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OBM Genetics

Review

Prospects for Cure in Wilson Disease

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Academic Editor: Khue Vu Nguyen

Special Issue: Treatment of Genetic Disease

OBM Genetics
2018, volume 2, issue 3
doi:10.21926/obm.genet.1803030

Received: July 18, 2018
Accepted: August 9, 2018
Published: August 23, 2018

Abstract

Wilson Disease is a monogenetic disorder of copper metabolism affecting the *ATP7B* gene. Treatment is lifelong and focuses on removal of copper to arrest disease progression and improve the clinical manifestations of copper toxicity. Currently the only cure is liver transplantation, however, lifelong monitoring and immunosuppressive medications are still needed afterwards. The possibility of introducing functional *ATP7B* gene through gene therapy provides an exciting potential option for achieving a more permanent cure without the need for additional therapy and medical monitoring.

Keywords

Wilson Disease; gene therapy; *ATP7B* gene; viral vectors; gene repair

1. Background

Progress over the last century has led from the formal recognition of Wilson disease (WD) as a neurologic disorder with associated liver disease [1] to the understanding that Wilson disease is an autosomal recessive inherited disorder of copper metabolism [2]. Even before the responsible gene for WD, *ATP7B,* was identified [3], it was known that excess copper, in particular in the liver and the central nervous system, underlay the pathophysiology of this disease and its wide array of clinical phenotype. As such, development of medical therapy focused on the removal of copper to arrest disease progression and even reverse some of the clinical manifestations of copper toxicity. Treatment efficacy was first shown for the copper chelating agent, dimercaptopropanol or British Anti-Lewisite (BAL) [4], and soon after for the first oral agent used to treat Wilson disease, d-Penicillamine [5], both increasing the urinary excretion of copper. Since there have been other additions to the treatment armamentarium, including the oral chelator triethylene-tetramine (trientine) that also increases urine copper excretion [5], and the use of zinc salts to induce endogenous metallothionein in enterocytes to block the intestinal absorption of dietary copper [6]. More recently, another chelator that enhances biliary copper excretion, tetrathiomolybdate, is undergoing clinical testing [7].

All current medical treatments for WD require lifelong therapy to maintain the health of the patient. As with treatment of any chronic disease, the requirement for daily therapy, sometimes with multiple daily dosages of medication apart from food, has led to non-adherence with treatment in up to half of patients and recurrent or new disease symptom or even death from liver failure in some non-adherent patients [8]. Furthermore, some patients experience side effects of treatment. The first therapy, BAL, was injected in 3 cc of oil and left painful abscesses at the injection site, and caused systemic reactions in some patients. There are known hypersensitivity reactions to the chelating agents, mostly for d-Penicillamine, as well as other systemic effects of d-Penicillamine including a lupus like syndrome or development of nephritis and nephrosis with proteinuria, and other dermatologic effects. There is also a potential for worsening of a patient's disease with the start of therapy in up to about 20% afflicted with neurologic or psychiatric symptoms at the outset of therapy with d-Penicillamine or even with trientine [8].

When liver transplant was first performed for WD for patients with a failing liver, it was known that the defective biliary copper excretion was central to the disease. Liver transplant provided further proof that the underlying defect resided in the liver (this is discussed in more detail below). However, liver transplant is not a perfect treatment, and though results of this life-saving procedure are excellent and has improved over the years, it still brings with it significant mortality and morbidity. Furthermore, following liver transplantation, lifelong medical treatment for preventing graft rejection is necessary. So, while liver transplant in effect does provide a kind of cure for WD, there is still a need for ongoing medical treatment and monitoring related to the graft, and therefore it is not a true "cure" in that it does not provide a complete or permanent treatment on its own.

So therein lies the rationale for considering treatments that may be a true "cure" for WD. The cure should provide the ability to prevent further hepatic copper accumulation and in the face of excess copper deposition, permit its safe removal from the liver and other sites in the body, without additional therapy. The possibility for achieving this is our subject of discussion.

2. *ATP7B* Gene as a Target

Wilson disease (WD) was discovered to be inherited in an autosomal recessive fashion. In 1993 the gene responsible for WD was identified as a transmembrane copper transporting P-type ATPase known as ATP7B [9]. The WD gene, *ATP7B*, is located on chromosome 13q14.3 and contains 20 introns and 21 exons, with a genomic length of 80kb [3, 10]. WD is thought to be a monogenic disorder with *ATP7B* as the only causative gene [3, 10], though other genetic and extragenetic factors contribute to modify the phenotype. There are now more than 600 known pathogenic variants of the *ATP7B* gene.

The ATP7B protein is responsible for the transport of copper across cellular membranes in cells where it is expressed [11]. ATP7B contains 1465 amino acids and the following functional domains: six copper binding domains, a phosphatase domain (A-domain), phosphorylation domain (P-domain, amino acid residues 971-1035), nucleotide-binding domain (N-domain, amino acid residues 1240-1291), and M-domain, which is comprised of eight hydrophobic transmembrane ion channels [12-14]. In hepatocytes (primary liver cells) where this protein is mostly expressed, ATP7B functions to transport copper into bile for excretion and incorporate copper into the ceruloplasmin peptide for secretion from the cell into the bloodstream.

Animal models of WD such as the Long-Evans Cinnamon rat that has a spontaneous deletion in the 3' terminal region of ATP7B [15] and ATP7B knockout mice that lack functional ATP7B [16, 17] show no ATP7B expression in the liver and have the typical biochemical and physiological alterations of dysfunctional biliary copper excretion with subsequent hepatic copper accumulation and decreased copper incorporation into ceruloplasmin that is found in human WD [3]. The *ATP7B* gene is most prominently expressed in the liver, and in particular in hepatocytes [10, 18], but is also expressed in smaller amounts in the brain, lung, kidney, mammary glands and placenta [19, 20]. Thus, introducing healthy hepatocytes with functional ATP7B through liver transplantation, cell transplant or by gene therapy corrects the disorder.

3. Liver Transplantation

One of the first liver transplants (LT) performed by Thomas Strazl in the USA in the early 1960s was for a patient with WD and acute liver failure (ALF) [21]. Transplanting this WD patient with a normal liver restored copper metabolism and cured the disease. Since then more than 500 transplants have been performed in the United States for [22] and in France 121 patients were transplanted for WD between 1985 and 2009 [23]. Liver transplantation allows for replacement of the defective or absent ATP7B transporter mainly expressed in hepatocytes. Transplantation in WD is life saving and curative by restoring biliary copper excretion and overall liver function. When liver transplants were first performed for WD, it was uncertain whether the removal of toxic copper from extrahepatic sites would occur. Evidence for the removal of extrahepatic copper by liver transplant alone comes from data on transplanted patients where there was resolution of Kayser-Fleischer rings (these disappeared with time) and improved neurologic function in most WD patients following liver transplantation [24]. Excellent outcomes and long-term survival are seen in LT patients with WD [22, 23, 25-28]. In an average period of 6 months following transplantation, there is normalization of parameters of copper metabolism, including serum ceruloplasmin, serum copper and urinary copper excretion. These findings indicate that restoring the defect within the liver by LT provides a cure, however, lifelong immunosuppressive medication

is necessary following LT to maintain graft viability. Furthermore, there is the morbidity and mortality associated with the surgery and complications from immunosuppressive therapy, including infection and other drug induced side effects.

4. How Much Liver Replacement Is Needed to Change the Phenotype ?

The knowledge that liver replacement by LT can restore copper metabolism begs the question of what percent of a normal functioning liver is required to prevent liver damage from copper in a patient with WD? Success of living donor LT (LDLT) using grafts that are heterozygous for WD and the use of auxiliary liver grafts (addition of a part of a liver to the patient while leaving their native liver intact) provide evidence that only a percent of normal ATP7B expression or a fraction of the total liver mass is needed to restore copper homeostasis. Heterozygosity for the WD gene mutation is associated with abnormal copper parameters in 28-35% of subjects. Expression of ATP7B is less in heterozygotes than in unaffected patients without the WD mutation, but there is adequate expression to not produce a clinically significant phenotype [29]. As most living donors are relatives, there has been interest in the safety of heterozygous donors. Data on LDLT for WD has demonstrated that the use of a living donor who is a heterozygote carrier for WD is safe and provides effective function in the recipient with WD [27, 30-32].

The notion that replacing abnormal hepatocytes can reverse the disease process in Wilson Disease led to translational studies in relevant animal models into the potential for hepatocyte transplantation for treating Wilson Disease. Initial work demonstrated that transplanted hepatocytes engraft, function and repopulate the liver extensively [33-35]. In a study by Yoshida et al hepatocytes of new born Long-Evans agouti (LEA) rats (with normal ATP7B function) were transplanted into livers of LEC rats before the onset of hepatic disease [36]. The study showed reduction in copper deposition and fewer deaths from hepatic failure in the animals that received normal functioning liver cells from the LEA rats [36]. Transplanted cells comprised 4-20% of hepatocytes expressing normal messenger RNA transcribed from the Wilson disease gene [36]. A study by Irani et al in the Long Evans Cinnamon (LEC) Rat Model of Wilson disease demonstrated that transplantation of normal hepatocytes resulted in gradual liver repopulation <25% at 6 weeks to 74-100% at 6 months or beyond [37]. Liver repopulation restored biliary copper excretion and reduced hepatic copper levels. In addition, histology was completely normal in LEC rats with extensive liver repopulation compared to untreated animals [37]. A study by Malhi et al in an LEC rat model demonstrated that early transplantation of hepatocytes resulted in increased bile copper excretion, reduced hepatic copper, and liver repopulation with transplanted cells eventually restoring copper homeostasis [15]. These studies suggested that approximately 40% hepatocyte repopulation is required to prevent liver damage from copper and increase ceruloplasmin [38]. Therefore, replacement of only part of the liver mass by hepatocyte transplantation or transduction of only a fraction of hepatocytes with ATP7B by gene therapy could protect against copper toxicity. However, hepatocyte transplantation in humans is limited by the availability of cells as well as the need to administer immunosuppression following cell transplantation.

5. Transducing ATP7B to Correct the WD Phenotype

Early studies demonstrated that hepatic abnormalities due to mutation of the *ATP7b* gene could be reversed by gene transfer in animal models. A study by Meng et al. in the LEC WD rat model demonstrated that the introduction of the human *ATP7B* transgene into fertilized oocytes of these animals [39] restored ceruloplasmin biosynthesis and biliary copper excretion. The data also showed that human ATP7B compensated for endogenous ATP7B protein deficiency in LEC rats [39]. This demonstrated that effective gene delivery could potentially result in a cure for WD, and development of appropriate gene delivery vectors that could target liver cells was needed.

Gene therapy involves the use of a "vector" to deliver DNA for expression in cells, and for WD the target is the primary liver cell or hepatocyte. The liver provides an excellent target for gene therapy as it is a well vascularized organ facilitating adequate delivery of viral vector, and there are some naturally occurring viral vectors with an affinity for uptake by primary liver cells. Integrating vectors incorporate their DNA permanently into the host chromosomes so that when cells replicate, the DNA will replicate with the chromosome. Concerns regarding the use of these vectors are the risk of insertional mutagenesis and immune responses directed against the expressed transgenes. Using episomal vectors where the DNA is not integrated into the host chromosome has the advantage of not risking disrupting essential host genes which may cause oncogenesis. Early gene therapy approaches using first generation adenoviral vectors were able to transduce the liver with ATP7B, but expression was lost due to lack of integration and the host immune response [40]. Thus gene expression that was adenovirus mediated initially achieved only a transient effect in improving copper homeostasis [41, 42]. Lentiviral vectors with *ATP7B* constructs that integrated into the host genome had a longer duration of expression but lacked liver tissue specificity and did not completely rescue liver abnormalities [43]. These studies did, however, provide evidence that gene therapy could be curative for WD if sufficient transgene expression of ATP7B in the liver can be achieved and maintained long-term.

An adeno-associated virus (AAV) is a nonenveloped virus that is replication-defective, does not cause human disease and induces only a very mild immune response. In the liver the AAV-induced immune response has been blunted by inhibition of expression of toll-like receptor 9 [44]. A big advantage of AAV vectors is the that they can target dividing and nondividing cells with a high efficiency [45]. More recently gene therapy using adeno associated viral vectors (AAV) has shown promising results in hemophilia B patients. It demonstrated that the human liver is permissive to transduction by AAV8 vectors and showed sustained expression of the therapeutic transgene for more than 5 years after a single administration of the AAV8 vector construct [46]. Although the hepatic transduction by AAV8 vectors shows promise for its potential use in the treatment of WD, it is uncertain whether the dosage of AAV8 vector required for correction of hemophilia will apply to Wilson disease. A study by Murillo et al demonstrated that administration of an AAV8 vector designed for expressing ATP7B under control of a liver specific promotor allowed for full restoration of copper homeostasis and provided a lasting therapeutic effect with correction of copper metabolism in a murine model of WD [47]. The study also demonstrated that a moderate dose of the AAV8 vector that is potentially feasible for use in humans, protected WD mice from copper toxicity and normalized biochemical and histological parameters [47]. Immunohistochemistry data supported that protection against toxicity was obtained with transduction below 100% suggesting that neighboring cells may be protected by hepatocytes

expressing ATP7B, as was suggested from data in previous studies using transplanted hepatocytes [15, 36, 37]. More studies will be needed to learn about levels of expression and administration under different conditions that might affect cell turnover. Further studies using truncated ATP7B proteins will also be important as the size of the *ATP7B* genome surpasses the optimal size for AAV packaging. This limits the use of large gene promotors or regulatory sequences. Another more recent study by Murillo et al identified a functional truncated version of the ATP7B protein that would allow introduction of additional regulatory elements as well as the therapeutic AAV vector genome [48].

6. The Use of Viral Vectors in Gene Therapy for Inherited Genetic Diseases

Several studies have assessed the use of viral vectors for human gene therapy for diseases such as severe combined variable immunodeficiency, hemophilia B, beta-thalassemia and a number of other inherited genetic and metabolic diseases. In 2011, Nathwani et al used the adeno-associated virus to express the human factor IX transgene to treat hemophilia B. Six patients were enrolled, and received varying doses of the adeno-associated virus that had the codon–optimized human factor IX transgene. The patients were not given immunosuppressive therapy with the administration of the adeno-associated virus. Results showed that there was an increase in the factor IX expression with peak expression at 8-12% of normal levels. Two of the participants however showed T-cell mediated injury of the transduced hepatocytes by the adeno-associated virus. These two patients required prednisolone but had resolution of the hepatocyte injury [49]. In a subsequent study, they evaluated the sustainability of the treatment and long-term side effects in 10 patients (6 of these patients were from the mentioned original study). Median follow up time was 3.2 years and results showed a sustained increase of factor IX levels from baseline, and no long term side effects. Some initially did have mild elevations in their liver enzymes and required prednisolone but had resolution within a median of 5 days on steroid treatment [46].

Another study demonstrating the use of viral vectors as a mode for gene therapy utilized the lentivirus for treatment of beta thalassemia. Recently, in a phase 1 and 2 study, 22 patients with transfusion dependent beta thalassemia were treated with lentiglobin BB305, a recombinant lentivirus that encodes hemoglobin A. Results showed a significant increase in hemoglobin A from 3.4 to 10 per deciliter and an overall hemoglobin increase from 8.2-13.7 per deciliter. There was a decrease in transfusion volume overall in 12 of the patients. Unlike the Natawani study, the patients enrolled in this study received myeloablative busulfan prior to treatment with the recombinant lentivirus. Side effects were similar to that of autologous stem cell transplantation with no long-term side effects seen up to approximately 3 years [50].

7. Limitations to Use of Viral Vectors for Gene Therapy

Despite the successes seen in these studies, there are still numerous barriers in the development and use of viral vectors as treatment for inherited genetic diseases. In 1999, during a phase I clinical trial, the use of adenovirus for the treatment of ornithine carbomyltransferase deficiency lead to the death of a patient [51]. Following that, in 2000, was the first successful study treating severe common variable immunodeficiency disorder with gene therapy via a viral vector in 9 patients. Unfortunately, four of the eleven patients afterwards developed a leukemia related illness [52].

Safety due to the induction of an immune response after treatment is a concern. As discussed in the Natawani and Thompson study, there was evidence of inflammatory hepatitis in several individuals. The variable outcomes in these studies illustrate that the interaction between the vector and the human immune system can sometimes lead to unexpected adverse consequences, which can potentially be severe or even fatal. The innate and adaptive immune response to the viral vector itself can lead to a severe inflammatory response, even when the vector has been stripped of all of their viral genes.

Another limitation to gene therapy can be the difficulty in targeted delivery of the virus to particular organs, even when the vector is directly injected into the organ of interest. As noted above, here the liver may be advantaged compared to other organs due to some already available vectors that target hepatocytes. However there can be a shortened viability of the virus due to neutralizing antibodies that have been pre-formed when the individual has been previously exposed to the wild type virus [45]. This can theoretically result in a sub-therapeutic dose of the vector being administered to treat the disease. In addition to issues related to targeting are concerns about the longevity of expression of non-integrating transgene carried by the viral vectors. This may arise from cell injury with increased cell turnover or by the natural turnover of cells of the tissue of interest. In this instance re-administration of the viral vector is required to sustain expression. However, development of neutralizing antibodies as noted above may limit re-administration. Various strategies are being developed to deal with this problem, including use of different serotype vector, artificial vectors thought not to generate immunity or immune modulation of the host. This remains an important area of study, and would apply to human trials for WD.

For integrating vectors, there also exists the theoretical potential for insertion of the gene into the patient's germline, which could impact fetal development. Furthermore, the technique is limited by the finite capacity of the virus for accommodating large transgenes [53]. As noted above, for WD there are options for using truncated ATP7B constructs that may still preserve appropriate function.

8. Gene Repair with CRISPR-Cas9 (Clustered Regularly Interspaced Palindromic Repeats)

Another form of gene therapy is the gene repair with the CRISPR-Cas9 system. CRISPR is a segment of DNA that is a short repetition of base sequences in the genome. It works in conjunction with the cas9 protein as an adaptive bacterial immune system. Cas9 is an endonuclease that cleaves the foreign DNA with the guidance of the CRISPR. The cleaved fragments of foreign DNA are then incorporated into CRISPR regions. When the virus attacks again, the CRISPR arrays allow the bacteria to recognize the virus. The RNA transcribed from the CRISPR array, crRNA, acts as a guide for the cas9 protein to recognize and cleave the viral DNA [54]. This regulatory mechanism has gained great interest with its potential use in treatment of inherited genetic diseases. The target RNA can be synthesized with a short guide that binds to the desired DNA sequence and cas9 enzyme. Once bound, the targeted DNA sequence can be cleaved and genetic sequences can be added or removed from the genome [55]. Thus far, this mechanism has been shown to be successful in repair of the CFTR gene in the intestinal stem cells of cystic fibrosis patients as well as hematopoietic cells obtained from patients with X-linked chronic

granulomatous disease [56, 57]. There have been promising results in targeting the MYBPC3 gene in hypertrophic cardiomyopathy in viable human embryos [58].

The first human trial with the use of CRISPR-cas9 is underway in the treatment of sickle cell disease. Although there is significant excitement regarding the potential treatments this method can provide for many inherited genetic diseases (including Wilson disease,) there are limitations. The CRISPR-cas9 system has limited ability to treat genetic diseases that are not monogenic and involve multiple mutations. In addition, ex-vivo studies have had a high frequency of off-target effects, and, as with viral vectors, there can be possible immune repercussions to the administration of the cas9 protein, which is a bacterial enzyme. The possibility of gene repair for WD is very exciting, however the large number of mutations of the gene and the need to test these individualized treatments on multiple patients to determine safety and efficacy suggest that this strategy for now may be limiting, and that gene introduction may be the first and more practical step towards curing WD.

9. Conclusion

There are multiple therapeutic approaches to WD, but gene therapy offers the first real “cure” for this disease. While viral mediated gene transfer for the treatment of Wilson disease has not yet been studied in humans, trials are likely to proceed in the near future. The current human trials for other genetic diseases highlight key features of an ideal recombinant virus for use in WD, include achieving an appropriate infectious titer, the ability to infect non-dividing cells and target primary hepatocytes, limited activation of immune response to the virus initially administered and the potential for re-administration of the virus with the transgene. Overall long-term safety and efficacy will need to be established, but given the excellent results in pre-clinical trials in animal models of WD, the probability of success is high.

Author Contributions

All authors contributed to the concept, writing and review of the manuscript.

Funding

Funding for MC was provided by a grant from the Wilson Disease Association.

Competing Interests

The authors have declared that no competing interests exist.

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