.

Gb3 is one of the potential glycosphingolipid receptors for Stx.[26] The cytotoxicity of Stx is highly selective toward cells that express Gb3 on the cell surface; and it is widely believed that endothelial cells expressing Gb3 are the main targets for Stx in STEC infection.[27,28] To prevent Stx cytotoxicity, Stx absorbents or binding neutralizers have been developed and were studied for clinical application.[29—31] In our present study, 1B-His binding with Gb3 was blocked by galactose but not mannose (Fig. 7). This data indicated that this 1B-His binding method can be applicable for the screening of Stx-binding neutralizer.

Another application of 1B-His binding was described in Fig. 8. The assay of Gb3 synthase activity was usually performed with radioactive materials.[32,33] Our present study indicated that a non-radioactive assay may be possible with the 1B-His binding assay, because the increase in Gb3 content was linear up to 2 h.

In this study, we established a new Gb3 assay method using a recombinant His-tagged Stx 1B-subunit. The data were reproducible and it was proved that a specific assay can be performed with Folch's lower fraction from HeLa cells. This simple and specific Gb3 assay method is very useful for the determination of effectiveness of various approaches for the treatment of Fabry disease. Recently, we described a new therapeutic strategy, active-site specific chaperone therapy for this disease.[34,35] The assay method established by this study will provide a crucial benefit for the preclinical and clinical trial of this treatment strategy. Knowledge of the physiological and pathological role of Gb3 will increase using this assay method.

Acknowledgments This work was supported in part by a research grant from the Ministry of Health, Labour and Welfare of Japan (S.I.).

References

1) Brady O. R., Gal A. E., Bradley R. M., Martensson E., Warshaw A. L., Laster L., *N. Engl. J. Med.*, **276**, 1163—1167 (1967).
2) Desnick R. J., Ioannou Y. A., Eng C. M., "The Metabolic and Molecular Bases of Inherited Disease," ed. by Scriver C. R., Beaudet A. L., Sly W. S., Valle D., McGraw-Hill, New York, 2001, pp. 3733—3774.
3) Riley L. W., Remis R. S., Helgerson S. D., McGee H. B., Wells J. G., Davis B. R., Hebert R. J., Olcott E. S., Johnson L. M., Hargett N. T., Blake P. A., Cohen M. L., *N. Engl. J. Med.*, **308**, 681—685 (1983).
4) Karmali M. A., Petric M., Lim C., Fleming P. C., Arbus G. S., Lior H., *J. Infect. Dis.*, **151**, 775—782 (1985).
5) Kaplan B. S., Cleary T. G., Obrig T. G., *Pediatr. Nephrol.*, **4**, 276—283 (1990).
6) Fraser M. E., Chernaia M. M., Kozlov Y. V., James M. N., *Nat. Struct. Biol.*, **1**, 59—64 (1994).
7) Lingwood C. A., Law H., Richardson S., Petric M., Brunton J. L., De Grandis S., Karmali M., *J. Biol. Chem.*, **262**, 8834—8839 (1987).
8) Waddell T., Head S., Petric M., Cohen A., Lingwood C., *Biochem. Biophys. Res. Commun.*, **152**, 674—679 (1988).
9) Jscewicz M., Feldman H. A., Donohue-Rolfe A., Balasubramanian K. A., Keusch G. T., *J. Infect. Dis.*, **159**, 881—889 (1989).
10) McCluer R. H., Williams M. A., Gross S. K., Meisler M. H., *J. Biol. Chem.*, **256**, 13112—13120 (1981).
11) Takahashi H., Hirai Y., Migita M., Seino Y., Fukuda Y., Sakuraba H., Kase R., Kobayashi T., Hashimoto Y., Shimada T., *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 13777—13782 (2002).
12) Wilcox W. R., Banikazemi M., Guffon N., Waldek S., Lee P., Linthorst G. E., Desnick R. J., Germain D. P., *Am. J. Hum. Genet.*, **75**, 65—74 (2004).
13) Touboul D., Roy S., Germain D. P., Bailler A., Brion F., Prognon P., Chaminade P., Laprevote O., *Anal. Bioanal. Chem.*, **382**, 1209—1216 (2005).
14) Zeidner K. M., Desnick R. J., Ioannou Y. A., *Anal. Biochem.*, **267**, 104—113 (1999).
15) Watanabe M., Matsuoka K., Kita E., Igai K., Higashi N., Miyagawa A., Watanabe T., Yanoshita R., Samejima Y., Terunuma D., Natori Y., Nishikawa K., *J. Infect. Dis.*, **189**, 360—368 (2004).
16) Ohshima T., Murray G. J., Swaim W. D., Longenecker G., Quirk J. M., Cardarelli C. O., Sugimoto Y., Pastan I., Gottesman M. M., Brady R. O., Kulkarni A. B., *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 2540—2544 (1997).
17) Folch J., Lees M., Sloane-Stanley G. H., *J. Biol. Chem.*, **226**, 497—509 (1957).
18) Kannagi R., Watanabe K., Hakomori S., *Methods Enzymol.*, **138**, 3—13 (1987).
19) Taki T., Ishikawa D., Ogura M., Nakajima M., Handa S., *Cancer Res.*, **57**, 1882—1888 (1997).
20) Ling H., Boodhoo A., Hazes B., Cummings M. D., Armstrong G. D., Brunton J. L., Read R. J., *Biochemistry*, **37**, 1777—1788 (1998).
21) Soltyk A. M., MacKenzie C. R., Wolski V. M., Hirama T., Kitov P. I., Bundle D. R., Brunton J. L., *J. Biol. Chem.*, **277**, 5351—5359 (2002).
22) Goins B., Freire E., *Biochemistry*, **27**, 2046—2052 (1988).
23) Sandvig K., Ryd M., Garred O., Schweda E., Holm P. K., van Deurs B., *J. Cell Biol.*, **126**, 53—64 (1994).
24) Hoffmann A., Roeder R. G., *Nucleic Acids Res.*, **19**, 6337—6338 (1991).
25) Pina D. G., Johannes L., *Toxicon*, **45**, 389—393 (2005).
26) Lingwood C. A., *Trends Microbiol.*, **4**, 147—153 (1996).
27) Kaye S. A., Louise C. B., Boyd B., Lingwood C. A., Obrig T. G., *Infect. Immun.*, **61**, 3886—3891 (1993).
28) Ohmi K., Kiyokawa N., Takeda T., Fujimoto J., *Biochem. Biophys. Res. Commun.*, **251**, 137—141 (1998).
29) Armstrong G. D., Fodor E., Vanmaele R., *J. Infect. Dis.*, **164**, 1160—1167 (1991).
30) Kitov P. I., Sadowska J. M., Mulvey G., Armstrong G. D., Ling H., Pannu N. S., Read R. J., Bundle D. R., *Nature* (London), **403**, 669—672 (2000).
31) Paton A. W., Morona R., Paton J. C., *Nat. Med.*, **6**, 265—270 (2000).
32) Taniguchi N., Yanagisawa K., Makita A., Naiki M., *J. Biol. Chem.*, **260**, 4908—4913 (1985).
33) Keusch J. J., Manzella S. M., Nyame K. A., Cummings R. D., Baenziger J. U., *J. Biol. Chem.*, **33**, 25308—25314 (2000).
34) Fan J.-Q., Ishii S., Asano N., Suzuki Y., *Nat. Med.*, **5**, 112—115 (1999).
35) Ishii S., Yoshioka H., Mannen K., Kulkarni A. B., Fan J. Q., *Biochim. Biophys. Acta*, **1690**, 250—257 (2004).

J Inherit Metab Dis (2006) 29:471–476
DOI 10.1007/s10545-006-0287-y

SSIEM SYMPOSIUM 2005

β-Galactosidase deficiency: An approach to chaperone therapy

Yoshiyuki Suzuki

Received: 30 November 2005 / Accepted: 2 February 2006

Summary We propose a new molecular therapeutic approach to lysosomal diseases with severe neurological manifestations. Some low-molecular-weight compounds, acting as competitive inhibitors of a lysosomal enzyme *in vitro*, were found to stabilize and restore catalytic activities of the enzyme molecule as a molecular chaperone. We started this trial first in Fabry disease (generalized vasculopathy) using galactose and 1-deoxygalactonojirimycin, and then in β-galactosidase deficiency disorders (β-galactosidosis) with generalized neurosomatic and/or systemic skeletal manifestations (GM_1-gangliosidosis and Morquio B disease), using a newly developed chemical compound *N*-octyl-4-epi-β-valienamine (NOEV). Administration of this chaperone compound resulted in elevation of intracellular enzyme activity in cultured fibroblasts from patients and genetically engineered model mice. In addition, substrate storage was improved after NOEV had been transported into the brain tissue via the blood–brain barrier. We hope this new approach (chemical chaperone therapy) will be useful for certain patients with β-galactosidosis and potentially other lysosomal storage diseases with central nervous system involvement.

Communicating editor: Jean-Marie Saudubray

Competing interests: None declared

Presented at the 42nd Annual Meeting of the SSIEM, Paris, 6–9 September 2005

Y. Suzuki (✉)
Clinical Research Center, International University of Health and Welfare, 2600-1 Kita-Kanemaru, Otawara 324-8501, Japan
e-mail: SuzukiY@iuhw.ac.jp

Introduction

GM_1-gangliosidosis (OMIM 230500) is a neurogenetic disease caused by mutations of the gene *GLB1* (3p21.33) that codes for lysosomal β-galactosidase (EC 3.2.1.23) with clinical onset at various ages (Suzuki et al 2001). The forms are classified as infantile, juvenile and adult forms. Another rare systemic bone disease, Morquio B disease, is also known also to be caused by different mutations of the same gene, resulting in β-galactosidase deficiency. Glycoconjugates with terminal β-galactose residues accumulate in tissues and body fluids from patients with these clinical phenotypes. Ganglioside GM_1 and its asialo derivative GA_1 accumulate in the GM_1-gangliosidosis brain. High amounts of oligosaccharides derived from keratan sulphate or glycoproteins are detected in visceral organs and urine from GM_1-gangliosidosis and Morquio B disease patients.

At present only symptomatic therapy is available for human β-galactosidosis patients. Allogeneic bone marrow transplantation did not modify subsequent clinical course or cerebral enzyme activity in a Portuguese water dog affected with GM_1-gangliosidosis (O'Brien et al 1990). Amniotic tissue transplantation was not effective in a patient with Morquio B disease (Tylki Szymanska et al 1985). Enzyme replacement therapy conducted for Gaucher disease and other lysosomal storage diseases is not available at present for β-galactosidosis. An experiment to inhibit GM_1 synthesis resulted in reduction of the GM_1 content in the mouse brain, but not of GA_1 (Kasperzyk et al 2004, 2005). More evaluation is necessary for the therapeutic trial of this type.

We tried to develop a new therapeutic approach to lysosomal storage diseases, particularly with the central nervous involvement. A molecular analysis revealed that some mutant proteins expressed in culture cells from Fabry patients

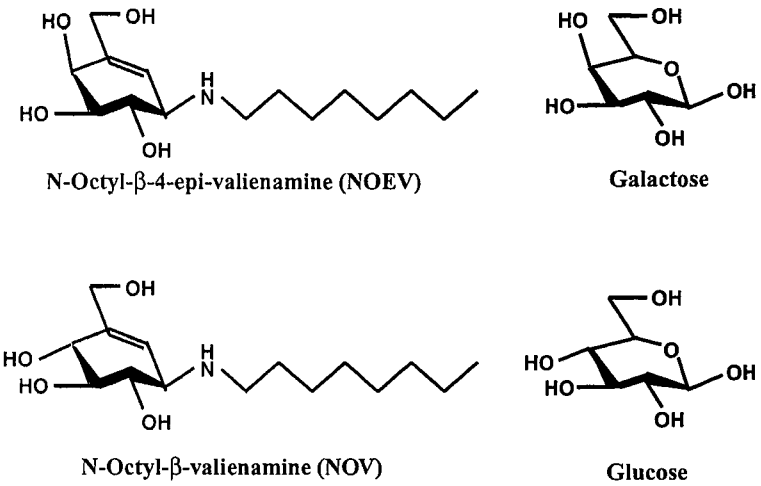

Fig. 1 Structure of NOEV and NOV. The compounds are analogues of galactose and glucose, respectively

do not exhibit the catalytic activity because of molecular instability of the molecule (Okumiya et al 1995a). Subsequently the unstable protein was found to have a defect in molecular folding and rapid degradation after biosynthesis (Ishii et al 1996). Zhang and colleagues (2000) reported the same result in a study of the mutant enzyme in an infantile GM_1-gangliosidosis patient. The R148S β-galactosidase mutation resulted in a major conformational change of the protein molecule with normal catalytic activity, and failed to reach the lysosome.

Simultaneously, trials to stabilize the mutant protein revealed that galactose in the culture medium was able to induce a high expression of the mutant α-galactosidase A gene in cultured lymphoblasts in both classical and atypical (cardiac) form of Fabry disease (Okumiya et al 1995b). This result prompted us to search for more potent inducers of mutant gene expression among commercially available chemical compounds structurally similar to galactose. 1-Deoxygalactonojirimycin (DGJ) was found to be the best candidate for a possible new molecular therapy of Fabry disease in cultured lymphoblasts and transgenic mice (Fan et al 1999).

Concept of chaperone therapy

There are three possible types of mutant gene expression in somatic cells.

1. No biosynthesis of the mutant protein.
2. Extremely low or completely deficient activity of the expressed mutant protein.
3. Expression of unstable mutant protein with normal or near-normal catalytic activity.

We tested these possibilities in Fabry disease, and found a surprisingly high percentage of the third possibility in Fabry disease and β-galactosidosis, although the rate of effectiveness depends on the definition of therapeutic effect in culture cell experiments (Iwasaki et al unpublished data). These mutant proteins are unstable at neutral pH in the endoplasmic reticulum/Golgi apparatus, and are rapidly degraded without appropriate molecular folding (Ishii et al 1996; Okumiya et al 1995a).

After galactose we found a commercially available compound, DGJ, for induction of enhanced mutant gene expression and enzyme activity of α-galactosidase A (Fabry disease; Fan et al 1999). Next, new chemically synthesized compounds were tried for this new approach: *N*-octyl-4-epi-β-valienamine (NOEV) for β-galactosidase (GM_1-gangliosidosis and Morquio B disease; Matsuda et al 2003) and *N*-octyl-β-valienamine (NOV) for β-glucosidase (Gaucher disease; Lin et al 2004) (Fig. 1).

Exogenous compounds that inhibit enzyme activity *in vitro* bind to the mutant enzyme intracellularly around the endoplasmic reticulum/Golgi apparatus, resulting in formation at neutral pH of a complex consisting of the mutant protein and chaperone compound. The catalytically active mutant gene is now stabilized, and the protein–chaperone complex is safely transported to the lysosome. The complex dissociates under the acidic conditions in the lysosome, and the mutant enzyme remains stabilized and its catalytic function is expressed (Fig. 2).

This strategy depends on the biological activity of the chaperone compound available for the study. In a previous study, we had to add a high dose of galactose (up to 200 mmol/L) in the culture medium of Fabry cells (Okumiya et al 1995b). This is obviously unnatural and deleterious to the function of somatic cells for long-term treatment, although a short-term human experiment demonstrated a positive therapeutic effect after high-dose intravenous galactose in a Fabry patient (Frustaci et al 2001).

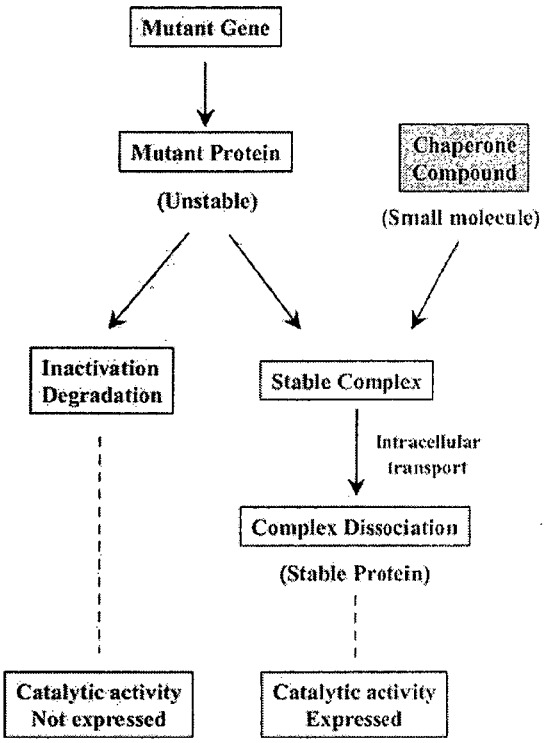

Fig. 2 Intracellular events induced by exogenous chemical chaperones supplied to the cell

NOEV was more efficient than DGJ for expression of β-galactosidase activity in GM_1-gangliosidosis as compared to α-galactosidase A activity in Fabry disease (Matsuda et al 2003; Tominaga et al 2001). Our calculation indicates that at least 10% normal enzyme activity is necessary for washout of the storage substrate in lysosomal diseases. The age of onset in patients expressing the enzyme activity above this level will be theoretically beyond the human lifespan (unpublished data).

Determination of intracellular chaperone concentration is technically not possible at present. We anticipate NOEV concentrations in human cells and animal tissues being much lower than the IC_{50} of NOEV *in vitro*. In fact, the NOEV concentration in the tissue culture medium was approximately equal to the IC_{50} in our cell culture experiments (Matsuda et al 2003).

NOV and NOEV: Chemical synthesis and characterization

Fortunately we found a commercially available compound, DGJ, for possible chaperone therapy of Fabry disease (Fan et al 1999). However, an extensive search for other galactose derivatives and analogous compounds did not reveal any material significantly active for β-linked galactose substrates. I happened to come across an inhibitor originally synthesized for chemical analysis of enzyme reactions catalysed by glucocerebrosidase (β-glucosidase)—NOV (Ogawa et al 1994, 1996, 1998; Tsunoda et al 1995). Gaucher disease is caused by deficiency of this enzyme. We then tried to develop chemical compounds related to this glucose derivative.

First, NOV was synthesized by chemical modification of the original glucocerebrosidase inhibitor, followed by replacing the ceramide moiety with simple aliphatic chains (Ogawa et al 1996, 1998). Subsequently, NOEV was synthesized by multistep epimerization of NOV at C4 (Ogawa et al 2002, 2004). Both NOV and NOEV (Fig. 1) were tested simultaneously, but characterization and evaluation of NOEV were quicker than for NOV simply because we had more experience in β-galactosidase and collected more clinical samples from patients with β-galactosidase deficiency.

NOEV is a potent inhibitor of lysosomal β-galactosidase *in vitro*. Addition of NOEV in the culture medium restored mutant enzyme activity in cultured human or murine fibroblasts at low intracellular concentrations, resulting in a marked decrease of intracellular substrate storage (see below). Its structure was assigned by a combination of COSY, TOCSY and HSQC NMR spectroscopy (Matsuda et al 2003). NOEV is stable at room temperature, and a is strong inhibitor of human β-galactosidase *in vitro*. It is freely soluble in methanol or dimethyl sulfoxide, and soluble in water up to 3–5 mmol/L at room temperature. Its molecular weight is 287.40. The IC_{50} is 0.2 μmol/L towards human β-galactosidase.

Effect of NOEV on cultured human and mouse fibroblasts expressing mutant human genes

In human fibroblast experiments, cells derived from juvenile and infantile GM_1-gangliosidosis patients expressed an increase of β-galactosidase activity after NOEV treatment (Iwasaki et al, unpublished data). Under the conditions of our study, we found two different types of response among the cells for analysis. Some cells responded to NOEV maximally at 0.2 μmol/L, such as R457Q, and others at 2 μmol/L, such as R201C and R201H. This result indicates that the molecular interaction between the chaperone compound and mutant protein is mutation-specific.

Mouse tissues expressing mutant human β-galactosidase showed essentially the same results (Matsuda et al 2003) (Fig. 3). However, the degree of enhancement was different in some mutations between the human and mouse cells. A 5- to 10-fold increase was observed for the R427Q mutation at 0.2 μmol/L of NOEV in the culture medium. A higher concentration (2 μmol/L) was necessary to reach the same degree with the R201C or R201H mutations (Iwasaki et al unpublished data). About one-third of the cells from patients with GM_1-gangliosidosis responded to this treatment; almost all patients with juvenile GM_1-gangliosidosis, and some of the patients with infantile GM_1-gangliosidosis, responded

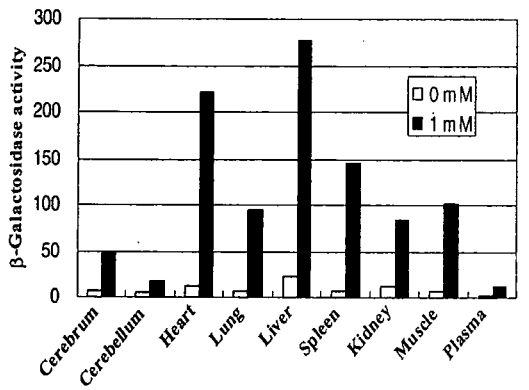

Fig. 3 Effect of NOEV on β-galactosidase activity in mouse tissues. Oral administration (*ad libitum*) of 1. mmol/L NOEV to 5-week old R201C mice for 5 weeks; each value is the mean of two experiments. The enzyme activity increased remarkably in all tissues examined, including cerebrum and cerebellum. Courtesy of Dr Junichiro Matsuda (National Institute of Infectious Diseases, Tokyo; currently National Institute of Biomedical Innovation, Osaka).

significantly. Almost the same or greater restorative effect was achieved with NOEV at 50-fold lower concentration than that with DGJ or *N*-butyldeoxygalactonojirimycin (Tominaga et al 2001).

After adding the mixture of gangliosides to the culture medium, intracellular GM_1 increased remarkably in R201C cells, but only slightly in the cells expressing the normal human gene. Incubation with NOEV significantly reduced GM_1 storage in the cells expressing the mutation R201C causing juvenile GM_1-gangliosidosis (Matsuda et al 2003).

Chaperone therapy on genetically engineered GM_1-gangliosidosis model mice

The R201C mice, expressing the human R201C-mutant β-galactosidase but lacking the endogenous mouse β-galactosidase (Matsuda et al 1997, 2003), had very low β-galactosidase activity in the brain (about 4% of the wild-type activity). They exhibited an apparently normal clinical course for the first 6 months after birth, followed by slowly progressive neurological deterioration, such as tremor and gait disturbance, during the next 9 months. Death occurred around 15 months of age due to malnutrition and emaciation.

Neuropathology revealed vacuolated or ballooned neurons, less abundant than in the knockout mouse brain described in our previous reports (Itoh et al 2001; Matsuda et al 1997). Cytoplasmic storage materials were present in pyramidal neurons and brainstem motor neurons, but not in neurons in the other areas of the brain.

Short-term oral administration of NOEV to the R201C model mouse (Matsuda et al 2003) resulted in significant enhancement of the enzyme activity in all the tissues examined, including the brain. Immunohistochemical staining revealed an increase in β-galactosidase activity and a decrease in GM_1 and GA_1 storage. However, mass biochemical analysis did not show the substrate reduction observed histochemically in these limited areas in the brain, probably because of the brief duration of treatment and only localized substrate accumulation at the early stage of the disease in this experiment. NOEV was found in significant amount in the central nervous system by mass spectrometric analysis, at the 30% level in the liver tissue in mice treated by oral administration of NOEV solution for 8 weeks (Kubo et al, unpublished data).

The experimental data to date are summarized in Table 1.

Neurological examination of genetically engineered GM_1-gangliosidosis model mice

We are currently trying to establish a system of neurological examination in the GM_1-gangliosidosis model mice that we prepared for the chaperone therapy experiments. This is essentially an application of the child neurology technique to the mouse species, using clinical observation, video monitoring, manual manipulation, and apparatus-assisted examination developed for neurological evaluation of mice and rats. We evaluate spontaneous movements, body and limb postures, behavioural patterns in an open field, primitive reflexes, postural reflexes, and equilibrium reactions. Data are being collected for normal (wild-type), transgenic and knockout mice, with or without NOEV administration. It is hoped that this systematic approach will be useful for monitoring the clinical course of a large number of genetically engineered model mouse strains for evaluating physiological roles of individual genes. Improvements of the posture and movements have been observed in some mice after NOEV

Table 1 Experimental data on biological activity of NOEV

1.	NOEV inhibits β-galactosidase activity *in vitro* at high concentrations.
2.	NOEV induces expression of mutant β-galactosidase activity *in situ* at low concentrations.
3.	The biological activity of NOEV is mutation-specific.
4.	NOEV is delivered through the blood–brain barrier by oral administration to disease model mice.
5.	Oral administration of NOEV induces expression of mutant β-galactosidase activity in the mouse brain.
6.	Oral administration of NOEV results in degradation of storage substrates.
7.	Oral administration of NOEV improves or prevents the central nervous system manifestations (preliminary data).
8.	Short-term oral administration of NOEV does not cause significant adverse effects to disease model mice.

administration for a few weeks in a preliminary experiment (Ichinomiya et al, in preparation).

Prospects

Chaperone therapy has two major advantages over enzyme replacement therapy: oral administration and accessibility to the brain. NOEV is a good candidate compound for this new therapeutic approach, particularly for the central nervous system pathology, as it is a small molecule that passes through the blood–brain barrier from the bloodstream, stabilizes mutant protein in neurons, and induces expression of enzyme activity. Clinical evaluation has not yet been completed, but we have some evidence that this compound could partially improve the disease progress in some mice after even a few months of low-dose administration in the early stage of the disease (Suzuki et al, unpublished data). We need long-term experiments to establish an optimal dose for prevention of clinical manifestations, accompanied by reduction of substrate storage, in these model mice. Possible adverse or toxic effects should be carefully evaluated before human clinical experiments are started.

We are aware that this new molecular approach is not justified for all patients with a single lysosomal enzyme deficiency disorder. Biosynthesis of a catalytically active enzyme is prerequisite in chemical chaperone therapy. Our initial survey indicates that 20–40% of β-galactosidosis (mainly GM_1-gangliosidosis) patients express unstable but catalytically active protein and respond to NOEV treatment in cultured fibroblasts. Patients of this type will be reasonable candidates for chemical chaperone therapy.

This strategy is in principle applicable to all lysosomal storage diseases if a specific compound is available for each enzyme in question. Special drug design technology may be needed to screen appropriate inhibitors. Bioinformatics analysis will develop a new aspect of molecular pathology in lysosomal storage diseases (Durand et al 2000; Fabrega et al 2000).

This study started with a lysosomal disease, and a number of related diseases became the target of this approach. Theoretically this principle can be applied to all other lysosomal diseases. At present our study is focused on diseases with storage of compounds with α- or β-linked glucose or galactose residues at the terminal end of the carbohydrate chain in the substrate molecule: α-glucosidase deficiency (glycogenosis II), β-glucosidase deficiency (Gaucher disease), α-galactosidase deficiency (Fabry disease), and β-galactosidase deficiency (β-galactosidosis; GM_1-gangliosidosis and Morquio B disease). We hope to extend this approach to the other lysosomal diseases in future if a specific chaperone compound is found for each disease.

Further, there may be other categories of diseases that are good targets of this approach if the molecular pathology in somatic cells has been studied and well understood in detail regarding mutant gene expression, mutant protein structure, intracellular transport of the protein, mechanism of functional expression, etc. It is hoped that studies in this direction will in future reveal new aspects of molecular therapy for inherited metabolic diseases with central nervous system involvement.

Acknowledgements This research was supported by grants from Ministry of Education, Culture, Science, Sports, and Technology of Japan (nos. 13680918, 14207106), and Ministry of Health, Labour and Welfare of Japan (nos. H10-No-006, H14-Kokoro-017, H17-Kokoro-019). I thank the following collaborators for this study: Junichiro Matsuda (National Institute of Infectious Diseases, Tokyo; currently National Institute of Biomedical Innovation, Osaka); Eiji Nanba, Katsumi Higaki and Kousaku Ohno (Tottori University, Yonago); Masayuki Itoh (National Institute of Neuroscience, Kodaira); Satoshi Ishii (The Tokyo Metropolitan Institute of Medical Science, Tokyo; currently Obihiro University of Agriculture and Veterinary Medicine, Obihiro); Jian-Qiang Fan (The Tokyo Metropolitan Institute of Medical Science, Tokyo; currently Mount Sinai School of Medicine, New York); Seiichiro Ogawa (Keio University, Yokohama); Masami Iiida (Seikagaku Corporation, Higashi-Yamato); Mieko Kurosawa, Hiroyuki Iwasaki, Hiroshi Watanabe and Satoshi Ichinomiya (International University of Health and Welfare, Otawara); and Roscoe O. Brady (National Institute of Neurological Disorders and Stroke, Bethesda).

References

Durand P, Fabrega S, Henrissat B, Mornon J-P, Lehn P (2000) Structural features of normal and mutant human lysosomal glycoside hydrolases deduced from bioinformatics analysis. *Hum Mol Genet* **9**: 967–977.

Fabrega S, Durand P, Codogno P, et al (2000) Human glucocerebrosidase: heterologous expression of active site mutants in murine null cells. *Glycobiology* **10**: 1217–1224.

Fan JQ, Ishii S, Asano N, Suzuki Y (1999) Accelerating transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nature Medicine* **5**: 112–115.

Frustaci A, Chimenti C, Ricci R, et al (2001) Improvement in cardiac function in the cardiac variant of Fabry's disease with galactose-infusion therapy. *N Engl J Med* **345**: 25–32.

Ishii S, Kase R, Okumiya T, Sakuraba H, Suzuki Y (1996) Aggregation of the inactive form of human α-galactosidase in the endoplasmic reticulum. *Biochem Biophys Res Commun* **220**: 812–815.

Itoh M, Matsuda J, Suzuki O, et al (2001) Development of lysosomal storage in mice with targeted disruption of the β-galactosidase gene: a model of human G_{M1}-gangliosidosis. *Brain Dev* **23**: 379–384.

Kasperzyuk JL, El-Abbadi MM, Hauser EC, d'Azzo A, Platt FM, Seyfried TN (2004) *N*-Butyldeoxygalactonojirimycin reduces neonatal brain ganglioside content in a mouse model of GM1-gangliosidosis. *J Neurochem* **89**: 645–653.

Kasperzyk JL, d'Azzo A, Platt FM, Alroy J, Seyfried TN (2005) Substrate reduction reduces gangliosides in postnatal cerebrum-brainstem and cerebellum in G_{M1} gangliosidosis mice. *J Lipid Res* **46**: 744–751.

Lin H, Sugimoto Y, Ohsaki Y, et al (2004) *N*-Octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured

ELSEVIER

Brain & Development 28 (2006) 482–486

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Fibroblast screening for chaperone therapy in β-galactosidosis

Hiroyuki Iwasaki [a], Hiroshi Watanabe [a], Masami Iida [b,1], Seiichiro Ogawa [c,2], Miho Tabe [d,3], Katsumi Higaki [e,4], Eiji Nanba [e,4], Yoshiyuki Suzuki [a,*]

[a] *Clinical Research Center, International University of Health and Welfare, 2600-1 Kita-Kanemaru, Otawara 324-8501, Japan*
[b] *Central Research Laboratories, Seikagaku Corporation, 3-1253 Tateno, Higashi-Yamato, Tokyo 207-0021, Japan*
[c] *Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan*
[d] *Biochemistry Section, Analysis Center for Medical Science, SRL Inc, 51 Komiya-machi, Hachioji 192-8535, Japan*
[e] *Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, 86 Nishi-machi, Yonago 683-8503, Japan*

Received 22 December 2005; received in revised form 31 January 2006; accepted 3 February 2006

Abstract

We performed screening of β-galactosidase-deficient fibroblasts for possible chemical chaperone therapy using *N*-octyl-4-epi-β-valienamine (NOEV) in patients with G_{M1}-gangliosidosis and Morquio B disease (β-galactosidosis). Fibroblasts were cultured with NOEV for 4 days and β-galactosidase activity was measured. Mutation analysis was performed simultaneously. Two separate criteria were set for evaluation of the chaperone effect: a relative increase of enzyme activity (more than 3-fold), and an increase up to more than 10% normal enzyme activity. Among the 50 fibroblast strains tested, more than 3-fold increase was achieved in 17 cell strains (34%), and more than 10% normal activity in 10 (20%). Both criteria were satisfied in 6 (12%), and either of them in 21 (42%). Juvenile G_{M1}-gangliosidosis was most responsive, and then infantile G_{M1}-gangliosidosis. This enhancement was mutation-specific. We estimate that the NOEV chaperone therapy will be effective in 20–40% of the patients, mainly in juvenile and infantile G_{M1}-gangliosidosis patients. A molecular design may produce mutation-specific chaperone compounds for the other disease phenotypes. This cellular screening will be useful for identification of human patients with β-galactosidase deficiency for chaperone therapy to be started in the near future.

Keywords: G_{M1}-gangliosidosis; β-Galactosidase; Gene mutation; *N*-Octyl-4-epi-β-valienamine; Chaperone therapy; Fibroblast

1. Introduction

Hereditary deficiency of lysosomal acid β-galactosidase (β-galactosidosis) causes two clinically distinct diseases in humans, G_{M1}-gangliosidosis and Morquio B disease [1]. The mode of inheritance is autosomal recessive. G_{M1}-gangliosidosis is a generalized neurosomatic disease occurring mainly in early infancy, and rarely in childhood or young adults. Morquio B disease is a rare bone disease without central nervous system involvement. Glycoconjugates with terminal β-galactose residues accumulate in tissues and urine from patients with these clinical phenotypes. Ganglioside G_{M1} and its asialo derivative G_{A1} accumulate in the G_{M1}-gangliosidosis brain. High amounts of oligosaccharides derived from keratan sulfate or glycoproteins are detected in visceral organs and urine from G_{M1}-gangliosidosis and Morquio B disease patients.

* Corresponding author. Tel./fax: +81 287 24 3229.
E-mail addresses: hiwasaki@iuhw.ac.jp (H. Iwasaki), watanabeh@iuhw.ac.jp (H. Watanabe), masami.iida@seikagaku.co.jp (M. Iida), sogawa379@ybb.ne.jp (S. Ogawa), m-tabe@srl.srl-inc.co.jp (M. Tabe), kh4060@grape.med.tottori-u.ac.jp (K. Higaki), enanba@grape.med.tottori-u.ac.jp (E. Nanba), SuzukiY@iuhw.ac.jp (Y. Suzuki).
[1] Fax: +81 42 563 5846.
[2] Fax: +81 45 566 1551.
[3] Fax: +81 426 48 4161.
[4] Fax: +81 859 34 8284.

0387-7604/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.braindev.2006.02.002

cells: a potential chemical chaperone therapy for Gaucher disease. *Biochim Biophys Acta* **1689**: 219–228.

Matsuda J, Suzuki O, Oshima A, et al (1997) β-Galactosidase-deficient mouse as an animal model for G_{M1}-gangliosidosis. *Glycoconjugate J* **14**: 729–736.

Matsuda J, Suzuki O, Oshima A, et al (2003) Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis. *Proc Natl Acad Sci USA* **26**: 15912–15917.

O'Brien JS, Storb R, Raff RF, et al (1990) Bone marrow transplantation in canine G_{M1} gangliosidosis. *Clin Genet* **38**: 274–280.

Ogawa S, Tsunoda H, Inokuchi J-i (1994) Synthesis of glucosylceramide analogues: imino-linked 5a-carbaglycosylceramides, potent and specific glucocerebrosidase inhibitors. *J Chem Soc Chem Commun* 1317–1318.

Ogawa S, Ashiura M, Uchida C, et al (1996) Synthesis of potent β-D-glucocerebrosidase inhibitors: *N*-alkyl-β-valienamines. *Bioorg Med Chem Lett* **6**: 929–932.

Ogawa S, Kobayashi Y, Kabayama K, Jimbo M, Inokuchi J-i (1998) Chemical modification of β-glucocerebrosidase inhibitor *N*-octyl-β-valienamine: synthesis and biological evaluation of *N*-alkanoyl and *N*-alkyl derivatives. *Bioorg Med Chem* **6**: 1955–1962.

Ogawa S, Kobayashi Matsunaga Y, Suzuki Y., (2002) Chemical modification of the β-glucocerebrosidase *N*-octyl-β-valienamine: synthesis and biological evaluation of 4-epimeric and 4-*O*-(β-D-galactopyranosyl) derivatives. *Bioorg Med Chem* **10**: 1967–1972.

Ogawa S, Sakata Y, Ito N, et al (2004) Convenient synthesis and evaluation of glycosidase inhibitory activity of α- and β-galactose-type valienamines, and some *N*-alkyl derivatives. *Bioorg Med Chem* **12**: 995–1002.

Okumiya T, Ishii S, Kase R, Kamei S, Sakuraba H, Suzuki Y (1995a) α-Galactosidase gene mutations in Fabry disease: heterogeneous expressions of mutant enzyme proteins. *Hum Genet* **95**: 557–561.

Okumiya T, Ishii S, Takenaka T, et al (1995b) Galactose stabilizes various missense mutants of α-galactosidase in Fabry disease. *Biochem Biophys Res Commun* **214**: 1219–1224.

Suzuki Y, Oshima A, Nanba E (2001) β-Galactosidase deficiency (β-galactosidosis): G_{M1}-gangliosidosis and Morquio B disease. In: Scriver CR, Beaud et al Sly WS, Valle D, eds; Childs B, Kinzler KW, Vogelstein B, assoc. eds. *The Metabolic and Molecular Bases of Inherited Disease*, 8th edn. New York: McGraw-Hill, 3775–3809.

Tominagá L, Ogawa Y, Taniguchi M, et al (2001) Galactonojirimycin derivatives restore mutant human β-galactosidase activities expressed in fibroblasts from enzyme-deficient knockout mouse. *Brain Dev* **23**: 284–287.

Tsunoda H, Inokuchi J-i, Yamagishi K, Ogawa S (1995) Synthesis of glycosylceramide analogs composed of imino-linked unsaturated 5a-carbaglycosyl residues: potent and specific gluco- and galactocerebrosidase inhibitors. *Liebigs Ann* 279–284.

Tylki Szymanska A, Maciejko D, Kidawa M, Jablonska Buda JU, Czartoryska B (1985) Amniotic tissue transplantation as a trial of treatment in some lysosomal storage diseases. *J Inherit Metab Dis* **8**: 101–104.

Zhang S, Bagshaw R, Hilson W, et al (2000) Characterization of β-galactosidase mutations Asp332→Asn and Arg148→Ser, and a polymorphism, Ser532→Gly, in a case of G_{M1} gangliosidosis. *Biochem J* **348**(Pt 3): 621–632.

At present only symptomatic therapy is available for human β-galactosidosis patients. Allogeneic bone marrow transplantation did not modify the subsequent clinical course or cerebral enzyme activity in a Portuguese water dog affected with G_{M1}-gangliosidosis [2]. Amniotic tissue transplantation was not effective in a patient with Morquio B disease [3]. Enzyme replacement therapy conducted for Gaucher disease and other lysosomal storage diseases is not available at present for β-galactosidosis.

Recently we reported results of a molecular approach (chemical chaperone therapy) for restoration of mutant α-galactosidase in Fabry disease. Galactose and its structural analog, 1-deoxygalactonojirimycin, enhanced residual enzyme activity in cultured human lymphoblasts from patients with α-galactosidase deficiency [4,5], and transgenic mouse tissues expressing a mutant enzyme causing Fabry disease [5,6]. Some mutant proteins are unstable at neutral pH in the endoplasmic reticulum/Golgi apparatus and are rapidly degraded without appropriate molecular folding [7,8]. Exogenously supplied chemical compounds that inhibit enzyme activity in vitro bind to the enzyme intracellularly to form a complex, stabilizing and transporting the catalytically active enzyme to lysosomes. The complex dissociates under the acidic condition in lysosomes, and the mutant enzyme remains stabilized and functional.

In a previous report we confirmed the effect of a new chemical compound *N*-octyl-4-epi-β-valienamine (NOEV) on cultured fibroblasts and model mice expressing a mutant β-galactosidase protein R201C [9]. In this study, we conducted screening of the patients with β-galactosidase deficiency for possible chaperone therapy in the near future.

2. Materials and methods

2.1. Chaperone compound NOEV

NOEV was synthesized by modification of a glucocerebrosidase inhibitor [9,10]. It is stable at room temperature and strongly inhibits human β-galactosidase in vitro. It is freely soluble in methanol or dimethylsulfoxide, and soluble in water up to 3–5 mM at room temperature. The molecular weight is 287.40.

2.2. Fibroblast culture

Fibroblasts from patients with β-galactosidase deficiency (G_{M1}-gangliosidosis or Morquio B disease) were stored in our laboratories, purchased from Coriell Cell Repositories (Camden, NJ, USA), or provided by the following colleagues at medical and scientific institutions: Mark Abramowicz and Patrick Van Bogaert (Brussels), Nils U. Bosshard (Zurich), Ernst Christensen (Copenhagen), Fatih Süheyl Ezgü (Ankara), Mirella Filocamo (Genova), Agata Fiumara (Catania), Erentraud Irnberger (Salzburg), Koji Inui (Osaka), Wim J. Kleijer (Rotterdam), Jana Ledvinova (Prague), Gert Matthijs (Leuven), Toshihiro Oura (Sendai), Alan Percy (Birmingham, AL), Konrad Sandhoff and Gerhild van Echten-Deckert (Bonn), George H. Thomas (Baltimore, MD), David A. Wenger (Philadelphia, PA), and Marie-Therese Zabot (Lyon). The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics, and harvested by scraping. They were collected by centrifugation, washed once with phosphate-buffered saline, and suspended in water. The cell suspension was sonicated, and used for enzyme assay (enzyme solution).

2.3. Enzyme assay

β-Galactosidase assay was performed on 96-well plates. The enzyme assay mixture consisted of 10 μl enzyme solution, with or without NOEV at the final concentration up to 5 μM, and 10 μl substrate solution containing 1 mM of 4-methylumbelliferyl-β-galactoside (Sigma, St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.5) and 0.1 M NaCl. After incubation for 1 h at 37 °C, the enzyme reaction was terminated by adding 0.2 M glycine–NaOH buffer (pH 10.7), and the liberated 4-methylumbelliferone was measured by fluorometry (excitation 355 nm; emission 460 nm) as described previously [11]. Protein was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

2.4. In vitro NOEV experiment

In this experiment human fibroblasts expressing normal (wild-type) β-galactosidase activity were used as an enzyme source, and NOEV was added to the enzyme assay mixture at final concentrations of 0–5 μM.

2.5. In situ NOEV experiments

Confluent fibroblasts (wild-type or mutant) were cultured in DMEM with or without NOEV (0, 0.2, 2, or 6 μM) on a 3.5-cm culture dish for a short-term experiment (4 days), or on a 10-cm culture dish for a long-term experiment up to 8 weeks. During the long-term culture the culture medium was changed regularly twice a week. Every 7–8 days, the cells were trypsinized, one-half was stored for enzyme assay and the other half was kept on culture.

For final harvesting and enzyme assay, the cells were scraped, collected by centrifugation, washed once with phosphate-buffered saline, suspended in water, and homogenized by sonication. The cell pellets were kept frozen at −80 °C until enzyme assay.

2.6. Gene mutation analysis

We tried to collect information about clinical and genetic data for each of the patients. However, information about phenotype and genotype was not always satisfactory. Enzyme deficiency was confirmed in our laboratory for all fibroblast strains in this study. Some cell strains without known genotype were subjected to gene mutation analysis [12]. After extraction of genomic DNA from human fibroblasts, each of the 16 exons with flanking sequence was amplified by polymerase chain reaction under the standard conditions. All exons except 1, 4, 7, and 9 were sequenced directly using ABI Prism 3100 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan). The amplified exons 1, 4, 7, and 9 were subjected to single strand conformation polymorphism [13]. Exons with aberrant bands were subcloned into pGEM-T vector (Promega, Madison, WI, USA) and sequenced.

3. Results

3.1. In vitro NOEV experiment

Addition of NOEV resulted in a dose-dependent inhibition of the normal human β-galactosidase activity in vitro (Fig. 1). It was reduced to 20% of the background activity at the concentration of 0.5 μM in the assay mixture. The IC_{50} was calculated as 0.125 μM.

3.2. Time course of enzyme activity in cultured fibroblasts in response to NOEV

The background enzyme activity was variable in patients with various clinical phenotypes. In general, the cells from late-onset patients showed higher residual enzyme activities. In some cell strains, the enzyme activity was significantly enhanced after incubation for 2–4 days with 0.2–2 μM NOEV in the culture medium (Fig. 2). The increase continued up to 7 days and then remained at the same level for 8 weeks (data not shown). The rate of cell proliferation remained the same as that for the cells without NOEV treatment.

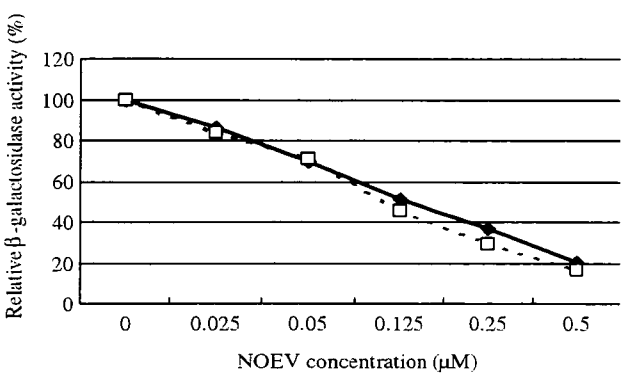

Fig. 1. Inhibition of β-galactosidase activity by NOEV in control human fibroblasts. NOEV was added to the enzyme assay mixture at final concentrations up to 0.5 μM. Inhibition of enzyme activity was dose-dependent. Each value is the mean of triplicate assays. (◆) normal control; (□) pathological control (dysostosis multiplex congenita).

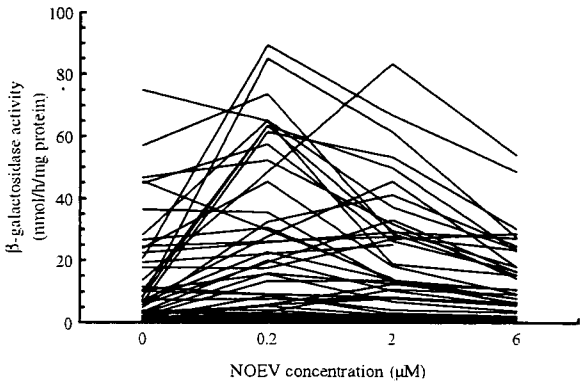

Fig. 2. β-Galactosidase activity after incubation for 4 days with or without NOEV. In some cell strains the enzyme activity was enhanced by 0.2–2 μM NOEV in the culture medium. Each value is the mean of triplicate assays. Two peaks of maximal activity were observed. Normal control values: mean 538 ± 230 nmol/h/mg protein; range: 220–1071 ($n = 19$), and 10% of the control mean: 54 nmol/h/mg protein.

3.3. NOEV effect and phenotype

Table 1 shows the cumulative summary of the cell study. The positive response was defined either as more than 3-fold increase, or as an increase up to 10% or more of the control mean (54 nmol/h/mg protein). The first condition was satisfied in 17 cell strains (34%), and the second condition in 10 (20%); both conditions were satisfied in 6 (12%), and either of them in 21 (42%). The

Table 1
NOEV effect and phenotype

Phenotype	Onset	Total	Positive response	
			>3-fold	>10%
G_{M1}-gangliosidosis	Infantile	31	10	2
	Juvenile	8	7	4
	Adult	7	0	4
Morquio B		3	0	0
Intermediate		1	0	0
		50	17	10

The fibroblasts were cultured in the medium containing 2 μM NOEV for 4 days, and the enzyme activity was assayed. The positive response was defined as a more than 3-fold increase (>3-fold), or as an increase up to more than 10% of the control mean (>10%). The background activity was 3–10% in adult G_{M1}-gangliosidosis, and the resulting relative increase was not high as compared to infantile or juvenile G_{M1}-gangliosidosis.

maximal enzyme activity was observed in two peaks either at 0.2 or 2 μM in most cell strains with positive response.

Juvenile G_{M1}-gangliosidosis was most responsive among the four clinical phenotypes tested in this study; relative increase in 7 of 8, and higher than normal 10% activity in 4 of 8. There was a relative increase of enzyme activity in 10 of 31 infantile G_{M1}-gangliosidosis cells in response to NOEV, but the enzyme activity reached more than the 10% normal level in only 2 cell strains. Adult G_{M1}-gangliosidosis and Morquio B disease apparently did not respond well to NOEV under the experimental conditions in this study. In general they showed relatively high residual enzyme activity, and the enzyme activity after NOEV treatment did not reach the 3-fold increase level, although an increase up to 10% of the normal control mean activity was achieved in four of seven cell strains.

3.4. NOEV effect and genotype

We collected more than 50 different β-galactosidase gene mutations [1]. In this study gene mutation analysis revealed several new or known mutations (data not shown). The effect of NOEV was genotype-specific (Table 2). Among the mutations examined, the amino acid substitution at 201 (R201C, R201H) causing juvenile G_{M1}-gangliosidosis responded maximally to NOEV at 2 μM, and the amino acid substitution at 457 (R457Q) causing infantile G_{M1}-gangliosidosis at 0.2 μM. The effect for these amino acid 457 or 201 mutations was confirmed in homozygous mutants. The response was less remarkable in compound heterozygotes with Q255H, V439G, Y57X, Y324C, or other mutations in human fibroblasts with β-galactosidase deficiency disorders (Table 2).

Table 2
NOEV effect and genotype

Mutation	Relative increase	Optimal NOEV concentration (μM)
R457Q	5- to 10-fold	0.2
R201C, R201H	5- to 10-fold	2
Q255H, V439G, Y57X, Y324C, others	2- to 6-fold	0.2–2
I51T, W273L, others	0.5- to 1.2-fold	–

The fibroblasts were cultured in the medium containing 2 μM NOEV for 4 days, and the enzyme assay was performed. The relative increase was calculated as compared to the activity without NOEV in the culture medium. In the homozygous mutants, the NOEV effect was clearly and unambiguously concluded under the conditions in this study; such as R457Q, R201C, or R201H (positive), and I51T or W273L (negative). No definite conclusion was possible for the optimal concentration of NOEV on the other mutations, such as Q255H, V439G, Y57X, Y324C, and others, because they were found as heterozygous with another known or unidentified mutation.

4. Discussion

Low molecular weight compounds for chemical chaperone therapy act as in vitro inhibitors at high concentrations and as in situ activators at low concentrations. We first demonstrated this apparently paradoxical phenomenon in Fabry disease [4–7], and then in G_{M1}-gangliosidosis [9] and Gaucher disease [14]. The mutant protein expressed in the cell does not exhibit catalytic activity because of a defect in molecular folding and rapid degradation after biosynthesis [8,15]. This principle was recently demonstrated in a patient with Fabry disease with deficiency of α-galactosidase A by infusion of galactose for a short period [16].

We synthesized a new chemical compound NOEV as a potent inhibitor of human β-galactosidase [10], and anticipated that it would be useful for chemical chaperone therapy of patients with β-galactosidase deficiency. Our previous study confirmed stabilization and restoration of the enzyme activity by this chaperone compound in the G_{M1}-gangliosidosis model mouse expressing the R201C mutation [9].

In this study, we tried a screening of patients with β-galactosidase deficiency for possible chaperone therapy using NOEV in the near future. Six cell strains in this study satisfied the two criteria for significant restoration of enzyme activity (3-fold increase and 10% of the control mean) to the level possibly sufficient for intraneural substrate degradation. We anticipate that the patients with the mutant genes satisfying one of two criteria in this study (at least 12% and at most 42%) will be good candidates for treatment and prevention of neurological manifestations during the course of the disease.

We postulate the lower limit of the enzyme activity for intracellular degradation of the substrates is 10% of the control mean (54 nmol/h/mg protein) based on our previous cell and tissue experiments (unpublished data). However, there are a few cell strains, particularly from adult G_{M1}-gangliosidosis patients, with the residual enzyme activity already at this level. We are fully aware that the above working hypothesis is based on in vitro experiments using fibroblasts (not neural cells) and a synthetic (not physiological) substrate for enzyme assays.

A few common mutations are known to cause specific phenotypes, such as R428H and R208C for infantile G_{M1}-gangliosidosis, R201C for juvenile G_{M1}-gangliosidosis, I51T for adult G_{M1}-gangliosidosis, and W273L for Morquio B disease [17–19]. In the present study the cells were collected randomly. However, the degree of efficacy in this study was dependent on the number of patients with common mutations causing individual phenotypes.

Under the conditions of our study, we found two different response types among the cells studied. Some cells responded to NOEV maximally at 0.2 μM and

the others at 2 μM. This result indicates that the molecular interaction between the chaperone compound and mutant protein is mutation-specific. We anticipate that a molecular design will be possible for synthesis of new chaperone molecules for mutation-specific activity in future.

A similar therapeutic trial but in the opposite direction has been reported by inhibition of substrate biosynthesis, substrate deprivation therapy, for Gaucher disease [20] and G_{M1}-gangliosidosis [21]. In the latter using the disease model mice, ganglioside G_{M1} was reduced in the brain but asialo-ganglioside G_{A1} was not. More studies are necessary for solid conclusion on the biochemical and clinical effects of this trial.

The purpose of our study is to develop a new drug for G_{M1}-gangliosidosis, an intractable neurogenetic disease in children and adults. Chemical chaperone therapy has two major advantages over enzyme replacement therapy currently in use for medical practice: oral administration and accessibility to the brain [9]. Biosynthesis of a catalytically active enzyme is a prerequisite for chemical chaperone therapy. Although this new molecular approach is not efficient in all patients with a single lysosomal enzyme deficiency disorder, it is important that prevention or treatment could be achieved even in some of the patients with an intractable progressive neurological disorder.

Acknowledgments

This research was supported by grants from Ministry of Education, Culture, Science, Sports and Technology of Japan (13680918 and 14207106), and Ministry of Health, Labour and Welfare of Japan (H10-No-006, H14-Kokoro-017, and H17-Kokoro-019).

References

[1] Suzuki Y, Oshima A, Nanba E. β-Galactosidase deficiency (β-galactosidosis): G_{M1}-gangliosidosis and Morquio B disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill; 2001. p. 3775–809.

[2] O'Brien JS, Storb R, Raff RF, Harding J, Appelbaum F, Morimoto S, et al. Bone marrow transplantation in canine G_{M1} gangliosidosis. Clin Genet 1990;38:274–80.

[3] Tylki Szymanska A, Maciejko D, Kidawa M, Jablonska Budaj U, Czartoryska B. Amniotic tissue transplantation as a trial of treatment in some lysosomal storage diseases. J Inherited Metab Dis 1985;8:101–4.

[4] Okumiya T, Ishii S, Takenaka T, Kase R, Kamei S, Sakuraba H, et al. Galactose stabilizes various missense mutants of α-galactosidase in Fabry disease. Biochem Biophys Res Commun 1995;214:1219–24.

[5] Fan JQ, Ishii S, Asano N, Suzuki Y. Accelerated transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. Nat Med 1999;5:112–5.

[6] Ishii S, Kase R, Sakuraba H, Taya C, Yonekawa H, Okumiya T, et al. α-Galactosidase transgenic mouse: heterogeneous gene expression and posttranslational glycosylation in tissues. Glycoconj J 1998;15:591–4.

[7] Okumiya T, Ishii S, Kase R, Kamei S, Sakuraba H, Suzuki Y. α-Galactosidase gene mutations in Fabry disease: heterogeneous expressions of mutant enzyme proteins. Hum Genet 1995;95:557–61.

[8] Ishii S, Kase R, Okumiya T, Sakuraba H, Suzuki Y. Aggregation of the inactive form of human α-galactosidase in the endoplasmic reticulum. Biochem Bophys Res Commun 1996;220:812–5.

[9] Matsuda J, Suzuki O, Oshima A, Yamamoto Y, Noguchi A, Takimoto K, et al. Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis. Proc Natl Acad Sci USA 2003;100:15912–7.

[10] Ogawa S, Kobayashi Matsunaga Y, Suzuki Y. Chemical modification of the β-glucocerebrosidase inhibitor *N*-octyl-β-valienamine: synthesis and biological evaluation of 4-epimeric and 4-*O*-(β-D-galactopyransyl) derivatives. Bioorg Med Chem 2002;10:1967–72.

[11] Sakuraba, Aoyagi T, Suzuki Y. Galactosialidosis (β-galactosidase-neuraminidase deficiency): a possible role of serine-thiol proteases in the degradation of β-galactosidase molecules. Clin Chim Acta 1982;125:275–82.

[12] Tominaga L, Ogawa Y, Taniguchi M, Ohno K, Matuda J, Oshima A, et al. Galactonojirimycin derivatives restore mutant human β-galactosidase activities expressed in fibroblasts from enzyme-deficient knockout mouse. Brain Dev 2001;23:284–7.

[13] Zhang H, Nanba E, Yamamoto T, Ninomiya H, Ohno K, Mizuguchi M, et al. Mutational analysis of TSC1 and TSC2 genes in Japanese patients with tuberous sclerosis complex. J Hum Genet 1999;44:391–6.

[14] Lin H, Sugimoto Y, Ohsaki Y, Ninomiya H, Oka A, Taniguchi M, et al. *N*-Octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease. Biochim Biophys Acta 2004;1689:219–28.

[15] Zhang S, Bagshaw R, Hilson W, Oho Y, Hinek A, Clarke JT, et al. Characterization of β-galactosidase mutations Asp332 → Asn and Arg148 → Ser, and a polymorphism, Ser532 → Gly, in a case of G_{M1} gangliosidosis. Biochem J 2000;348(Pt. 3):621–32.

[16] Frustaci A, Chimenti C, Ricci R, Natale L, Russo MA, Pieroni M, et al. Improvement in cardiac function in the cardiac variant of Fabry's disease with galactose-infusion therapy. N Engl J Med 2001;345:25–32.

[17] Yoshida K, Oshima A, Shimmoto M, Fukuhara Y, Sakuraba H, Yanagisawa N, et al. Human β-galactosidase gene mutations in G_{M1}-gangliosidosis: a common mutation among Japanese adult/chronic cases. Am J Hum Genet 1991;49:435–42.

[18] Oshima A, Yoshida K, Shimmoto M, Fukuhara Y, Sakuraba H, Suzuki Y. Human β-galactosidase gene mutations in Morquio B disease. Am J Hum Genet 1991;49:1091–3.

[19] Mosna G, Fattore S, Tubiello G, Brocca S, Trubia M, Gianazaa E, et al. A homozygous missense arginine to histidine substitution at position 482 of the β-galactosidase in an Italian infantile G_{M1}-ganglosidosis patient. Hum Genet 1992;90:247–50.

[20] Heitner R, Elstein D, Aerts J, Weely S, Zimran A. Low-dose *N*-butyldeoxynojirimycin (OGT 918) for type I Gaucher disease. Blood Cells Mol Dis 2002;28:127–33.

[21] Kasperzyk JL, d'Azzo A, Platt FM, Alroy J, Seyfried TN. Substrate reduction reduces gangliosides in postnatal cerebrum–brainstem and cerebellum in G_{M1} gangliosidosis mice. J Lipid Res 2005;46:744–51.

特集 小児疾患の早期発見とその対応—長期予後の向上をめざして—
II．各論—健診・検査後の対応とその評価—

1．新生児スクリーニング
10）今後検討されるべき疾患
①ライソゾーム病

東京慈恵会医科大学　小児科　井田博幸（いだ ひろゆき）

KEY WORDS▶ スクリーニング，リピドーシス，ムコ多糖症，ポンペ病

はじめに

ライソゾームは細胞内小器官のひとつである。細胞内で産生された，あるいは細胞外から取り込まれた高分子化合物を分解するための加水分解酵素を多く内包している。この加水分解酵素をコードする遺伝子に変異が起こると，種々の酵素蛋白の異常が起こり，酵素活性が失われる。この結果，酵素に特異的な基質がライソゾーム内に蓄積して細胞の機能障害を引き起こし，個体の病気として表現される。これら疾患群を総称してライソゾーム病と呼ぶ。本稿ではライソゾーム病におけるスクリーニングの基本的考え方，スクリーニング対象，方法，結果などについて現在までの報告について概説する。

I．ライソゾーム病におけるスクリーニングの意義

1．診断が困難である：個々の疾患頻度が少ないので医師がライソゾーム病の診断を思い浮かべにくい。また，確定診断に必要な検査が一般レベルで行われておらず，大学の研究室レベルで行われていることが多い。これらの事実はライソゾーム病の診断を困難にしている。

2．治療法の進歩により死亡率の減少が望める：従来，ライソゾーム病には根治療法が存在しなかったが，近年の医学の進歩により酵素補充療法，骨髄移植などの根治療法が開発され，実地臨床上，行われるようになり明らかな臨床効果が証明されている。

3．保因者の同定により罹患率を減少させることができる：ライソゾーム病は遺伝病なので遺伝カウンセリングにより発症率をコントロールできる。すなわち，出生前診断により罹患児の発生を減少させることができる。

4．治療効果を上げるために不可逆性変化が生じる前に診断する必要がある：前述したようにライソゾーム病の診断は困難なので，症状が進行した時点で診断されることも少なくない。不可逆性の変化が起こってしまっては治療を行っても，満足な効果を得られない。特にムコ多糖症における骨変形，ファブリ病における腎不全などが臨床上の重要な不可逆性変化である。

II．ライソゾーム病のスクリーニングの基本的考え方

1．スクリーニングの対象

〒105-8461　東京都港区西新橋3-25-8