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Human recombinant palmitoyl-protein thioesterase-1 (PPT1) for preclinical evaluation of enzyme replacement therapy for infantile neuronal ceroid lipofuscinosis

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ABSTRACT

Infantile neuronal ceroid lipofuscinosis (INCL, also known as Haltia–Santavuori disease) is a lysosomal storage disorder of infants and children characterized by blindness, seizures and a progressive neurodegenerative course. Recent clinical trials have involved neural stem cells and gene therapy directed to the central nervous system; however, enzyme replacement therapy has never been addressed. In the current paper, we describe the production of human recombinant PPT1 (the defective enzyme in INCL) by standard methods in Chinese Hamster Ovary (CHO) cells. The enzyme is largely mannose 6-phosphorylated as assessed by mannose 6-phosphate receptor binding (80% bound) and taken up rapidly by immortalized patient lymphoblasts, where clearance of PPT substrates was demonstrated (EC_{50} of 0.25 nM after overnight incubation). When injected intravenously into PPT1-deficient mice, the clearance of recombinant human PPT1 from plasma was rapid, with a half-life of 10 min. Most of the injected dose was distributed to the kidney and liver and potentially corrective levels were also observed in heart, lung and spleen. Brain uptake was minimal, as expected based on experience with other intravenously administered lysosomal enzymes. The enzyme may be useful as an adjunct to central nervous system-directed therapies and could be used as a starting point for modifications designed to improve brain delivery.

Introduction

Deficiency of the lysosomal enzyme palmitoyl-protein thioesterase-1 (PPT1; EC 3.1.2.22)[1] causes a human lysosomal storage disorder, designated ceroid lipofuscinosis, neuronal-1 (CLN1) characterized by progressive blindness, cognitive and motor deterioration, psychiatric disturbances, and seizures, leading to a chronic vegetative state [1]. The onset of the disease varies from infancy to adulthood [2–4]. The pathology is notable for widespread yellow–brown pigment deposition (lipofuscin) with the ultrastructural appearance of granular osmiophilic deposits (GROD) under electron microscopy. Storage material is found throughout most tissues. The disease is not typical of lysosomal storage disorders affecting the brain in that severe neuronal loss and volume contraction (rather than volume expansion due to storage material) is observed [5].

Enzyme replacement therapy has not previously been developed for PPT1 deficiency because peripherally administered enzyme is expected to have only limited access to the brain. However, this has not been tested formally. In the current paper, we confirm that human recombinant PPT1 enzyme produced in a CHO cell line has minimal distribution to the brain but seems to be well tolerated when administered via tail vein to PPT1-deficient mice. Potentially corrective levels of enzyme were achieved in peripheral organs that were tested, including the heart, which may be important clinically [6].

Materials and methods

High-level expression of PPT1 in CHO cells

A fragment corresponding to the entire coding region of the human PPT1 sequence (nucleotides 233–1153 of NM_000310 (GenBank)) was generated by polymerase chain reaction amplification from pCMV5-hPPT1 [7] and cloned into XhoI sites in the polylinker region of expression vector pMSXND1 [8]. The nucleotide sequence

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[1] *Abbreviations used:* INCL, infantile neuronal ceroid lipofuscinosis; PPT1, palmitoyl-protein thioesterase-1; CHO, Chinese Hamster Ovary; CLN1, ceroid lipofuscinosis, neuronal-1; GROD, granular osmiophilic deposits; FBS, fetal-bovine serum; MU-6S-Palm-βGlc, 4-methylumbelliferyl-6-thiopalmitoyl-β-D-glucoside; PBS, phosphate-buffered saline; HP-TLC, high performance-thin layer chromatography; Man 6-P, mannose 6-phosphate.

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of the forward primer used was 5′-GCG ATA CTC GAG ATG GCG TCG CCC GGC TGC CTG TGG CTC TTG-3′ and the reverse primer, 5′-GCG ATA CTC GAG TCA TCC AAG GAA TGG TAT GAT GTG GGC-3′. The open reading frame was sequenced to confirm fidelity of the amplification. High-level expression in CHO cells was obtained essentially as described [9] by multiple rounds of selection in methotrexate [10]. Briefly, CHO cells (obtained from the ATCC, #CCL-61™) were maintained in F-12 medium (Invitrogen) supplemented with 10% (v/v) fetal-bovine serum (FBS) and were transfected with pMSXND1-hPPT1 using FuGENE 6 (Roche). Cells were selected with G418 and resistant clones isolated using cloning cylinders. Subclones were analyzed for secretion of PPT1 by measuring enzyme activity in conditioned medium using a fluorescence assay based on hydrolysis of an artificial substrate, 4-methylumbelliferyl-6-thiopalmitoyl-β-D-glucoside (MU-6S-Palm-βGlc) [11]. Highest producing clones were grown in nucleotide-deficient α-minimum essential medium (Invitrogen) containing 0.25 μM methotrexate. When resistant colonies appeared, they were expanded, aliquots frozen, and the remaining cells subjected to 2- to 3-fold higher concentration of methotrexate. This process was repeated for a total of 6 cycles, reaching a final maximum methotrexate concentration of 30 μM, after which further increases did not lead to higher enzyme production. The final cell line produced was designated CHO-hPPT1 and has been deposited at the National Cell Culture Center (Minneapolis, MN).

Purification and characterization of recombinant human PPT1

CHO-hPPT1 cells were maintained in nucleoside-deficient α-minimum essential medium (Gibco, Catalog No. 32561) containing 30 μM methotrexate, and were supplemented with 10% dialyzed FBS (Gemini, Catalog No. 100–106, lot A12A01X) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. For production of recombinant protein, confluent adherent CHO-hPPT1 cells were split 1:2 and grown in F-12 medium (Invitrogen) supplemented with 10% FBS to confluence. For recombinant protein production, cells were washed with phosphate-buffered saline (PBS) three times and cultured in serum-free medium (Hyclone CDM4CHO, SH30557) for 14 days, with medium collected and replenished with fresh medium every two days. Typically, 30 dishes each containing 5 ml of medium were used. Collected medium was centrifuged at 400*g*, filtered through a 0.2 μm filter unit to remove debris and floating cells, then concentrated approximately 20-fold using a stirred cell equipped with a YM-10 membrane (Millipore) at 4 °C, and snap-frozen until further use.

Concentrated medium (about 30 ml) was dialyzed overnight using a dialysis cassette (20 K molecular weight cut-off, Pierce) against two changes of Mono-S equilibration buffer (50 mM Na acetate, 25 mM NaCl, 2 mM EDTA, pH 5.0; 4 l per change) and centrifuged for 20 min at 8000 rpm in an SLA-1500 rotor (34,155g_{max}). The resulting supernatant (40 ml) was loaded onto a Mono-S 10/100 GL column (1 × 10 cm, 8 ml bed volume) at a flow rate of 2 ml/min and washed with 40 ml of equilibration buffer. Enzyme activity was eluted with a 160-ml linear gradient of increasing NaCl concentration (25–350 mM) in the same buffer. Fractions (4 ml) containing enzyme activity were pooled, concentrated to 5 mg/ml and exchanged into PBS containing 1 mM EDTA and 1 mM β-glycerol phosphate by repeated rounds of dilution and concentration using a Centricon-10 concentrator.

Uptake of PPT1 and hydrolysis of cysteinyl lipids by PPT1-deficient lymphoblasts

A PPT1-deficient immortalized lymphoblastoid cell line (UT8–01) was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). For metabolic labeling experiments, 200 ml of log-phase patient lymphoblastoid cells (0.5–0.75 × 10^6 cells/ml) were pulsed-labeled with [^{35}S] cysteine for 4 h [12], washed, and then incubated overnight in unlabeled cysteine-containing "chase" medium containing various amounts of purified PPT1 in the presence of 5 mM mannose (control) or mannose 6-phosphate (Man 6-P). For time course studies, the labeled cells were incubated for up to 16 h with 0.008 U/ml (0.54 μg/ml) of PPT1 added to the culture medium (1 U = 1 μmol of MU-6S-Palm-βGlc hydrolyzed per min). The cells were harvested into microcentrifuge tubes and washed twice with 0.5 ml of cold PBS and pelleted for 10 s at maximum speed in a microcentrifuge at 4 °C, followed by chloroform–methanol extraction and analysis by high-performance-thin layer chromatography (HP-TLC) as previously described [12].

Plasma and tissue clearance of PPT1 administered by tail vein injection

Adult PPT1 knockout mice at 8–16 weeks of age of either sex were injected via tail vein with human PPT1 at concentrations of 0 (vehicle), 2.5, 7.5, or 25 mg/kg of human recombinant PPT1 (15 U/mg, in a total volume of 250 μl, four mice per group). Blood (50 μl) was collected at the times indicated from the submandibular vein into Eppendorf tubes containing 1 μl of 0.5 M EDTA at the time indicated, mixed well, centrifuged at 4000 rpm for 10 min in a tabletop microfuge, and the supernatant plasma was snap frozen in liquid nitrogen. At 2, 6, 24, and 72 h after injection, mice were anesthetized with Avertin (250 mg/kg) and perfused with 20 ml of heparinized saline through the left ventricle over a period of 20 min, and brains, hearts, lungs, livers, kidneys, and spleens were snap frozen in liquid nitrogen. For PPT1 assays, plasma was diluted as appropriate and assayed directly, and tissues were homogenized in 5–10 volumes of 50 mM Tris–HCl, 100 mM NaCl, 2 mM EDTA, pH 7.0, using a Polytron homogenizer, centrifuged for 1 h at 100,000*g*, and the supernatant assayed according to [11]. All enzyme assays were conducted under conditions where the increase in fluorescent intensity was linear with respect to time and concentration and in comparison with known standards (purified PPT1 and 4-methylumbelliferone). Typically, each assay contained 10–30 μg of protein and incubations were carried out from 15 min to 3 h. Protein content was determined using the Dc protein assay (BioRad).

Results

Production of recombinant human PPT1 in CHO cells

The full-length human PPT1 cDNA was cloned into a plasmid expression vector containing the dihydrofolate reductase resistance marker and used to transfect CHO cells, which were selected by multiple stepwise increases in methotrexate concentration over a period of nine months to achieve a very high and stable level of enzyme expression and secretion. Clones selected following this procedure secreted from 200 to 250 μg of PPT1 enzyme per ml of culture medium and could be grown in continuous culture in serum-free medium for up to 21 days. The enzyme was stable during concentration and dialysis, and was purified to homogeneity using a Mono-S cation exchange column (Fig. 1). Final enzyme activity was 15 U/mg (1 U = 1 μmol of MU-6S-Palm-βGlc hydrolyzed per min). Approximately 50 mg of purified enzyme was obtained from 600 ml of serum-free medium collected over 14–21 days. We have performed at least three such collections with essentially equivalent results.