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Modification of aspartoacylase for potential use in enzyme replacement therapy for the treatment of Canavan disease

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ABSTRACT

Canavan disease is a fatal neurological disease without any effective treatments to slow the relentless progress of this disorder. Enzyme replacement therapy has been used effectively to treat a number of metabolic disorders, but the presence of the blood–brain-barrier presents an additional challenge in the treatment of neurological disorders. Studies have begun with the aim of establishing a treatment protocol that can effectively replace the defective enzyme in Canavan disease patients. The human enzyme, aspartoacylase, has been cloned, expressed and purified, and the surface lysyl groups modified through PEGylation. Fully active modified enzymes were administered to mice that are defective in this enzyme and that show many of the symptoms of Canavan disease. Statistically significant increases in brain enzyme activity levels have been achieved in this animal model, as well as decreases in the elevated substrate levels that mimic those found in Canavan disease patients. These results demonstrate that the modified enzyme is gaining access to the brain and functions to correct this metabolic defect. The stage is now set for a long term study to optimize this enzyme replacement approach for the development of a treatment protocol.

1. Introduction

Canavan disease (CD)[2] is a fatal, genetically transmitted neurodegenerative disorder that was first identified by brain pathology [1], and was subsequently shown to be a medical disorder that is distinct from the previously identified leukodystrophies and other demyelinating diseases [2]. Symptoms of CD appear in early infancy and typically progress very rapidly. The early symptoms include loss of motor skills, loss of muscle control, and megalocephaly. Progression of the disease leads to paralysis, blindness, and epileptic seizures, with death usually occurring within the first decade of life [3]. A deficiency in aspartoacylase (ASPA, EC3.5.1.15) activity caused by mutations in the *aspA* gene has been implicated as the cause of CD [4]. Analysis of DNA isolated from CD patients has identified numerous mutants resulting in low activity or the complete loss of aspartoacylase activity [5], with several common mutations prevalent in different ethnic groups [6,7]. This enzyme plays a critical role in brain metabolism, the deacetylation of *N*-acetyl-L-aspartic acid (NAA) to produce acetate and aspartate, and appears to be the only enzyme in brain that can effectively metabolize NAA.

NAA is one of the most abundant amino acid metabolites in the brain, but its precise biological function is still uncertain. Among the diverse roles suggested for NAA includes functioning as a storage form and source of acetyl groups in neurons [8] that could then either serve as precursors for the fatty acid biosynthesis necessary for myelin production [9,10], or could be transported for extramitochondrial fatty acid biosynthesis [11]. A myriad of other hypotheses have also been advanced that support the need for functionally viable aspartoacylase in the developing brain. While the precise role of NAA has not been defined, it has been conclusively demonstrated that the concentration of this metabolite must be controlled within a narrow range for optimal health. This control requires a balance between the activities of aspartate *N*-acetyltransferase and aspartoacylase, the enzymes responsible for the synthesis and degradation of NAA. Low brain NAA levels are a hallmark of neuronal injury and death. A decrease in neuronal NAA concentration has been observed in many neurodegenerative diseases, including epilepsy [12], multiple sclerosis [13], myotrophic lateral sclerosis [14], and Alzheimer's disease [15]. In contrast, it is the elevated levels of NAA found in CD patients that leads to the symptoms of this disorder.

Several gene replacement therapy trials have been approved for the treatment of infants with defective aspartoacylase [16,17], and some limited success has been seen in slowing the relentless progress of this disease. The primary barriers that must be overcome in gene therapy are the successful incorporation of the replacement gene into

Abbreviations: ASPA, aspartoacylase; CD, Canavan disease; ERT, enzyme replacement therapy; NAA, *N*-acetyl-L-aspartate; PEG, polyethylene glycol.

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the patient's genomic DNA and the expression and control of the protein product. A potential alternative approach that would bypass these issues is direct administration of the properly functioning protein. However, for enzyme replacement therapy (ERT) to succeed a different set of barriers must be surmounted. Injection of foreign proteins into a patient will cause an immune response, the half-life of injected proteins can be quite short, and to be most effective the protein must become localized in the cells where the deficiency is manifest.

Surface modifications of proteins can decrease the immunogenicity of foreign proteins. The introduction of polyethylene glycol (PEG) polymers through covalent modification of exposed protein functional groups has been shown to decrease the immune response to these modified proteins and increase their circulation half-life [18]. Recent studies have demonstrated the efficacy of ERT in an animal model of the metabolic disorder phenylketonuria in which mutations in the gene that encodes for phenylalanine hydroxylase result in the inability to metabolize phenylalanine. Treatment of recombinant phenylalanine hydroxylases with several PEGs that are terminated in reactive functional groups lead to modified forms of these enzymes that retain full catalytic activity and achieve increased stability [19]. Injection of these PEGylated enzymes into a mouse model with defective phenylalanine hydroxylase results in enhanced *in vivo* catalytic activity, decreased serum phenylalanine levels, and significantly reduced immunogenicity when compared to control studies with the untreated enzyme [20]. Administration of a PEGylated form of adenosine deaminase is a well established treatment therapy for children with severe combined immune deficiency caused by a defect in purine metabolism [21]. Achieving similar success with aspartoacylase will be needed to consider ERT as a viable approach for the treatment of CD.

An additional potential complication in the use of ERT for the treatment of neurological disorders is the presence of a blood–brain barrier which serves as the gatekeeper controlling access to the neurological system in higher organisms. This structure provides a physical barrier, by means of tight junctions composed of membrane proteins and lipids that seal the gaps between endothelial cells, a chemical barrier that regulate the transport of material through these cells and pump foreign substances away from the brain, and a metabolic barrier that hydrolyzes and inactivates toxic compounds. Thus the blood–brain barrier effectively protects that brain against foreign substances, but also limits access to many therapeutic agents designed to treat neurological disorders. The modified replacement enzyme must be able to traverse this barrier if it is to function to overcome the metabolic defect that is responsible for CD.

In this study we have examined the effect of PEGylation on the catalytic activity and the protein stability of aspartoacylase. Several PEGylated forms of this enzyme have been injected into mice that are deficient in aspartoacylase. Statistically significant increases in brain aspartoacylase activity and decreases in NAA levels have now been achieved with this approach, demonstrating the capability of modified aspartoacylase to cross the blood–brain barrier.

2. Materials and methods

2.1. Materials

The plasmid containing the *acy2* gene was transformed into KM71H *Pichia pastoris* cells following the directions in the Easy Select Pichia Transformation kit (Invitrogen). Reactive PEG reagents were purchased from NOF (Japan) and the protease inhibitor cocktail was obtained from Sigma. Mice were from the Canavan original black strain [22].

2.2. Purification of aspartoacylase

Aspartoacylase was expressed in a *P. pastoris* cell line using standard protocols, with methanol induction of the *acy2* gene under control of an alcohol oxidase promoter [12,13]. Fifteen grams of cells obtained from 4 l of growth media were resuspended in 50 ml of buffer containing 20 mM potassium phosphate, pH 7.4, 20 mM imidazole, 5% glycerol and 0.5 M NaCl. Fifty microliters of protease inhibitor cocktail was added to the lysate to inhibit proteases and the cells were mechanically disrupted by using a bead beater. The crude supernatant was loaded on a 10 ml Fast flow Nickel Sepharose column (Amersham Biosciences) using an Akta Explorer 100 chromatography system. After washing the column with buffer containing a low level of imidazole (20 mM) to remove any non-specifically adsorbed proteins the enzyme was eluted with a linear imidazole gradient (from 20 to 400 mM). The active fractions were pooled and dialyzed into 50 mM Hepes, pH 7.5, with 1 mM dithiothreitol (DTT) and loaded onto an anion-exchange column (Source 15Q). The enzyme was eluted from this column using a linear salt gradient (from 0 to 0.5 M NaCl), yielding from 5 to 10 mg of highly purified enzyme. The purified enzyme was dialyzed into 50 mM Hepes, pH 7.5, 1 mM DTT and 0.1 M NaCl for immediate use or storage at −80 °C.

2.3. Activity measurements

Aspartoacylase activity was measured by a coupled assay previously developed for this enzyme [23]. In this assay, carried out in the presence of 0.75 mM of *N*-acetyl-L-aspartate in a reaction buffer containing 50 mM Hepes, pH 7.5, and 10 mM $Mg(OAc)_2$, the aspartic acid produced by the deacetylation reaction of aspartoacylase is deaminated by using an excess of L-aspartase as the coupling enzyme. Production of the resulting fumarate product was followed at 240 nm ($\varepsilon = 2.53\ mM^{-1}\ cm^{-1}$) using a Cary 50 UV-visible spectrophotometer.

2.4. PEGylation of aspartoacylase

Aspartoacylase samples (0.8 mg per reaction) were treated with different PEG reagents containing terminal activating aldehyde or succinimidyl groups attached with either carboxymethyl or succinyl linkers. Linear 2 kDa, 5 kDa, 10 kDa, 20 kDa and branched 40 kDa PEG molecules were added to the reaction mixtures in varying enzyme to polymer ratios from 1:2 to 1:64 and incubated pH 7.5 or 8.3 and at temperatures ranging from 4 to 37 °C. Aliquots were removed from the reactions at different time points, quenched by the addition of excess lysine and then frozen for subsequent analysis. For the enzyme distribution studies aspartoacylase was treated with fluorescein-labeled 5 kDa PEG. The enzyme samples were each concentrated by using a 30 kDa spin concentrator to remove the excess PEG. After completion of the reaction, followed by several buffer exchanges, the sample was concentrated to 1 mg/ml in a buffer containing 50 mM Hepes, pH 7.5, 1 mM DTT, 0.1 M NaCl. PEGylated enzyme samples that were used in the animal studies were further purified by binding and elution from an anion-exchange column (Source 15Q) with a linear NaCl gradient to remove any residual unmodified enzyme.

Aspartoacylase was also directed labeled by treatment with fluorescein dye dissolved in DMSO at a final dye to protein ratio of 1:10 (w/w) with the reaction run at 20 °C for 1 h. After spin concentration to remove any unreacted dye the extent of modification was determined by the A_{495}/A_{280} ratio.

2.5. Animal treatment protocol

Canavan, black original strain mice [22] were used for the animal studies, and the studies were conducted with five groups of four mice each, two males and two females per groups. All mice were humanely handled in accord with IACUC protocol and policies. All KO/KO genotypes were examined twice, with mice genotyped before weaning and again at harvest time. Treatment of all mice began at 23 days of age and consisted of two doses of enzyme per week from age 3 to 5 weeks and then one dose per week from age 6 to 10 weeks.