ORIGINAL PAPER

Generation and characterization of recombinant feline β-galactosidase for preclinical enzyme replacement therapy studies in GM1 gangliosidosis

**Tatiana I. Samoylova · Douglas R. Martin ·
Nancy E. Morrison · Misako Hwang ·
Anna M. Cochran · Alexandre M. Samoylov ·
Henry J. Baker · Nancy R. Cox**

Abstract Lysosomal β-galactosidase is required for the degradation of GM1 ganglioside and other glycolipids and glycoproteins with a terminal galactose moiety. Deficiency of this enzyme leads to the lysosomal storage disorder, GM1 gangliosidosis, marked by severe neurodegeneration resulting in premature death. As a step towards preclinical studies for enzyme replacement therapy in an animal model of GM1 gangliosidosis, a feline β-galactosidase cDNA was cloned into a mammalian expression vector and subsequently expressed in Chinese hamster ovary (CHO-K1) cells. The enzyme secreted into culture medium exhibited specific activity on two synthetic substrates as well as on the native β-galactosidase substrate, GM1 ganglioside. The enzyme was purified from transfected CHO-K1 cell culture medium by chromatography on PATG-agarose. The affinity-purified enzyme preparation consisted mainly of the protein with approximate molecular weight of 94 kDa and displayed immunoreactivity with antibodies raised against a 16-mer synthetic peptide corresponding to C-terminal amino acid sequence deduced from the feline β-galactosidase cDNA.

Keywords GM1 gangliosidosis · β-galactosidase · Enzyme replacement therapy

T. I. Samoylova (✉) · D. R. Martin · N. E. Morrison · M. Hwang · A. M. Cochran · H. J. Baker ·
N. R. Cox
Scott-Ritchey Research Center, College of Veterinary Medicine, Auburn University,
Auburn, AL 36849, USA
e-mail: samoiti@vetmed.auburn.edu

A. M. Samoylov
Department of Pathobiology, College of Veterinary Medicine, Auburn University,
Auburn, AL 36849, USA

Introduction

Lysosomal storage disorders (LSDs) comprise a family of more than 40 distinct inherited human and animal diseases, each caused by deficient activity of a specific acid hydrolase which normally catabolizes macromolecules such as sphingolipids, glycosaminoglycans, glycogen, or glycoproteins (Platt and Walkley 2004). Even though individually rare, LSDs as a group have a prevalence of one per approximately 7,700 live births (Meikle et al. 1999) and together constitute a significant health risk and burden on society. The gangliosidoses are LSDs affecting ganglioside/glycolipid/glycoprotein catabolism, resulting in severe, progressive neurological degeneration and premature death. GM1 gangliosidosis is caused by a mutation of the GLB1 gene which results in deficient activity of the lysosomal enzyme β-galactosidase required to catabolize GM1 ganglioside and other glycolipids and glycoproteins with a terminal galactose moiety.

Feline GM1 gangliosidosis is nearly an identical replica of the human disease and provides an ideal model to develop methods and test hypothesized benefits of enzyme replacement therapy, identify limitations of this therapy, and define mechanisms of therapeutic benefit (Baker et al. 1971). The cat disease is a naturally occurring inherited disease which is exceptionally well characterized with respect to clinical presentation, genetics, morphology, biochemistry, pathogenesis of brain, hepatic and thymic diseases, molecular characterization of the mutations and responses to therapy (Baker et al. 1979; Baker et al. 1982; Cox et al. 1998; Cox et al. 1999; Martin et al. 2002). Domestic cats are a favored species for experimental neurology and provide several important advantages for preclinical therapeutic studies. Unlike rodents, cats can be evaluated readily by routine clinical procedures and are large enough to permit frequent sampling of tissues and body fluids. Also, the cat brain is 100 times larger than the mouse brain and only 10–20 times smaller than the brain of a child (Vite et al. 2003), thereby providing a closer approximation of the therapeutic challenges of treating children affected with lysosomal diseases.

Enzyme replacement therapy (ERT) is a strategy for correction of LSDs by systemic administration of the active enzyme to replace the defective hydrolase. From the circulation, enzymes are taken up by cells via receptor-mediated endocytosis and transported to the lysosome, where they hydrolyze the accumulated undegraded substrate, as reviewed by Grabowski and Hopkin (2003). It is the most successful therapeutic approach for many non-neuronopathic LSDs. This approach is especially attractive because the pathogenesis of these disorders, including GM1 gangliosidosis, can be effectively prevented with only a small increase in enzymatic levels above the deficient state. Since Food and Drug Administration (FDA) approval of the first ERT for Gaucher type I disease in 1991, enzyme replacement-based therapies have been developed for several other LSDs (Neufeld 2004). ERT therapeutics manufactured by Genzyme for Fabry disease and for MPS I (by Genzyme/BioMarin Pharmaceutical) were approved by the FDA in 2003. In 2005, an enzyme preparation for MPS VI (marketed by BioMarin Pharmaceutical) also was approved. Clinical trials are underway for several other non-neuronopathic LSDs.

Although non-central nervous system (CNS) organs can be treated effectively by a variety of methods that provide functional enzymes to diseased cells, the blood-

brain barrier (BBB) severely limits the entry of enzyme molecules into the CNS from systemic circulation, preventing therapeutic benefit. Currently, there is no effective therapeutic option for neuronopathic lysosomal diseases, including the gangliosidoses. However, high doses of enzymes injected intravenously or intrathecally have been shown to reverse nervous system pathology and improve function in several lysosomal diseases that affect brain (Dickson et al. 2007; Dunder et al. 2000; Matzner et al. 2005; Roces et al. 2004; Vogler et al. 2005). While derived from different LSDs, these reports have important features in common: optimized regimes of infusion of a missing functional enzyme that involve high enzyme dose/long-duration combinations leading to positive changes in brain pathology. Taken together, these reports encourage the use of such an approach for the treatment of other lysosomal diseases affecting brain.

This study includes generation and characterization of feline β-galactosidase, thus providing a basis for preclinical therapeutic trials in cats with GM1 gangliosidosis. The outcomes of such trials are expected to present valuable information for the application of ERT to children.

Materials and methods

Cloning of feline ß-galactosidase cDNA and transfection of CHO-K1 cells

A feline β-galactosidase clone was amplified from normal feline brain cDNA with *Pfu* Turbo DNA polymerase (Stratagene, Cedar Creek, TX, USA) and primers fβ-gal 5′utr (5′-AGAGGCTGGAGGATGGACTT-3′) and fβ-gal stop (5′-CCATCAGA-CACGGTCCCATC-3′). The 2,025 base pair amplicon (100 ng) was polished for 30 min with 0.125 units *Taq* DNA polymerase to add the A overhang required for cloning into expression vector pCR3.1 (Invitrogen, Carlsbad, CA, USA). Cloning proceeded at 15°C overnight according to the manufacturer's instructions, and DH5α competent bacteria (Invitrogen) were transformed by heat shock. Integrity of the fβ-galactosidase clone was verified by automated fluorescent DNA sequencing.

The CHO-K1 cell line derived as a subclone from the parental Chinese hamster ovary (CHO) cell line was purchased from American Type Culture Collection (ATCC CRL-9618) (Manassas, VA, USA). Cells were cultured in Ham's F12K medium (Fisher Scientific, Pittsburgh, PA, USA) with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, and 10% fetal calf serum (FCS) (HyClone, Logan, UT, USA). Cells were calcium phosphate-transfected according to standard procedures with the plasmid containing the full-length feline β-galactosidase cDNA, using 20 μg plasmid per 5×10^5 cells. Transfected cells (designated fCHO-K1) were selected with 600 μg/ml active G418 (Invitrogen). The mixed population of transfected cells possessed high enzyme specific activity and, therefore, was used for enzyme production without further subcloning.

Production of feline ß-galactosidase in fCHO-K1 cells

To maximize production of the enzyme, fCHO-K1 cells were cultured at 37°C in Ham's F12K medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate and 10%

FCS until cells were ~80–90% confluent. Medium was then changed to a serum-free medium (CHO-S-SFM II, Invitrogen) specifically designed for CHO cells, and flasks were incubated at 30°C in 5% CO_2 for 6 days. The conditioned medium containing β-galactosidase was then collected, filtered with a 0.45 μm filter and frozen at −20°C.

Detection of ß-galactosidase in fibroblast cell cultures

Two cell types developed previously in our laboratory were used for these studies: primary fibroblasts isolated from a normal cat and primary fibroblasts isolated from a cat affected with GM1 gangliosidosis. Immortalization of primary fibroblasts was achieved by calcium-phosphate transfection with plasmid pSV3-DHFR (ATCC), which contains the large T antigen of Simian virus 40 (Martin et al. 2004). Fibroblast cell cultures were grown in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen) with sodium bicarbonate adjusted to 1.5 g/l and 10% FCS for 48 h and reached ~70% confluency. The medium was replaced with the appropriate medium or treatment (see legend to Fig. 1) for an additional 48 h. Cells were rinsed with PBS and fixed in 1% paraformaldehyde at 4°C for 10 min and then rinsed four times with PBS. To localize β-galactosidase, cells were incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining solution (500 μg/ml X-Gal, 1 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide) in 0.05 M citrate-phosphate buffer (0.05 M citric acid monohydrate, 0.05 M disodium phosphate heptahydrate, 0.1 M sodium chloride, pH 3.8) for approximately 5 h at 37°C.
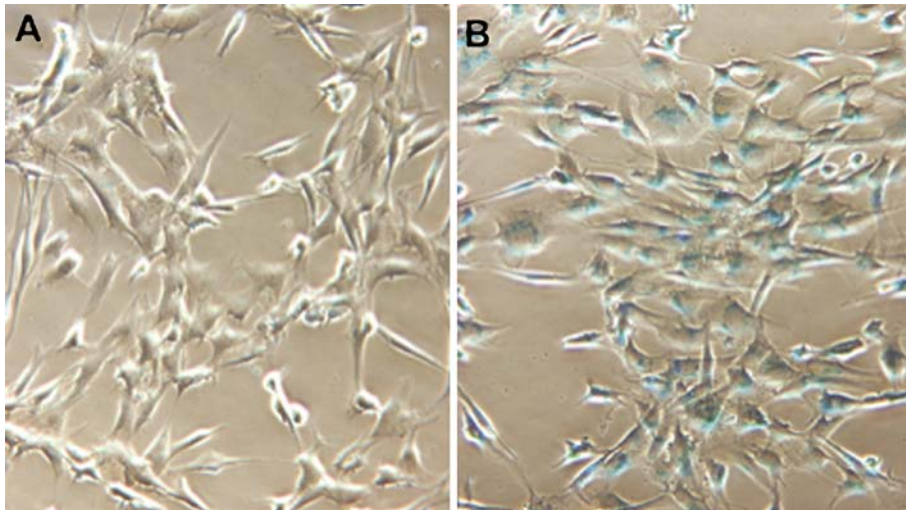

Fig. 1 Internalization of fß-galactosidase into GM1 fibroblasts. A GM1 fibroblasts cultured in fresh growth medium. B GM1 fibroblasts cultured in conditioned medium from transfected fCHO-K1 cells. After treatment, cells were in culture for 48 h prior to reacting with X-Gal staining solution. Blue staining inside GM1 fibroblasts (B) indicates that ß-galactosidase from conditioned medium was internalized by the cells and was enzymatically active on X-Gal substrate. Note: color figures are available on the publication website

Fluorogenic assays of ß-galactosidase activity

Synthetic substrate 4-methylumbelliferyl-β-D-galactopyranoside (4-MU-Gal) was used to determine the amount of ß-galactosidase enzymatic activity in any given sample. *In conditioned medium.* Prior to analysis, samples of conditioned medium were thawed at 4°C and centrifuged at 16,000×*g* for 10 min. 4-MU-Gal was heated at 37°C for 30 min and centrifuged at 16,000×*g*, for 5 min at room temperature. To assay ß-galactosidase activity, 20 μl sample aliquots were incubated with 100 μl of 4-MU-Gal substrate at 37°C in citrate-phosphate buffer (0.05 M citric acid monohydrate, 0.05 M $Na_2HPO_4 \cdot 7H_2O$, 0.1 M NaCl, pH 3.8) for 1 h. The enzymatic reaction which liberates 4-MU from its synthetic substrate was terminated by the addition of 3 ml cold glycine carbonate buffer (0.17 M glycine, 0.17 M sodium carbonate, pH 10.0) and fluorescence was read on a Synergy HT plate reader (Bio-Tek, Winooski, VT, USA). The ß-galactosidase specific activity was calculated in nmol 4 MU/h/mg protein. *In cell lysates.* Cells were grown to 80–90% confluence, rinsed with PBS and collected by trypsinization followed by centrifugation. To lyse cells, 0.1% Triton X-100 in water was added to the pellets and cells were disrupted by aspiration with 22 G needle. The samples were spun for 5 min at 16,000×*g* at 4°C. The supernatants were used for determination of ß-galactosidase activity as above for conditioned medium.

Purification of ß-galactosidase from fCHO-K1 conditioned medium

Feline β-galactosidase was purified from the conditioned medium of fCHO-K1 cells by affinity chromatography on a *p*-aminophenylthio-*β*-D-galactopyranoside (PATG) agarose (Sigma-Aldrich, St. Louis, MO, USA) column as described by Zhang et al. (1994). Briefly, a 3 ml substrate-affinity column of PATG-agarose was prepared using a 5 ml syringe. First, the column was washed with 10 bed volumes of binding buffer (20 mM sodium acetate buffer, pH 4.3, 0.1 mM DTT, 300 mM NaCl). After that, 130 ml of the conditioned medium was allowed to percolate freely though the column at a flow rate of 0.3 ml/min. Then, the column was washed with 200 ml of binding buffer and β-galactosidase was eluted from the column with 10 ml of elution buffer (10 mM phosphate, pH 7.0, 0.1 mM DTT, 1 M NaCl, 0.1 M γ-D-galactonolactone (Sigma)). To remove γ-D-galactonolactone, the eluate was immediately subjected to dialysis against phosphate buffer, pH 7.0. All procedures were performed at 4°C. After the dialysis, the eluate was concentrated with 30 kDa Centricon devices (Millipore), evaluated for β-galactosidase activity and protein content, and stored at −80°C until further use.

Ganglioside analysis by thin layer chromatography

These experiments were performed to test hydrolytic activity of feline β-galactosidase on its native substrate, GM1 ganglioside. A modified version of an assay from Callahan and Gerrie (Callahan and Gerrie 1975) was used. Conditioned medium from fCHO-K1 cells was concentrated 10-fold with Centricon Ultracel YM-50 (Millipore). The conditioned medium or purified feline β-galactosidase at amounts shown in Fig. 5 were mixed with the reaction buffer (12.5 μM sodium

acetate pH 5.0, 4.65 mM sodium taurocholate, 0.1% Triton X-100, 1 μM sodium chloride) containing 40 μg GM1 ganglioside (Matreya, Pleasant Gap, PA, USA) and final assay volume adjusted to 100 μl with water. All samples were incubated for 2 h at 37°C. Assay reactions were stopped with the addition of 1 ml chloroform/methanol (2:1, *v:v*). The reaction mixtures were shaken and then centrifuged for 10 min at 4°C. In each sample, the aqueous layer was discarded and the organic fraction dried under air. Dried samples were resolubilized in 50 μl chloroform/methanol (2:1, *v:v*) and stored at −20°C.

Extracted samples were applied to pre-coated silica gel 60 high-performance thin layer chromatography (TLC) plates (10×10 cm; Whatman), which were heat-activated for 30 min at 110°C. Ganglioside standards (2.5 μg each) (Matreya) and the samples were loaded onto the plate and developed with a chloroform-methanol-0.4% calcium chloride in water solution of 11:9:2 (*v/v/v*). Plates were dried and sprayed with fresh resorcinol reagent, covered with a clean glass plate and heated for 20 to 30 min at 100°C for color development.

Generation and purification of feline anti-β-galactosidase monoclonal antibody (fβgal 654)

An immunogenic peptide for creation of a monoclonal antibody to feline lysosomal β-galactosidase was designed based on the published feline cDNA sequence (AF006749, GenBank). Peptide fβgal 654 (CGH PLP DLS DRD SGW DRV) corresponds to feline β-galactosidase residues 654–669 with CG added to the amino terminus for conjugation of adjuvant. Although the peptide location (the extreme carboxyl terminus) is similar to a previously published anti-human β-galactosidase antibody (Okamura-Oho et al. 1996; Zhang et al. 1994), little amino acid homology exists at the carboxyl termini of feline and human β-galactosidase. The peptide was synthesized at >95% purity, analyzed for net peptide content, and conjugated to keyhole limpet hemocyanin (KLH) by Global Peptide Services LLC (Fort Collins, CO, USA). Female Balb/c mice were immunized intramuscularly with the following emulsification: 100 μl KLH-peptide at the concentration of 1 mg/ml, 100 μl aluminum hydroxide gel adjuvant (Alhydrogel 85, Accurate Chemicals/Superfos Biosector, Denmark) at 0.4 mg/ml, and 10 μg CpG-1826 (McCluskie et al. 2002) (5′- TCC ATG ACG TTC CTG ACG TT -3′, synthesized with a phosphorothioate backbone) (Integrated DNA Technologies, Coralville, IA, USA). Two subsequent immunizations were performed as above, but with 50 μg KLH-peptide. The final immunization was performed with 25 μg unconjugated peptide alone.

Isolation of spleen cells from immunized mice, fusion with myeloma cells and subsequent isolation of hybridoma clones were performed according to standard procedures by the Auburn University Hybridoma Facility, Auburn, Alabama. Hybridoma supernatants were screened by Western blotting for selection of the best antibody clone (isotype IgG2b).

Protein gel electrophoreses and Western blot analysis

Two types of protein gel electrophoresis were performed. First, proteins were separated in a denaturing gel to demonstrate sample purity and determine molecular

weights of purified polypeptides. For denaturing electrophoresis, samples were incubated with a loading buffer containing SDS and β-mercaptoethanol. Gels were stained with Coomassie Blue R-250. Second, proteins were separated in a native gel to preserve β-galactosidase activity for detection with 4-MU-Gal substrate. For native electrophoresis, samples were mixed with native loading buffer and gels were run in Tris-glycine running buffer without SDS. Gels were soaked in the citrate-phosphate buffer (outlined above for β-galactosidase activity) for 30 min at RT and reacted with 4-MU-Gal substrate for 1 h at 37°C. Broad and low range molecular weight standards were used. Sample loading buffers and running buffers for both types of electrophoresis as well as precast gels and molecular weight standards were purchased from BioRad (Hercules, CA, USA).

For Western blots with monoclonal antibody fβgal 654, proteins from SDS-polyacrylamide gels were transferred to nitrocellulose membranes and blocked in 7.5% nonfat dry milk in PBS with 0.05% Tween-20 for 2 h at room temperature. A 90-min incubation of hybridoma supernatant (1:5) was followed by incubation with goat anti-mouse IgG/IgM secondary antibody conjugated to horseradish peroxidase (1:120,000–1:150,000, Pierce). The Western blot was developed with SuperSignal West Dura chemiluminescent substrate (Pierce, Rockford, IL, USA).

Results

For enzyme production, the feline β-galactosidase cDNA in mammalian expression vector pCR3.1, was calcium phosphate transfected into CHO-K1 cells. Conditioned medium obtained from a mixed population of transfected cells was harvested, evaluated for specific β-galactosidase activity and used further for fβ-galactosidase purification. In an assay with 4-methylumbelliferyl-β-D-galactopyranoside (4-MU-Gal) as β-galactosidase substrate, the cells transfected with fβ-galactosidase (named fCHO-K1) showed significantly elevated specific enzymatic activity in conditioned medium compared to untransfected CHO-K1 cells (Table 1).

To demonstrate the ability of fβ-galactosidase to be internalized by GM1 cells and to be enzymatically active within the cells, monolayer cultures of enzyme-deficient GM1 fibroblasts were incubated with fβ-galactosidase-containing medium from fCHO-K1 cells. GM1 fibroblasts treated with the conditioned medium showed a strong β-galactosidase histochemical reaction with X-Gal as substrate while untreated cells did not. Staining inside GM1 fibroblasts indicates that fß-galactosidase from fCHO-K1 conditioned medium was internalized by the cells

Table 1 β-galactosidase specific activity in conditioned medium from two different types of CHO-K1 cells (untransfected and transfected with feline β-galactosidase cDNA)

Cells	β-gal specific activity
CHO-K1 untransfected	13.1±0.3
fCHO-K1 transfected	2361.3±93.6

The enzyme specific activity was measured in triplicate in independent experiments using 4-MU-Gal as β-galactosidase substrate. The mean specific activity±SD is shown in nmol 4 MU/h/mg protein (t-test, P=<0.001).

and reacted with the substrate producing a blue-colored product localized to the lysosomal compartment (Fig. 1). Moreover, high β-galactosidase specific activity was detected in fß-galactosidase-treated cells using another synthetic substrate, 4-MU-Gal (Table 2). β-galactosidase specific activity in the treated GM1 cells comprised 36% of normal cat fibroblast enzyme activity.

Subsequently, feline β-galactosidase was purified from the conditioned medium of fCHO-K1 cells by affinity chromatography on a PATG agarose column. The conditioned medium was allowed to percolate freely though the substrate-affinity column of PATG-agarose and β-galactosidase was eluted from the column with a buffer containing γ-D-galactonolactone. To remove γ-D-galactonolactone, the eluate was immediately dialysed against pH 7.0 phosphate buffer. After the dialysis, the eluate was concentrated and evaluated for β-galactosidase activity and protein content. Specific β-galactosidase activity in the final preparation was approximately 240,000 nmol 4 MU/h/mg protein which is comparable to the activity of human β-galactosidase purified from conditioned medium from CHO17 cells in the laboratory of Dr. J. Callahan, whose protocol we used (Zhang et al. 1994).

Proteins in the final fβ-galactosidase preparation were characterized by SDS-PAGE, native protein electrophoresis, and Western blot analysis. The most abundant protein in the preparation had the molecular weight ~94 kDa, as judged by comparison with protein standards (Fig. 2, SDS-PAGE). This likely is the precursor fβ-galactosidase secreted by transfected fCHO-K1 cells into culture medium. Additionally, proteins were separated in a native gel to preserve β-galactosidase activity for detection with 4-MU-Gal substrate. For native electrophoresis, samples were mixed with native loading buffer and gels were run in Tris-glycine running buffer, without SDS. Gels soaked in the citrate-phosphate buffer and reacted with 4-MU-Gal substrate are shown in Fig. 3. The results demonstrated the presence of an active enzyme of the same molecular weight in both, unpurified (fCHO-K1 conditioned medium, lane 3) and purified (lane 4) β-galactosidase, preparations. Western blot analysis of CHO-K1 and fCHO-K1 proteins was performed using an antibody raised against a synthetic peptide corresponding to a 16-residue C-terminal amino acid sequence deduced from the feline β-galactosidase cDNA. The analysis clearly showed the presence of the 94 kDa polypeptide in conditioned medium from transfected CHO-K1 cells and in purified protein, while conditioned medium from untransfected CHO-K1 cells did not demonstrate any reaction (Fig. 4). An immunoreactive polypeptide of the same molecular weight was detected within the transfected cell lysate as well.

Table 2 β-galactosidase specific activity in GM1 fibroblasts treated with conditioned medium from transfected fCHO-K1 cells

| Cells	β-gal specific activity
GM1 fibroblasts treated	158.1±2.0
GM1 fibroblasts not treated	6.1±0.3
Normal fibroblasts	437.2±5.6

The enzyme specific activity was measured using 4-MU-Gal as β-galactosidase substrate. The mean specific activity ± SD is shown in nmol 4 MU/h/mg protein (t-test, P=<0.001).

Fig. 2 SDS-PAGE of proteins from CHO-K1 cell conditioned medium. *Lane 1* low range molecular weight markers (Bio-Rad), *lane 2* total proteins (10 μg) from untransfected CHO-K1 cell medium, *lane 3* total proteins (10 μg) from fCHO-K1 cell medium (without purification), *lane 4* proteins (1.5 μg) purified from fCHO-K1 cell medium by PATG affinity chromatography
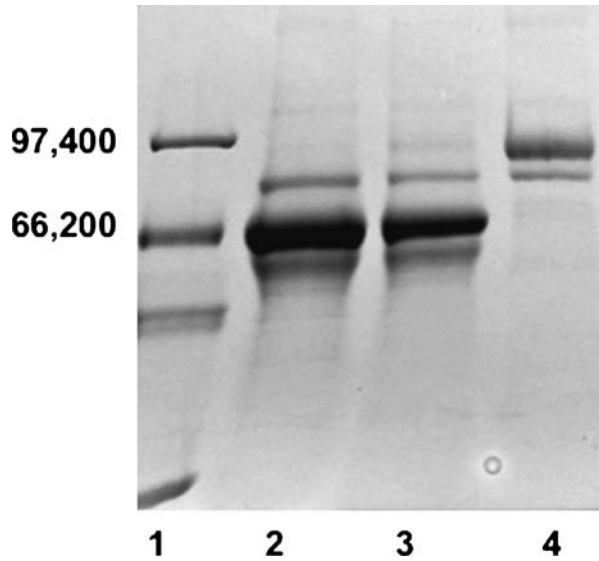
For clinical applications, generated fβ-galactosidase must be functional on the natural substrate, GM1 ganglioside. To test the ability of fβ-galactosidase to hydrolyse the natural substrate, GM1 ganglioside was reacted with different doses of non-purified (fCHO-K1 conditioned medium) or purified fβ-galactosidase. The resultant mixtures were resolved using TLC. As seen in Fig. 5, the enzyme was able to cleave GM1 ganglioside to produce the downstream reaction product, GM2 ganglioside. The increasing doses of fCHO-K1 conditioned medium added to the substrate resulted in increased GM2, showing dose dependency of the reaction. Addition of purified fβ-

Fig. 3 Native electrophoresis and enzymatic activity of proteins from CHO-K1 cell conditioned media. *Lane 1* ß-galactosidase from bovine liver (positive control, Sigma), *lane 2* total proteins (10 μg) without purification from untransfected CHO-K1 cell medium, *lane 3* total proteins (10 μg) without purification from fCHO-K1 cell medium, *lane 4* proteins (1.5 μg) purified from fCHO-K1 cell medium by affinity chromatography. Samples were mixed with native loading buffer and the gel was run in Tris-glycine buffer, without SDS. The gel was soaked in the citrate-phosphate buffer and reacted with 4-MU-Gal substrate. The bands were visualized using 312 nm Transilluminator FBTI88 (Fisher)
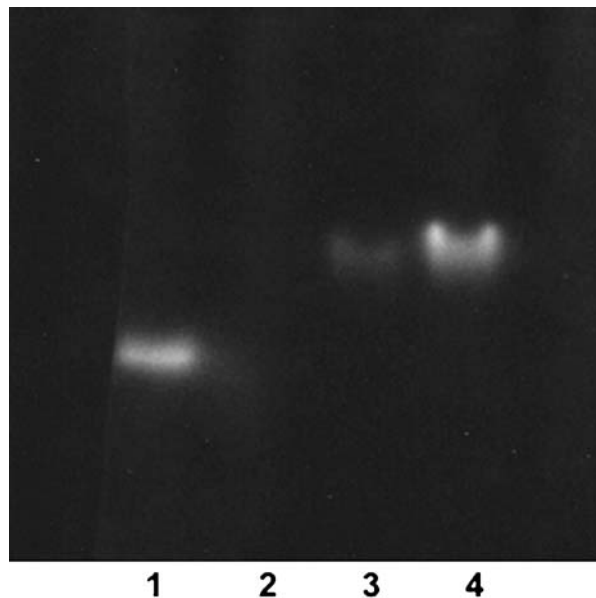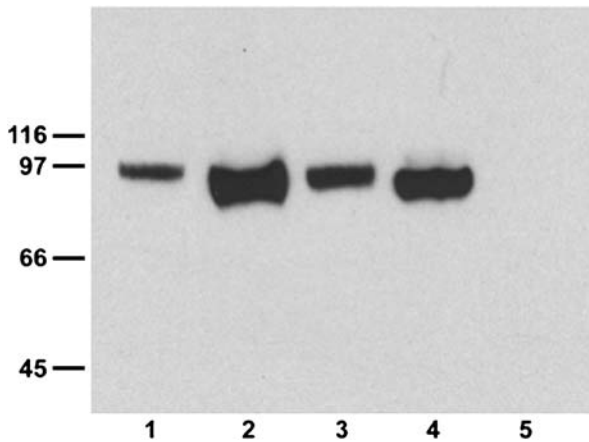
Fig. 4 Western blot analysis of CHO-K1 cell lysates and conditioned media. CHO-K1 cells were transfected with a full-length feline β-galactosidase cDNA, and protein was subjected to SDS-PAGE in a 10% polyacrylamide gel. The blot was probed with a monoclonal antibody (fβgal 654) to the extreme carboxyl terminus of fβ-galactosidase. *Lane 1* total cell lysate from transfected CHO-K1 cells (10 μg). *Lane 2* conditioned medium from transfected CHO-K1 cells affinity-purified with *p*-aminophenylthio-β-D-galactopyranoside agarose (33.5 ng). *Lane 3* conditioned medium from transfected CHO-K1 cells concentrated by centrifugal filtration (0.75 μg). *Lane 4* lysate from feline GM1 gangliosidosis skin fibroblasts after transfection by the same expression plasmid used for transfection of CHO-K1 cells (positive control, 10 μg). *Lane 5* untransfected CHO-K1 cell lysate (negative control, 20 μg). Molecular size standards (in kDa) are shown on *left side of blot*, with precise molecular weights as follows: 116.3, 97.4, 66.2, 45.0
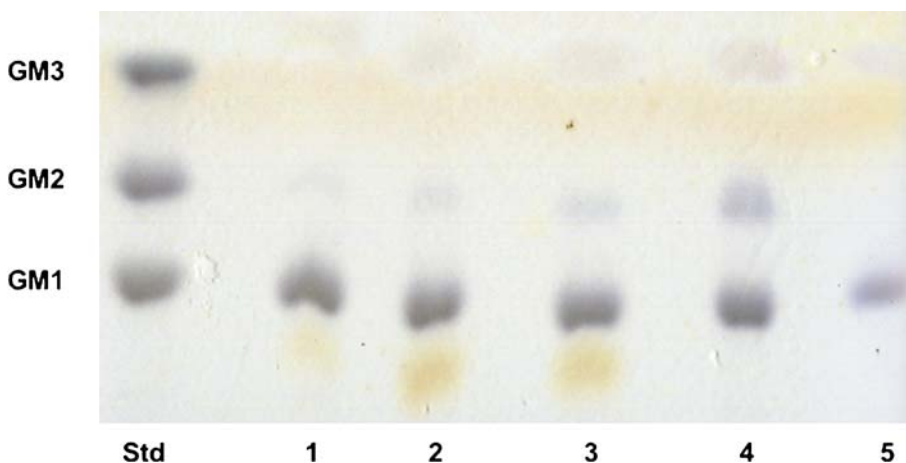

Fig. 5 Thin-layer chromatogram of GM1 ganglioside reacted with fß-galactosidase. GM1 ganglioside was incubated in 100 μl assay mixture (see "Materials and methods") containing different amounts of concentrated fCHO-K1 conditioned medium or purified fß-galactosidase. *Lane 1* GM1 treated with 5 μl fCHO-K1 medium, *lane 2* GM1 treated with 10 μl fCHO-K1 medium, *lane 3* GM1 treated with 20 μl fCHO-K1 medium, *lane 4* GM1 treated with 10 μl of purified fß-galactosidase, *lane 5* untreated GM1 control

galactosidase to the reaction mixture led to a similar effect, indicating that the enzyme is able to cleave the natural substrate. To examine this further, GM1 fibroblasts (characterized by abnormal accumulation of externally loaded GM1) were treated with fβ-galactosidase-containing conditioned medium and gangliosides were extracted and separated by TLC. The treatment resulted in reduction of GM1 and increased amounts of GM2 and GM3 gangliosides (not shown). Taken together, the enzyme was shown to be fully functional on GM1 substrate.

Discussion

Each lysosomal storage disease is rare and requires a disease-specific enzyme replacement treatment. This does not make the development of therapies for individual diseases any less significant, since many of them (especially those with neurological involvement) are lethal or result in drastically reduced quality of the patients' lives, requiring long-term, extensive care. Most of the enzyme preparations used for LSDs are human recombinant proteins made in CHO cells. In this study, CHO-K1 cells were designed to produce fβ-galactosidase for potential use in preclinical trials in a cat model of GM1 gangliosidosis which closely resembles the human disease. The enzyme was generated by affinity purification of conditioned medium from CHO-K1 cells transfected with feline β-galactosidase cDNA. Subsequent characterization demonstrated the purified enzyme's specific activity on various substrates and immunoreactivity with a monoclonal antibody to feline β-galactosidase.

Specific activity of the enzyme in conditioned medium when measured with 4-MU-Gal as β-galactosidase substrate was on average 2,361 nmol/h/mg protein which is similar to that for CHO cells transfected with human β-galactosidase cDNA reported by Zhang et al. (1994). The produced enzyme was effectively internalized by GM1 feline fibroblasts, as shown with X-Gal staining and thin layer chromatography of the fβ-galactosidase-treated cells. Specific enzymatic activity inside GM1 fibroblasts treated with fβ-galactosidase was determined with 4-MU-Gal substrate to comprise 36% of normal fibroblast activity. As for most lysosomal storage diseases, the amount of enzyme needed to correct the storage in GM1 gangliosidosis is theorized to be low (<10% of the normal level).

Feline β-galactosidase expressed by transfected CHO-K1 cells was purified in a one-step procedure and characterized as to molecular weight and specific activity. The molecular weight of the major polypeptide in the affinity-purified protein preparation was shown to be approximately 94 kDa by SDS-PAGE. This band is thought to represent the feline precursor β-galactosidase secreted by transfected fCHO-K1 cells into culture medium. The human precursor β-galactosidase (88 kDa), secreted by permanently transfected CHO cells, was described previously (Zhang et al. 1994). The difference in molecular weights between human and feline precursors might be explained by differences in glycosylation/post-translational processing or by the approach taken for molecular weight determination. The diffuse appearance of the band might indicate the presence of fβ-galactosidase polypeptides of diverse glycosylation levels. A faint band corresponding to one of the proteins in CHO-K1 cell conditioned medium was also detected by SDS-PAGE. This minor band appeared in at least three fβ-galactosidase preparations obtained independently

(not shown) and could represent a polypeptide that is associated with β-galactosidase that cannot be removed with the affinity purification method used. Further purification might be considered; however, it should be carefully balanced with the loss of specific enzyme activity which decreases with each additional purification step. The native electrophoresis that uses 4-MU-Gal as the β-galactosidase substrate demonstrated the presence of a single diffuse band in the purified preparation as well as in fCHO-K1 conditioned medium.

Several approaches have potential for delivery of the missing enzyme across the BBB into GM1-affected brain to reduce storage material and improve brain function. The BBB can be breached with high-dose enzyme replacement therapy (Dunder et al. 2000; Roces et al. 2004). The most promising results following high dose ERT in neuronopathic lysosomal diseases were obtained in the Sly laboratory (Vogler et al. 2005). The researchers studied the distribution of recombinant human β-glucuronidase and reduction in storage by weekly doses of up to 40 mg/kg administered intravenously to mucopolysaccharidosis type VII mice over 1–13 week periods. It was shown that, if given in high doses over a sufficient duration of treatment (4 mg/kg/week for 13 weeks or 20 mg/kg/week for 4 weeks), enzyme reached the brain parenchyma and produced clearance of CNS lysosomal storage. Mice receiving 20 mg/kg once weekly for four weeks had on average 2.5% of normal activity in brain. The results indicate that functional β-glucuronidase can be delivered across the BBB of mature mice by using high doses of enzyme. As a whole, the studies substantiate the applications of the high enzyme dose/long duration approaches for treatment of other lysosomal diseases that involve the CNS.

Mechanisms by which lysosomal enzymes cross BBB and reduce storage in the CNS remain to be determined. Two most frequently discussed options for enzyme delivery across BBB are M6P/IGF2R-mediated transport (Urayama et al. 2004; Vogler et al. 2005) and transport by phagocytic cells that take up a portion of enzyme infused at high doses (Vogler et al. 2005). A third possibility is that enzyme is taken up by a variety of mechanisms collectively termed the extracellular pathways that allow small amounts of large molecules to enter the CNS. The examples of therapeutics which are likely to enter CNS by such mechanisms are antibodies directed against amyloid beta protein and erythropoietin used in the treatment of stroke (Banks 2004).

In summary, the current study reports successful generation and purification of feline β-galactosidase for pre-clinical experiments in the well-characterized cat model of GM1 gangliosidosis. High-dose ERT in the feline GM1 model will provide valuable insight into the utility of this strategy for human patients.

References

Baker HJ, Lindsey JR, McKhann GM, Farrell DF (1971) Neuronal GM1 gangliosidosis in a Siamese cat with beta-galactosidase deficiency. Science 174:838–839

173

Baker HJ, Reynolds GD, Walkley SU, Cox NR, Baker GH (1979) The gangliosidoses: comparative features and research applications. Vet Pathol 16:635–649

Baker HJ, Walkley SU, Rattazzi MC, Singer HS, Watson HL, Wood PA (1982) Feline gangliosidoses as models of human lysosomal storage diseases. In: Desnick RJ, Patterson DF, Scarpelli DG (eds) Animal Models of Inherited Metabolic Diseases. Alan R. Liss, New York, pp 203–212

Banks WA (2004) Are the extracellular pathways a conduit for the delivery of therapeutics to the brain? Curr Pharm Des 10:1365–1370

Callahan JW, Gerrie J (1975) Purification of GM1-ganglioside and ceramide lactoside beta-galactosidase from rabbit brain. Biochim Biophys Acta 391:141–153

Cox NR, Ewald SJ, Morrison NE, Gentry AS, Schuler M, Baker HJ (1998) Thymic alterations in feline GM1 gangliosidosis. Vet Immunol Immunopathol 63:335–353

Cox NR, Morrison NE, Sartin JL, Buonomo FC, Steele B, Baker HJ (1999) Alterations in the growth hormone/insulin-like growth factor I pathways in feline GM1 gangliosidosis. Endocrinology 140:5698–5704

Dickson P, McEntee M, Vogler C, Le S, Levy B, Peinovich M, Hanson S, Passage M, Kakkis E (2007) Intrathecal enzyme replacement therapy: successful treatment of brain disease via the cerebrospinal fluid. Mol Genet Metab 91:61–68

Dunder U, Kaartinen V, Valtonen P, Vaananen E, Kosma VM, Heisterkamp N, Groffen J, Mononen I (2000) Enzyme replacement therapy in a mouse model of aspartylglycosaminuria. FASEB J 14:361–367

Grabowski GA, Hopkin RJ (2003) Enzyme therapy for lysosomal storage disease: principles, practice, and prospects. Annu Rev Genomics Hum Genet 4:403–436

Martin DR, Cox NR, Hathcock TL, Niemeyer GP, Baker HJ (2002) Isolation and characterization of multipotential mesenchymal stem cells from feline bone marrow. Exp Hematol 30:879–886

Martin DR, Krum BK, Varadarajan GS, Hathcock TL, Smith BF, Baker HJ (2004) An inversion of 25 base pairs causes feline GM2 gangliosidosis variant. Exp Neurol 187:30–37

Matzner U, Herbst E, Hedayati KK, Lullmann-Rauch R, Wessig C, Schroder S, Eistrup C, Moller C, Fogh J, Gieselmann V (2005) Enzyme replacement improves nervous system pathology and function in a mouse model for metachromatic leukodystrophy. Hum Mol Genet 14:1139–1152

McCluskie MJ, Weeratna RD, Payette PJ, Davis HL (2002) Parenteral and mucosal prime-boost immunization strategies in mice with hepatitis B surface antigen and CpG DNA. FEMS Immunol Med Microbiol 32:179–185

Meikle PJ, Hopwood JJ, Clague AE, Carey WF (1999) Prevalence of lysosomal storage disorders. JAMA 281:249–254

Neufeld EF (2004) Enzyme replacement therapy. In: Platt FM, Walkley SU (eds) Lysosomal Disorders of the Brain. Oxford University Press Inc., New York, pp 327–338

Okamura-Oho Y, Zhang S, Hilson W, Hinek A, Callahan JW (1996) Early proteolytic cleavage with loss of a C-terminal fragment underlies altered processing of the beta-galactosidase precursor in galactosialidosis. Biochem J 313(Pt 3):787–794

Platt FM, Walkley SU (2004) Lysosomal defects and storage. In: Platt FM, Walkley SU (eds) Lysosomal Disorders of the Brain. Oxford University Press, New York, pp 32–49

Roces DP, Lullmann-Rauch R, Peng J, Balducci C, Andersson C, Tollersrud O, Fogh J, Orlacchio A, Beccari T, Saftig P, von Figura K (2004) Efficacy of enzyme replacement therapy in alpha-mannosidosis mice: a preclinical animal study. Hum Mol Genet 13:1979–1988

Urayama A, Grubb JH, Sly WS, Banks WA (2004) Developmentally regulated mannose 6-phosphate receptor-mediated transport of a lysosomal enzyme across the blood-brain barrier. Proc Natl Acad Sci USA 101:12658–12663

Vite CH, Passini MA, Haskins ME, Wolfe JH (2003) Adeno-associated virus vector-mediated transduction in the cat brain. Gene Ther 10:1874–1881

Vogler C, Levy B, Grubb JH, Galvin N, Tan Y, Kakkis E, Pavloff N, Sly WS (2005) Overcoming the blood-brain barrier with high-dose enzyme replacement therapy in murine mucopolysaccharidosis VII. Proc Natl Acad Sci USA 102:14777–14782

Zhang S, McCarter JD, Okamura-Oho Y, Yaghi F, Hinek A, Withers SG, Callahan JW (1994) Kinetic mechanism and characterization of human beta-galactosidase precursor secreted by permanently transfected Chinese hamster ovary cells. Biochem J 304(Pt 1):281–288