Research Article

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Long-term metabolic correction of Wilson's disease in a murine model by gene therapy

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Background & Aims: Wilson's disease (WD) is an autosomal recessively inherited copper storage disorder due to mutations in the *ATP7B* gene that causes hepatic and neurologic symptoms. Current treatments are based on lifelong copper chelating drugs and zinc salts, which may cause side effects and do not restore normal copper metabolism. In this work we assessed the efficacy of gene therapy to treat this condition.

Methods: We transduced the liver of the *Atp7b*$^{-/-}$ WD mouse model with an adeno-associated vector serotype 8 (AAV8) encoding the human ATP7B cDNA placed under the control of the liver-specific α1-antitrypsin promoter (AAV8-AAT-ATP7B). After vector administration we carried out periodic evaluation of parameters associated with copper metabolism and disease progression. The animals were sacrificed 6 months after treatment to analyze copper storage and hepatic histology.

Results: We observed a dose-dependent therapeutic effect of AAV8-AAT-ATP7B manifested by the reduction of serum transaminases and urinary copper excretion, normalization of serum holoceruloplasmin, and restoration of physiological biliary copper excretion in response to copper overload. The liver of treated animals showed normalization of copper content and absence of histological alterations.

Conclusions: Our data demonstrate that AAV8-AAT-ATP7B-mediated gene therapy provides long-term correction of copper metabolism in a clinically relevant animal model of WD providing support for future translational studies.

Keywords: Adenoassociated virus (AAV); ATPase copper transporting beta polypeptide (ATP7B); Copper metabolism; Gene transfer.

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Abbreviations: AAV, Adenoassociated virus; ATP7B, ATPase copper transporting beta polypeptide; TGN, trans-Golgi network.

Introduction

Wilson's disease (WD) is a rare autosomal recessive inborn error of copper metabolism caused by mutations in the gene that encodes the ATPase copper transporting beta polypeptide, *ATP7B*. Copper is a potentially toxic metal but it is essential for a wide number of physiological functions acting as a co-factor of a variety of enzymes [1]. After its intestinal absorption, copper is transported to hepatocytes where it binds to ATP7B located in the membrane of the trans-Golgi network (TGN). This large transmembrane protein is in charge of transferring the metal to copper-dependent enzymes. Loading of copper into ceruloplasmin is essential for the ferroxidase activity of this enzyme and constitutes an important secretory pathway for the metal, as 95% of copper present in the plasma of healthy individuals is bound to ceruloplasmin [1,2]. In response to increased cytosolic copper concentration, ATP7B translocates from TGN to the apical membrane of hepatocytes to facilitate copper excretion into the bile [3]. WD affects approximately 1 in 30,000 people worldwide. The genetic defect causes progressive copper accumulation in the liver during the first decade of life followed by copper deposition in the brain if the stores continue to grow. The disease is commonly heralded by liver damage which may evolve to neurologic dysfunction if it is left untreated. Current therapy is aimed at reducing copper stores and preventing its re-accumulation. With adequate treatment, the progression of the disease is usually halted and symptoms improve. Drugs approved for WD

treatment are zinc salts and copper chelators such as penicillamine and trientine [4,5]. Zinc acts by blocking the absorption of copper in the intestinal tract and by inducing the expression of metallothionein in enterocytes. This cysteine-rich protein is an endogenous chelator of metals that binds copper in the enterocyte and inhibits its entry into the portal circulation. This action both depletes accumulated copper and prevents its reaccumulation. Copper chelators act by ligating copper and promoting its urinary excretion but they do not restore the normal copper metabolism, i.e. they do not increase serum ceruloplasmin nor enhance biliary copper excretion. In fact copper chelators by removing copper from the stores and transferring it to blood before urinary excretion may facilitate deposition of the metal in the brain, resulting in exacerbation of neurological symptoms [6]. Another limitation of current therapies for WD is that they should be given lifelong which implies problems derived from side effects and treatment adherence [5]. Furthermore, a recent study of a large cohort of patients on chelation therapy showed that although half of the patients fully recovered or improved, 15% deteriorated, 8% required liver transplant and 7.4% died [7]. Thus, alternative therapeutic approaches, particularly those capable of offering a permanent correction of the disorder, like gene or cell therapy, are desirable.

Translational research in WD has been aided by the existence of relevant animal models. Long-Evans Cinnamon (LEC) rats harbor a spontaneous deletion in the 3' terminal region of the ATP7B [8]. More recently, *Atp7b* knockout mice were developed [9,10]. These animals show no ATP7B expression in the liver and exhibit the typical biochemical and physiopathological alterations found in human WD, except for neurological manifestations. Abnormalities include high copper excretion in the urine, low holoceruloplasminemia, high serum transaminase levels and increased liver copper concentration with associated hepatocellular damage [9,10].

Proof of concept studies performed in LEC rats showed that introduction of a functional *ATP7B* gene in transgenic rats resulted in hepatic expression of the protein, with restoration of holoceruloplasmin biosynthesis and biliary copper excretion [8]. Early gene therapy approaches using first-generation adenoviral vectors expressing ATP7B to LEC rats achieved therapeutic effects, with restoration of serum active holoceruloplasmin and elevation of copper content in stool [11]. However, improvements were transient due to the short duration of transgene expression achieved by this kind of vectors. So far, *in vivo* use of long-term expression vectors like lentivirus was unable to completely reverse the liver alterations present in adult WD animals, probably due to inefficient hepatic transduction [12]. These evidences suggest that gene therapy could become a curative treatment for WD if sufficient transgene expression of ATP7B can be maintained in the liver for a long period of time. Gene therapy using adeno-associated viral vectors (AAV) is nowadays the most promising therapy for the correction of genetic disorders. Clinical proof of concept has been reported in hemophilia B patients, showing sustained expression of the therapeutic transgene for more than 5 years after a single intravenous administration of an AAV8 vector, with excellent tolerance [13]. In this study we describe the construction and characterization of an AAV8 vector designed for liver-specific expression of ATP7B and its long-lasting therapeutic effects in a relevant mouse model of WD.

Material and methods

Animals and animal manipulation

The generation and characterization of *Atp7b*$^{-/-}$ mice on the hybrid C57BL/6 × 129S6/SvEv background (Jackson Laboratories) was previously described by Dr. Svetlana Lutsenko [9,10]. Mice were bred and maintained under pathogen-free conditions and genotyped at 3 weeks of age according to the original protocol [9]. Treatment with AAV vectors were performed in male mice at 6 weeks of age by intravenous injection. For urine and feces collection, mice were placed for 24 h into metabolic cages (Tecniplast s.p.A.; Buguggiate, VA, Italy) and received food and water ad libitum. Liver samples were collected from euthanized mice for copper determination, histological analysis and for nucleic acid extraction. For the copper overload experiments, mice were fasted overnight and then they received an intraperitoneal injection of 100 μg $CuSO_4$ before starting feces collection. The experimental design was approved by the Ethical Committee for Animal Testing of the University of Navarra.

Construction of AAV vector genomes

The plasmids used in this study were AAV-pA1AT-ATP7B-sPolyA, AAV-pA1AT-dLuciferase-sPolyA and AAV-AlbEnh-pA1AT-EGFP-BGHPolyA. They contain the genome of the AAV vectors AAV8-AAT-ATP7B, AAV8-AAT-dLuc and AAV8-EalbAAT-EGFP, respectively. The expression cassette contained in the AAV8-pA1AT-ATP7B-sPolyA plasmid consisted of: 1) human *ATP7B* coding sequence (CDS) (GenBank accession number U03464); 2) the liver-specific human α1-antitrypsin promoter (pA1AT) [14]; and 3) a synthetic polyadenylation signal (sPolyA) [15]. Gene synthesis of the human *ATP7B* followed by sPolyA was carried out by GenScript (Piscataway, NJ 08854 USA) to obtain the plasmid pUC57-ATP7B-sPolyA. Human pA1AT was isolated from ssAAV8-AlbEnh-pA1AT-EGFP-BGHPolyA plasmid by PCR amplification using the following primers: 1) pA1AT forward primer 5′CTGGTCTAGAACGCGTCGCCACCCCCTCCACCTTGG 3′; 2) pA1AT reverse primer 5′ATCATGATGCGGCCGCTTCACTGTCCCAGGTCAGTG 3′. The PCR product was then digested with XbaI and NotI restriction enzymes and inserted into the same sites of pUC57-ATP7B-sPolyA. Then, the expression cassette containing A1AT-ATP7B-sPolyA was subcloned into the shuttle pAAV-MCS vector using MluI and SmaI sites. The expression cassette contained in the AAV8-pA1AT-dLuciferase-sPolyA plasmid consisted of: 1) the destabilized luciferase gene (the luciferase open reading frame fused to the rapid degradation domain of mouse ornithine decarboxylase); 2) the pA1AT promoter; and 3) the sPolyA. The destabilized luciferase gene (dLuciferase) was released from pdLucFXR plasmid (provided by Dr. Tomas Aragon, CIMA) (GenBank accession number AY603759) using NruI and BamH1. The resulting fragment was ligated in the blunted NotI and BamH1 restriction sites of puc57-pA1AT-sPolyA. Finally, the expression cassette pA1AT-dLuciferase-sPolyA was removed from pUC57 using MluI and SmaI and ligated in the MluI and PmlI restriction sites of the shuttle pAAV-MCS vector. The expression cassette contained in the AAV8-AlbEnh-pA1AT-EGFP-BGHPolyA plasmid consisted of: 1) the enhanced green fluorescent protein gene (EGFP); 2) the mouse albumin enhancer (AlbEnh) linked to the pA1AT promoter [14]; and 3) the bovine growth hormone polyadenylation signal. In all the constructs the expression cassette was flanked by both AAV2 wild-type ITRs.

Serum ceruloplasmin assay

To determine oxidase activity from serum ceruloplasmin, o-dianisidine dihydrochloride was used as substrate. Chloride was removed from the samples prior to analysis to avoid interference with enzymatic activity. This was accomplished by precipitating serum proteins with saturated ammonium sulfate solution. After 10,000 rpm centrifugation for 5 min at room temperature, clear supernatants containing the chloride were removed with a pipette. The pellets were resuspended then in 0.1 M sodium acetate buffer pH5.0 and added to a 96-well plate. Total oxidase activity of the samples was measured by adding 2.5 mg/ml of dimethoxybenzidine dihydrochloride (o-dianisidine; Sigma-Aldrich Co., MO, USA). Then, samples were incubated at 30 °C for 90 min until reaction was stopped by the addition of 9 M sulfuric acid to each well. Finally, absorbance was measured at 540 nm.

Metal-responsive element luciferase reporter assay

A reporter plasmid (pGL3-4MRE-LUC) was used to assess the functional copper export capacity of the ATP7B transgene cloned in our AAV vector. This plasmid