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Intrathecal enzyme replacement therapy improves motor function and survival in a preclinical mouse model of infantile neuronal ceroid lipofuscinosis

Jui-Yun Lu [a,c], Hemanth R. Nelvagal [d], Lingling Wang [a,c], Shari G. Birnbaum [b], Jonathan D. Cooper [d], Sandra L. Hofmann [a,c,*]

[a] Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390-8593, USA
[b] Department of Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX 75390-8593, USA
[c] Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, TX 75390-8593, USA
[d] Pediatric Storage Disorders Laboratory, Department of Basic and Clinical Neuroscience, King's Health Partners Centre for Neurodegeneration, James Black Centre, Institute of Psychiatry, Psychology & Neuroscience, King's College London, 125 Coldharbour Lane, London SE5 9NU, UK

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ABSTRACT

The neuronal ceroid lipofuscinoses (NCLs) are a group of related hereditary lysosomal storage disorders characterized by progressive loss of neurons in the central nervous system resulting in dementia, loss of motor skills, seizures and blindness. A characteristic intralysosomal accumulation of autofluorescent storage material occurs in the brain and other tissues. Three major forms and nearly a dozen minor forms of NCL are recognized. Infantile-onset NCL (CLN1 disease) is caused by severe deficiency in a soluble lysosomal enzyme, palmitoyl-protein thioesterase-1 (PPT1) and no therapy beyond supportive care is available. Homozygous *Ppt1* knockout mice reproduce the known features of the disease, developing signs of motor dysfunction at 5 months of age and death around 8 months. Direct delivery of lysosomal enzymes to the cerebrospinal fluid is an approach that has gained traction in small and large animal models of several other neuropathic lysosomal storage diseases, and has advanced to clinical trials. In the current study, *Ppt1* knockout mice were treated with purified recombinant human PPT1 enzyme delivered to the lumbar intrathecal space on each of three consecutive days at 6 weeks of age. Untreated PPT1 knockout mice and wild-type mice served as additional controls. Four enzyme concentration levels (0, 2.6, 5.3 and 10.6 mg/ml of specific activity 20 U/mg) were administered in a volume of 80 μl infused over 8 min. Each group consisted of 16–20 mice. The treatment was well tolerated. Disease-specific survival was 233, 267, 272, and 284 days for each of the four treatment groups, respectively, and the effect of treatment was highly significant ($p < 0.0001$). The timing of motor deterioration was also delayed. Neuropathology was improved as evidenced by decreased autofluorescent storage material in the spinal cord and a decrease in CD68 staining in the cortex and spinal cord. The improvements in motor function and survival are similar to results reported for preclinical studies involving other lysosomal storage disorders, such as CLN2/TPP1 deficiency, for which intraventricular ERT is being offered in clinical trials. If ERT delivery to the CSF proves to be efficacious in these disorders, PPT1 deficiency may also be amenable to this approach.

1. Introduction

The neuronal ceroid lipofuscinoses are a group of neurodegenerative disorders characterized by widespread accumulation of autofluorescent storage material in lysosomes and a progressive loss of neurons in the central nervous system [1]. Deficiency in the enzyme palmitoyl protein thioesterase-1 (PPT1; EC 3.1.2.22) causes the autosomal recessive storage disorder infantile neuronal ceroid lipofuscinosis, or CLN1 disease [2]. This enzyme is a small, globular hydrolase of the α/β type that removes fatty acids from cysteine residues in lipid-modified proteins during lysosomal protein degradation [3]. Over 60 mutations in the *PPT1/CLN1* gene in NCL patients have been described [4]. Complete absence of enzyme activity results in severe neurodegeneration occurring in infancy and is characterized by progressive cognitive and motor deterioration, blindness, and seizures leading to premature death [2]. Childhood and adult onset cases are associated with missense mutations that allow for varying levels of residual enzyme activity in the range of 2–7% [5–7]. Hematopoietic stem cell transplantation has had no effect on the course of affected infants [8]. No effective treatment is available and

Abbreviations: ERT, enzyme replacement therapy; CLN1, ceroid lipofuscinosis, neuronal-1; CLN2, ceroid lipofuscinosis, neuronal-2; LSD, lysosomal storage disorder; MU-6S-Palm-βGlc, 4-methylumbelliferyl-6-thiopalmitoyl-β-D-glucoside; NCL, neuronal ceroid lipofuscinosis; PPT1, palmitoyl-protein thioesterase-1; TPP1, tripeptidyl peptidase-1.

* Corresponding author at: Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-8593, USA.
E-mail address: sandra.hofmann@utsouthwestern.edu (S.L. Hofmann).

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various approaches, including small molecule, enzyme replacement, and gene and cellular therapies are the subject of preclinical studies [9].

The *Ppt1* knockout mouse demonstrates the major features associated with the human disease, including autofluorescent storage and manifestations such as seizures, decline in motor performance and reduced lifespan [10,11]. The mice live about 235 days in the absence of treatment [10,12]. We have previously shown that PPT1 administered weekly via the tail vein is effective in clearing visceral storage, but has no impact on motor deterioration or survival when the treatment is started after blood brain barrier closure in the mouse, which occurs at 3 weeks of age [13]. About a three-week survival advantage and delay in onset of motor symptoms was shown when treatment was started at birth, suggesting that the brain would be responsive to ERT if appropriate access could be achieved.

In addition to infantile NCL, several of the other NCLs (CLN2, 5 and 10) are caused by deficiencies of soluble lysosomal enzymes that can be supplied exogenously to cells through the mannose 6-phosphate receptor pathway. Classic late-infantile NCL (CLN2 disease) is caused by deficiency in the soluble lysosomal protease, tripeptidyl peptidase-1 (TPP1). Treatment of TPP1 knockout mice with recombinant TPP1 enzyme (10 mg/ml) delivered to the cerebrospinal fluid via lumbar intrathecal injection (given on three consecutive days) in mice at 4 weeks of age prolonged lifespan of CLN2 knockout mice from 16 to 23 weeks [14]. Based on these encouraging results in mice, the approach was successfully applied to the TPP1-deficient dog model [15–17] and has progressed to a human clinical trial (ClinicalTrials.gov ID# NCT01907087). In the current study we have used the same protocol to treat Ppt1 knockout mice with human recombinant PPT1 and show a dose-dependent positive effect on motor function, brain pathology and survival.

2. Materials and methods

2.1. Human recombinant PPT1

Human recombinant PPT1 was prepared from an overproducing Chinese Hamster Ovary (CHO) clonal cell line as described [18] except that the enzyme was exchanged into an artificial CSF buffer at the final step (aCSF, 148 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 0.9 mM $MgCl_2$, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, pH 7.2). Enzyme was concentrated using a stirred cell equipped with a YM-10 membrane (Millipore) at 4 °C. Solutions for injections were free of endotoxin (<0.06 EU/ml) as determined using a limulus amebocyte lysate assay (Lonza, #50-648U). All injections in this study were from the same enzyme preparation. The specific activity of this lot was 20 U/mg (where 1 U = 1 μmol of 4-methylumbelliferyl-6-thiopalmitoyl-β-D-glucoside (MU-6S-Palm-βGlc) hydrolyzed per minute [19]). 4-methylumbelliferyl-6-thiopalmitoyl-β-D-glucoside was obtained from Moscerdam Substrates. Mannose 6-phosphate receptor binding was 85% as determined by a column-binding assay [18]. 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 20–50 μg of protein and incubations were carried out from 30 min to 1 h. Protein content was determined using the Dc protein assay (BioRad). Other reagents were obtained from Sigma-Aldrich unless otherwise noted.

2.2. Affinity-purified polyclonal antibodies

Three New Zealand White rabbits were each immunized with 500 μg of purified human recombinant PPT1. The antigen was injected intradermally (final volume 1 ml) in Freund's complete adjuvant (Difco, #263910). An IgG fraction was prepared from preimmune serum by Protein A agarose chromatography. Human PPT1 antibody IgG was affinity-purified using the AminoLink Plus Immobilization Kit (Thermo Scientific, #44894) following the manufacturer's instructions.

2.3. Intrathecal mouse injections

All procedures were carried out under an Institutional Animal Care and Use Committee-approved protocol at the University of Texas Southwestern Medical Center. *Ppt1* knockout mice [10] were maintained as homozygous breeding stock on a C57BL/6 background, housed in a barrier facility and received food and water ad libitum. Treatment groups were assigned randomly from littermates born within a 2–3 day window after timed mating and group housed. All assessments were carried out by individuals blinded with respect to treatment group and genotype. Concurrent groups of untreated Ppt1 knockout mice and wild-type C57BL/6 mice were maintained for comparison but were not included in the analysis for treatment effects. Each treatment group consisted of between 16 and 20 mice at about 6 weeks of age. Six weeks of age is in the presymptomatic period of Ppt1 knockout mice [13] and well after the permeability transition for lysosomal enzymes to reach the brain from the systemic circulation in the neonatal mouse [20]. For the enzyme replacement therapy, mice were anesthetized with isoflurane delivered through an inhalation system (EZ-Anesthesia Classic System, Braintree). Animals were shaved to expose the skin around the lumbar region and injected between vertebrae L5 and L6 using a 30-gauge needle (Becton Dickinson) oriented toward L4 as described [14]. The needle was connected to a 100 μl gas-tight Hamilton syringe (#81075) by a short length of silastic tubing (0.3 mm inner diameter, Dow Corning, #2415496). The dose (vehicle or enzyme) was administered at 10 μl per min using an NE-300 syringe pump (New Era Pump Systems).

2.4. Rotarod assessments of motor performance

For motor coordination testing, mice were tested on a Rotarod (model 755, IITC Life Science Inc., Woodland Hills, CA) at 3, 5, 6, 7, 8 and 9 months of age and the latency to fall in seconds recorded. Trials were terminated after a maximum of 60 s. At each age, mice underwent a pre-test trial on a stationary rod, followed by two test trials on the constant speed Rotarod (3 rpm) for each of three consecutive days. The maximum latency to fall in the two test trials on the final day of testing was reported as the final outcome measure. A two-factor (treatment group and time of measurement) ordinary ANOVA model was used to analyze data for vehicle, 2.6, 5.3, and 10.6 mg/ml PPT1 dosing groups for months 3, 5, 6 and 7. Ordinary one-way ANOVA (treatment group) was used to analyze month 8 for the 2.6, 5.3 and 10.6 mg/ml treatment groups (as all mice in the vehicle group had died by month 8).

2.5. Survival

Mice were assessed weekly for body weight and more frequently for general health. Mice were sacrificed when a loss of greater than 10% of highest weight recorded was noted for two consecutive weeks, when animals could not right themselves, or were moribund (poorly responsive to tactile stimulation). The observer was blinded as to assignment of treatment groups. The Kaplan–Meier log-rank test for trend was used to determine whether there was an overall significant difference between the treatment groups.

2.6. Tissue processing, neuropathology and immunohistochemistry

Mice were anesthetized with Avertin and perfused transcardially with cold heparinized physiological saline followed by freshly prepared 4% formaldehyde in PBS, pH 7.4. Whole mice were then post-fixed in 4% formaldehyde for a further 48 h and transferred to 50 mM Tris. Spinal cords were separated from the brainstem just below the foramen magnum and dissected from the surrounding bone and musculature. Brains