Molecular Therapy and Gene Therapy for Hurler Syndrome

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Abstract

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disease which leads to systemic disease, including progressive neurodegeneration, mental retardation and death before the age of 10 years. MPS I results from deficiency of α-L-iduronidase (IDUA) and subsequent accumulation of glycosaminoglycans (GAG). IDUA enzyme acitivity is an essential assessment for research and diagnostic testing of MPS I disease. Due to different parameters (reaction time, temperature and substrate concentration) used by different labs, the enzyme levels of a certain sample varied. To solve the inconsistency of IDUA enzyme assays in this field, a standardized protocol of IDUA enzyme assay was established through adjustment by Michaelis-Menten equation (Chaper 1). In clinical practice, MPS I disease is treated by enzyme replacement therapy (ERT) and bone marrow transplantation (BMT). Clinical ERT with intravenous IDUA reverses some aspects of MPS I disease and ameliorates others. However, neurologic benefits are thought to be negligible because the blood-brain barrier (BBB) blocks enzyme from reaching the central nervous system (CNS). To address this question, high-dose IDUA (11.6 mg/kg, once per week, 4 weeks) was administered to adult MPS I mice. IDUA enzyme activity in cortex of injected mice increased to 97% of that in wild type mice ($p<0.01$). GAG levels in cortex were reduced by 63% of that from untreated MPS I mice ($p<0.05$). Water T-maze tests showed that the learning abnormality in MPS I mice was surprisingly reduced ($p<0.0001$). These results demonstrated the efficacy of high dose ERT in treating neurological diseases in MPS I mice (Chapter 2). Previous study in our lab showed that a single administration of lentiviral vector in neonatal MPS I mice can achieve significant metabolic correction and neurological improvements. To further improve the efficacy of lentiviral gene therapy, a total of 9 constructs were designed by codon optimization, and different combination of promoters and enhancers. The transgene expression of these 10 constructs was compared after transfection into HEK 293FT cells, and 5 constructs with the highest IDUA expression were identified (Chapter 4). These results pave the way for

developing a directly applicable clinical trial of human lentiviral gene therapy for MPS I disease, and also provide evidence for vector design for treating other lysosomal diseases.

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Chapter 1

Background and Significance

1. Overview

The mucopolysaccharidoses (MPSs) are a group of inherited metabolic disorders, which belong to the large family of more than 40 identified lysosomal diseases (1). MPSs are caused by mutations in genes encoding lysosomal enzymes, which can degrade glycosaminoglycans (GAGs, formerly called mucopolysaccharides), long chain of sugar carbohydrates. Based on the types of deficient enzyme and accumulated GAGs, 11 types and subtypes of MPS diseases have been distinguished.

Deficiency of these enzymes can lead to subsequent lysosomal accumulation of GAG storage materials (2-5). This causes progressive tissue damage, and thereby severe dysfunction of various organs, including heart, lung, bone, joint, and central nervous system (CNS). The major symptoms of MPS diseases include cardiopulmonary disease, growth delay, skeletal dysplasias, organomegaly and shortened lifespan. Moreover, patients with severe MPS I, II, III, and VII exhibit progressive neurodegeneration and mental retardation.

Current available therapies for treating the MPS diseases include hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT). However, HSCT has a high rate of mortality (10-15%) and severe morbidity. ERT is of limited use due to the need for frequent, life long, and expensive (>$200,000 per year) treatments. Further, ERT has negligible neurological improvements because the blood-brain-barrier (BBB) prevents the intravenously infused enzyme from entering the brain.

1. Description of MPS diseases

2.1 MPS I

MPS I is associated with an incidence of approximately 1 out of 100,000 and results from mutations in gene encoding the lysosomal enzyme α-L-iduronidase (IDUA) (5-7). Deficiency of IDUA gives rise to progressive lysosomal

accumulation of heparan and dermatan sulfate in tissues (5). The symptoms of MPS I start to manifest in the childhood. Based on the severity of symptoms, MPS I can be divided into three subtypes, from severe (Hurler syndrome) to intermediate (Hurler-Scheie syndrome) to mild (Scheie syndrome) (5, 6). Patients with Scheie or Hurler-Scheie diseases have symptoms including growth delay, aortic valvular disease, skeletal dysplasias, corneal clouding and joint stiffness (5). In addition to more severe forms of these symptoms, patients with Hurler syndrome also have growth delay, hepatosplenomegaly, unique facial features, hydrocephalus, mental retardation and neurodegeneration. Moreover, Hurler syndrome patients usually die before the age of 10 years old. ERT with 0.58 mg/kg of laronidase (Aldurazyme) per week has been available for patients and can treat non-neurological symptoms of MPS I patients. Also, HSCT has demonstrated its efficacy in treating some aspects of Hurler syndrome (7).

2.2 MPS II

MPS II (Hunter syndrome) is caused by deficiency of the enzyme iduronate-2-sulfatase (IDS) and subsequent lysosomal accumulation of heparan and dermatan sulfate (5). MPS II has an X-linked recessive inheritance, with an incidence of approximately 1 out of 150,000 (8, 9).

The initial symptoms of MPS II include ear infections, abdominal and hernias that are quite common to babies, making it more difficult for early diagnosis. Within the first 2 years, most MPS II patients begin to exhibit growth delay, hearing loss, coarse facial features, cardiovascular problems, respiratory problems, and neurocognitive disabilities (10-12). Similarly, MPS II can be divided into two subtypes based on the severity of symptoms: mild and severe. The severe form is usually associated with neurodegeneration and death within less than 15 years, while some patients with the mild form can survive up to their 50s or 60s. ERT with idursulfase has been shown to provide MPS II patients metabolic correction.

2.3 MPS III

MPS III (Sanfilippo syndrome) is a rare autosomal recessive lysosomal disease, which is caused by deficiency in one of the enzymes degrading GAG heparan sulfate. The incidence of MPS III is approximately 1 out of 70,000. MPS III can be divided into 4 subtypes based on deficiency of different enzymes: Sanfilippo syndrome types A, B, C, and D (5). The 4 different enzymes responsible for degradation of GAGs are listed as followed: heparan sulfate sulfamidase (SGSH), α-N-acetylglucosaminidase (NAGLU), N-acetylglucosamine 6-sulfatase (GNS) and heparan sulfate acetyl-CoA: α-glucosaminide N-acetyltransferase (HGSNAT). In spite of the differences in nature of enzymes, the clinical manifestations of the 4 subtypes are almost indistinguishable. Among all MPS diseases, MPS III has the mildest physical symptoms, only including hearing loss, coarse facial features, heptatomegaly and umbilical hemia (13, 14). Also, patients with MPS III can usually live into adolescence or early adulthood. However, MPS III disease is usually associated with severe neurological manifestations. Currently, there is no available ERT for MPS III disease, and the treatment remains largely supportive.

2.4 MPS IV

The incidence of MPS IV (Morquio syndrome) is estimated to be 1 out of 200,000 to 300,000. MPS IV can be divided into two subtypes based on the deficient enzymes galactose 6-sulfate sulfatase (Type A) and beta-galactosidase (Type B), which are responsible for degrading the keratan sulfate sugar chain. Clinical manifestations of these two subtypes are similar, however, Type B is usually associated with milder symptoms. Patients with MPS IV usually appear healthy at birth, which makes it difficult for early diagnosis. The symptoms of MPS IV include abnormal heart and skeletal development, hypermobile joints, bell-shaped chest, spinal cord compression and short stature. Unlike other MPS diseases, MPS IV does not affect intelligence and neurocognitive abilities. Patients with MPS IV usually can live into late childhood, and some with mild

disease may survive into their 70s. Currently, ERT with elosulfase alfa (VIMIZIM) has been available for MPS IV patients.

2.5 MPS VI

MPS VI (Maroteaux-Lamy syndrome) results from deficiency of arylsulfatase B (ARSB) (15, 16), and subsequent lysosomal accumulation of dermatan sulfate and sulfated oligosaccharides. MPS VI has a wide spectrum of severity, which is based on the amount of residual ARSB activity. MPS VI patients share similar symptoms with Hurler syndrome, but do not have abnormal intellectual development. The onset of MPS VI disease is early in life, with one of the initial symptoms being a prolonged period of learning how to walk. Most patients have severe skeletal abnormalities, including short stature, joint stiffness and spinal stenosis. In terms of the lifespan, MPS VI patients typically die due to cardiac malfunction in early adulthood (5). Currently, ERT with galsulfase (Naglazyme) has been available for MPS VI patients and has successfully achieved improvements in growth and joint movements.

2.6 MPS VII

MPS VII (Sly syndrome) is an autosomal recessive inherited disease due to deficiency of ß-glucuronidase (GUSB) activity and subsequent lysosomal accumulation of heparan sulfate, dermatan sulfate, and chondroitin sulfate. The incidence of MPS VII is approximately 1 out of 250,000. MPS VII shares similar features with MPS I disease. Mild patients exhibit hepatosplenomegaly and skeletal dysplasias without intellectual impairments, and can even survive into their 50s (5). Patients with more severe MPS VII disease usually have hepatosplenomegaly, corneal clouding, dysostosis multiplex, mental retardation, coarse facial features, hearing loss and early death.

2.7 MPS IX

MPS IX (Natowicz syndrome) is caused by mutations in the gene encoding hyaluronidase (17, 18). The deficiency of active hyaluronidase leads to lysosomal accumulation of hyaluronan. Up to 2001, only one MPS IX patient has been reported. The symptoms of MPS IX include painful swelling of the masses, growth delay, and coarse facial features. No neurological impairments have been identified with MPS IX disease.

2. Molecular biology of MPS diseases

GAGs are long unbranched polysaccharides consisting of repeating disaccharide units of an acidic sugar (uronic or iduronic acid) and an N-acetylated amino sugar. Based on type of sugars and linkeage, number and location of sulfate groups, GAGs can be classified into several types: hyaluronic acid/hyaluronan, chondroitin sulfate, keratan sulfate, dermatan sulfate, heparin and heparan sulfate. GAGs are essential components of extracellular matrix and can provide tissues with resilence under pressure. For degradation, extraceullar GAGs are endocytosized into cells, transported to lysosomes and broken down by specific enzymes. Deficiency of enzyme involved in GAG degradation leads to lysosomal diseases (see details in Figure 1).

Lysosomal enzyme is synthesized in the endoplasmic reticulum (ER), and mannose and mannose 6-phosphate (M6P) residues are added in the Golgi apparatus. Approximately 90 % of enzyme is targeted to lysosome by mannose 6-phosphate receptors (M6PRs), while the rest is secreted and endocytosed mainly by ubiquitously distributed M6PRs and mannose receptors (MRs) on macrophages (19, 20). Deficiency of enzyme adding M6P residues to lysosomal enzyme leads to inclusion-cell (I-cell) disease. Without M6P residues, lysosomal enzyme cannot be targeted to lysosomes, and are instead secreted out of cells. Therefore, lysosome cannot function normally to degrade sustances (e.g. oligosaccharides, lipids, and glycosaminoglycans), resulting in characteristic ‘inclusion cells’. The I-cell phenotype is also observed when IDUA is

overexpressed (up tp 250 fold of normal levels) by retroviral vector transduction into cells. It may result from saturation of M6P mediated lysosome-targeting pathway by high level of IDUA, which leads to reduced amount of other enzymes delivered to lysosome(21). These results suggest that when treating lysosomal diseases with gene therapy, transgene expression should be controlled within certain limits because extremely high levels of one enzyme may induce the 'I-cell' like phenotype.

Lysosomal enzyme delivery across the BBB in neonatal mice is through M6PRs, which are down-regulated after first two weeks of life in mice (22). Therefore, it is extremely difficult to treat the CNS pathology of MPS diseases after the neonatal period. However, it has been shown in MPS VII that when mannose and M6P residues were eliminated from the lysosomal enzyme, plasma half-life was dramatically prolonged from 11 minutes to 18.5 hours (23). Also, enzyme diffusion was more efficient, manifested by the wide spread GAG storage reduction in multiple organs including brain (23). Although the mechanism of how these enzymes targeted to the lysosome remains unclear, the therapeutic benefits are significant and not exclusive to the CNS. Also, when blocking the MR-mediated clearance system by knocking out MR, or by infusing competitive inhibitors of mannose, wider distribution and enhanced clearance of storage in other difficult-to-reach sites for enzyme, e.g. bone and retina, was observed (20). Although neither knocking out ubiquitously expressed MR nor using toxic MR inhibitors is feasible in human patients, making mutant enzyme that cannot be bound by MR might be a good choice to achieve better enzyme diffusion.

3. Neuropathology of MPS diseases

Patients with severe MPS I, II, III, and VII exhibit neurological impairments. Currently, the mechanism of neurological disease is not well understood. Accumulation of GM2 and GM3 gangliosides in brain has been identified (24-28), suggesting their potential roles in neurological pathogenesis.

GM2 and GM3 gangliosides have been shown to be upregulated in several types of MPS diseases (29, 30). Although the mechanism how GM2 and GM3 contribute to neuropathogenesis is not elucidated, it has been related with altered synaptic connectivity and reinitiation of dendrite growth (31). In addition, overexpression of GM3 synthase can lead to reactive oxygen species (ROS) production and even death of neuronal cells (32). Also, it has been suggested that cell signaling, apoptosis, astrogliosis inflammation and oxidative damage may be involved (33). Astrogliosis were identified in brain of MPS III patients, which was consistent with the fact that inflammatory pathways were upregulated by inhibiting macrophage migration inhibitor factor (Mif) (35, 36). More interestingly, there is evidence that some signaling pathways may contribute to the neuropathogenesis of MPSs. Fibroblast growth factor 2 (FGF-2) is a cytokine involved in tissue neurogenesis and morphogenesis (37), which can also promote neuronal survival (38). When incubated with FGF-2, multipotent adult progenitor cells (MAPCs) can differentiate into neurons and glia cells (39). Abnormal heparan sulfate molecules have been shown to interfere with interactions between FGF-2, FGF receptors, and heparan sulfate molecules. Furthermore, these abnormal interactions can reduce proliferation and survival of MAPCs (34). In light of these facts, the perturbation of heparan sulfate and FGF interaction may play a role in neuropathogenesis of MPS diseases. In addition, it has been shown that apoptosis may contribute to the neuropathology of MPS IIIB (35). Collectively, the mechanisms of neurological impairments in MPS I, II, III, and VII have not been elucidated, and remain a heated topic in this field.

4. Current Therapies

Treatment of MPSs is mainly based on the phenomenon of cross-correction: lysosomal enzyme infused or secreted from transduced cells reaches other tissues through blood circulation (40). This is possible because approximately 10% of the lysosomal enzymes produced in a cell will be secreted and recaptured by other cells. Circulating lysosomal enzymes are mainly taken up by

cells via M6PRs and/or MRs (41). Therefore, only a relatively small number of cells expressing the lysosomal enzyme are sufficient to correct many other cells (42). Besides the efficiency of receptor-mediated endocytosis, cross-correction depends on the efficiency of secretion of lysosomal enzymes from the cells in which they are produced (43).

Although extensive studies on developing novel therapies for MPS diseases have been conducted, only a couple of therapeutic options are available to patients. Currently, there are HSCT and ERT for treating MPS diseases. Further, gene therapies or small molecule therapies are under development and will potentially be applied in clinical trials.

4.1 Enzyme Replacement Therapy

ERT is intravenously administering human recombinant lysosomal enzyme into patients. Currently, ERT is available for only three MPS types: I, II and VI (44, 45). It seems that the results of ERT depend on the severity of disease at the onset of treatment. Generally, following treatment with ERT, patients exhibit decrease in urine GAG levels, resolution of hepatoslenomegaly, and cardiopulmonary improvements as shown by 6 minute walk test (6MWT) within the first 6 months of treatment (46).

However, more than 50% of patients treated with ERT develop antibodies to the recombinant proteins. ERT is of limited use due to the need for frequent, life long, and expensive (>$200,000 per year) treatments. Further, ERT has negligible neurological improvements because the BBB prevents the intravenously infused enzyme from entering the brain (47-52).

In terms of neurological improvements, intrathecal ERT has provided promising results, especially for treating spinal cord compression (53, 54). Following intrathecal infusion of IDUA into MPS I dogs, high levels of IDUA activity and

GAG reduction have been observed in the spinal, cervical, and lumbar meninges. Furthermore, in MPS I dogs, infusion of IDUA into the cisterna magna resulted in reduction of storage material in glia, perivascular cells, and neurons (49, 55). However, these studies were limited to animal, adult or non-neuronopathic pediatric patients. In light of this, it is essential to investigate whether intrathecal ERT can be safely applied in MPS children.

Intrathecal ERT has been conducted in MPS I dogs and successfully reduced GAGs in the CNS (55, 57).The rationale of intrathecal ERT is that in the cerebrospinal fluid (CSF), a small amount of the lysosomal enzyme can represent large concentration gradients when compared with the affinity constants for the enzyme receptors on neurons, glia and other cells. In light of the very large concentration gradients, a tiny amount of enzyme can penetrate the ependymal layer and result in sufficient uptake by neurons and glia at the end sites.

4.2 Hematopoietic Stem Cell Transplantation

It has been shown that MPS diseases can be treated by HSCT. The underlying mechanism might be that donor-derived HSCs differentiate into macrophages in spleen and lung, Kupffer cells in liver, and microglial cells in brain (57, 58). The donor-derived cells then can provide enzyme to deficient cells via metabolic cross-correction. Untill now, there are no randomized controlled trials (RCT) with BMT or HSCT for MPS diseases, therefore, our discussion here will be mainly based on anecdotal descriptions. In 1980, the first BMT for the treatment of MPS disease was performed on a child with MPS I (59). Since then, approximately 500 children have received transplantation for MPS I, II, III, IV, VI, and VII (60). Stabilization of the disease or deceleration of the disease progress was successfully achieved in patients. However, it has been suggested that some MPS diseases, like MPS III, are generally significantly less responsive to BMT or HSCT (61). Moreover, good timing is essential for achieving significant

improvements in patients, effective treatment for MPS I disease can be achieved when transplantation was performed in patients below 2.5 years old of age (62).

Prior to transplantation, patients usually receive treatment with cyclophosphamide and either busulphan or total body irradiation (63-65) to create space for engraftment. In Hurler syndrome, it seems that success of engraftment depends on cell dose (65, 66-68). In addition to graft rejection, which decreases the rate of long term survival following treatment, patients are also subject to HSCT-related mortality due to graft-versus-host disease (GVHD), sepsis, or pneumonia. The rate of GVHD was reported to be between 35% and 50% while the mortality rate due to transplant complications is between 11% and 45% (63, 67). As relevant procedures have been improved and wide use of cord blood, risk for these complications has gradually decreased.

Several months after transplantation, there are improvements in spleen, liver, lung and upper respiratory system. Also, improvements in hydrocephalus and neurocognitive disability have been reported in many patients after transplantation. Moreover, symptoms in growth, corneal clouding and development of the musculoskeletal system were at least partially reduced, and improvements in joints and hearing were observed in many patients. Also, although cardiac valve deformities still persist, there is a significant decrease in cardiac hypertrophy (60). More importantly, after transplantation, overall survival (OVS) rate of Hurler syndrome patient ranges from 50 to 85%, and some patients can live into their 30s. The age of treatment and severity of diseases is important for neurological outcomes of transplantation (63, 66, 68). Earlier treatment is more beneficial for reduction in GAG and secondary storage materials GM2 and GM3 ganglioside within brain (27). However, most patients still continue to display low IQ and neurocognitive disability after transplantation (69, 70).

4.3 Small molecules

4.3.1 Substrate reduction therapy

Substrate reduction therapy (SRT) offers a novel strategy for treating MPS diseases. Failure of relevant metabolic pathways leads to accumulation of the substrate (GAG) and MPS diseases. SRT solves this problem by reducing the level of substrate to a point where residual degradative activity is sufficient to prevent substrate accumulation. It was naturally expected that a decrease in efficiency of GAG synthesis might lead to deceleration of the storage process and improvements in cellular functions (71). In the optimal condition, SRT could restore the balance between GAG synthesis and degradation. In fact, SRT has been introduced as a therapeutic option for treating other lysosomal diseases, for instance, Gaucher disease (72) or Niemann-Pick C disease (73). Miglustat (N-butyldeoxynojirimycin), the inhibitor of glucosylceramide synthase, has been applied as a drug for the aforementioned diseases. Since it can decelerate glycosphingolipids synthesis, miglustat is expected to be effective in reducing glycosphingolipids storage. In spite of accumulatiing evidence that positive effects of miglustat treatment in clinical trials (74, 75), its efficacy in Gaucher disease remains to be well defined (76). As to MPS diseases, there are various ways to inhibit GAG synthesis, with the most straightforward method using specific chemical inhibitors of GAG synthesis. However, identification of non-toxic inhibitors of enzymes involved in GAG synthesis is extremely difficult. Therefore, a search for indirect inhibitors of GAG synthesis has been conducted (77). Rhodamine B, a staining fluorescent dye, has been shown to decelerate GAG synthesis indirectly of MPS IIIA and MPS VI cells *in vitro* (78).

Epidermal growth factor (EGF), when bound by its specific receptor, can initiate a signal transduction pathway leading to expression of certain genes that encode enzymes responsible for GAG synthesis. Genistein, a natural isoflavone, can interfere with this pathway by inhibiting phosphorylation of EGFR (79). Therefore, genistein can significantly inhibit GAG synthesis of MPS I, MPS II, MPS IIIA and MPS IIIB fibroblasts *in vitro* (79-82). Silencing the expression of genes encoding

GAG synthetases is another option to reduce the efficiency of GAG production. Currently, several strategies employing the RNA interference (RNAi) mechanism have been developed. For instance, short interfering RNA (siRNA) oligonucleotides were used to reduce mRNA levels of four genes including GALTI, GALTII, XYLT1 and XYLT2, whose products are responsible for GAG synthesis (83). Additionally, short hairpin RNA (shRNA) molecules, targeting EXTL2 and EXTL3 genes, whose expression was also required for GAG synthesis, have been employed.

4.3.2 Enzyme chaperone

Under physiological conditions, chaperones restore the native conformation of misfolded proteins. It has been estimated that approximately 30% of proteins misfold and/or aggregate, and are rapidly degraded (84). In MPS diseases, missense mutations and in-frame deletions may cause polypeptide misfolding, however, they may not or only slightly affect the functionally essential domains of the enzyme. This offers a chance to use pharmacological chaperones to facilitate the stabilization of misfolded proteins and thus treat MPS diseases.

Adding N-(n-nonyl) deoxynojirimycin (NN-DNJ) to fibroblasts from a Gaucher patient resulted in a 2-fold increase in the enzyme activity of glucocerebrosidase (10). Another chaperone, 1-deoxygalactonojirimycin, was added to Fabry fibroblasts with missense mutations. After treatment for over 100 days, there was a remarkable reduction of GB3 storage (85). In GM2-gangliosidosis (Tay–Sachs and Sandhoff disease), the chronic adult forms have approximately 5% of normal enzyme activity, while some unaffected individuals have only 10% of normal levels. Interestingly, almost all disease-associated missense mutations do not affect the active sites but only cause retention and accelerated degradation. By adding hexosaminidase inhibitors to cultured fibroblasts from an adult GM2-gangliosidosis patient, a significant increase in the level of hexosaminidase A could be achieved (86). Collectively, small molecule chaperones can be effective

for treating various lysosomal disorders caused by mutated but yet catalytically active enzymes.

4.4 Gene Therapy

Gene therapy is the most promising strategy for treating MPS diseases because some vectors can transduce or transfect a variety of tissues after a single systemic administration (87). Further, gene therapy offers the possibility of comprehensive and affordable treatment of all aspects of MPS diseases. However, on the one hand, viral vectors have drawbacks including expense and ease of manufacturing (88).On the other hand, non-viral vectors are limited of use by their inability to efficiently enter cells and penetrate the BBB.

Many preclinical studies have been conducted to investigate the efficacy of gene therapy vectors to treat MPS diseases. Both non-viral and viral vectors have been employed in these studies, which include minicircles, Sleeping Beauty (SB) transposons, retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAV). Also, different routes of administration have been investigated including *ex vivo* transduction and *in vivo* infusions through intravenous, intrathecal, intraparenchymal, intracisternal and hydrodynamic injections.

4.4.1 Non-viral methods

Two non-viral gene therapy strategies have been tested in mouse models of MPS diseases. The first is transposons, e.g. the SB transposon system, which can provide sustained transgene expression in mammalian cells (89-91). The SB transposon system is a plasmid-based gene transfer system, which was constructed by engineering of extinct DNA sequences found in salmonid fish (89, 90, 92). The transposon and SB transposase are the main component of the SB system. And the transposon is composed of around 230 bp inverted repeat/direct repeat sequences (IR/DRs) and expression cassette encoding the gene of interest (GOI) with promoter sequence. When delivered into host cells, with the

help of IR/DRs, SB transposase can excise the transposon from the plasmid vector. Then, the excised transposon can randomly integrate into the host genome (88), allowing for stable, long-term transgene expression. Compared with viral vector, the advantages of using the SB transposon system for gene therapy include the cheap expense, ease of use, lessimmunogenicity, low risk of contamination with replication-competent viruses, and large capacity (88). However, although no adverse events have been identified, there is still concern about insertional mutagenesis. The SB gene therapy has been shown to be effective in mouse models of MPS I and MPS VII (93, 94).

Another potential nonviral vector is minicircles, which are circular DNA molecules only containing the transgene expression cassette by removal of most plasmid backbone sequences (95, 96). Minicircles can provide sustained episomal transgene expression, successfully avoiding safety concern about insertional mutagenesis. The disadvantage of minicircles is that episomes are not necessarily carried to the daughter cells after cell divisions. Currently, minicircles have been employed to treat MPS I in mice (97).

However, non-viral vectors cannot effectively transduce cells in the CNS, regardless of the administration routes. In light of this, gene therapy with minicircles cannot treat neurological diseases of MPSs.

4.4.2 Viral gene therapy

4.4.2.1 Retrovirus

Retroviruses are a group of viruses with two identical strands of RNA as genetic materials. The retroviral genome contains three genes, *gag*, *pol*, and *env*, which encode proteins necessary for viral life cycle. These sequences are flanked by long terminal repeats (LTRs) responsible for reverse transcription and integration.

The *pol* gene encodes reverse transcriptase, which can produce a double stranded DNA (dsDNA) copy of the viral RNA genome (98) upon entry into host cells. After converted into dsDNA and transported to the nucleus, the viral DNA can integrate into the host genome with the help of the viral integrase. Transcription and translation of the integrated viral gene sequence by the host cell machinery can lead to production of viral genomes and proteins for completing the viral life cycle. For gene therapy application in MPS diseases, retroviral vectors have been engineered by replacing the *gag, pol* and *env* genes by a gene encoding a therapeutic lysosomal enzyme. After integration of the therapeutic gene into the host genome, long-term transgene expression can be achieved in target cells.

Retroviruses were the first vectors used for gene therapy of MPS diseases (99). Due to safety concern about insertional mutagenesis, self-inactivating retroviruses (SIN-RVs) were thereby designed (100, 101). SIN-RVs achieved lower levels transgene expression and were less efficient in correcting clinical symptoms, however, this can be overcome by increasing the dose. However, retrovirus gene therapy is still with safety concern about insertional mutagenesis. More importantly, it has been suggested that retroviral vectors can yield neurological improvements even though they do not cross the BBB. However, the retroviral vector is limited of use by their inability to transduce non-dividing cells, which is non-ideal for transducing brain tissue.

4.4.2.2 AAV

AAV is a single-stranded DNA (ssDNA) virus, which contains two open reading frames (ORFs), *rep* and *cap*, flanked by inverted terminal repeat sequences (ITRs). *Rep* is responsible for encoding proteins necessary for the viral life cycle. *Cap* encodes VP1, VP2, and VP3, which can assemble to form the viral capsid (102-104). To generate AAV vectors for gene therapy, the rep and cap sequences are replaced by an expression cassette including a promoter and the

gene of interest (GOI). Three plasmids (one carrying the ITRs and the GOI, one carrying *cap* and *rep*, and one carrying helper proteins) are co-transfected into cultured cells. The viral genome is packaged into capsid proteins produced within the transduced cells. Then, AAV virions can be purified from cell lysates with affinity columns, density gradients or ion exchange purification methods (105, 106). AAV serotype 2 (AAV2) was the first serotype applied to create rAAV vectors (107, 108) and widely used in clinical trials worldwide. More interestingly, AAV9 has been shown to be able to cross the BBB and transduce neurons and astrocytes throughout the CNS (109-113). Therapeutic effects with AAV9-mediated gene therapy have been demonstrated in mice (111), cats (110, 114), dogs (115), pigs (116) and non-human primates (117).

AAV has become a powerful option as gene therapy vectors for several reasons. Firstly, AAV is non-pathogenic and can transduce a variety of tissues and cell types including brain, liver, heart, and muscle (116-119). Secondly, AAV can maintain stable transgene expression for up to 18 months in various animal models including mice, dogs, and hamsters. Thirdly, the frequency of AAV integrating events is very low since the viral *rep* gene has been removed (119, 122). The low frequency of integration events reduces the possibility of insertional mutagenesis, making it more appealing than integrating vectors.

However, AAV shares drawbacks of most viral vectors including the high cost of delivery, variable efficiencies of production and storage and immunogenicity. In addition, gender bias has been reported in mice and dogs treated by AAV gene therapy. Approximately 2 to 13 fold higher activities were measured in liver and serum of males compared with females after intravenous administration of AAV serotypes 2, 5, 8 and 9 (123-126). Another big issue in its clinical transition is immune response. After intravenous administration, exposure to AAV vectors can activate $CD8^+$ T-cell responses against the viral capsid in a dose-dependent manner (127-129). Also, neutralizing antibodies to the viral capsid or the

expressed protein may hinder transduction of peripheral organs (130-132). Since AAV9 can transduce antigen-presenting cells (APCs), these vectors can also trigger antigen specific immune responses which lead to elimination of transgene expression (132).

4.4.2.3 Lentivirus

Compared to retroviral vectors, the advantage of the lentiviral vectors is that they can transduce non-dividing cells, rendering them suitable for transducing terminally differentiated cells including neurons (133). However, since lentiviruses integrate semi-randomly into the host genome (134), there is still safety concern about insertional mutagenesis.

For treating MPS diseases, HIV-based lentiviral vectors have been engineered to deliver genes of interest into target cells achieves metabolic correction and even partial amelioration of neurological pathology (133-136). However, since all these studies cannot completely and effectively reduce neuropathology, we need improve the potency of lentiviral vector to treat the CNS pathology. Previous study in our lab has demonstrated that a single administration of lentiviral vector carrying IDUA gene can partially correct MPS I disease in a murine model. However, it was calculated that treatment of an infant would need approximately 40 L of this lentiviral vector. Although through further concentration, it could be feasible to inject this amount into human patients, a more potent lentiviral vector should be developed. Based on codon optimization and different combinations of promoters and enhancers, we have designed 9 more constructs and aim to compare the efficacy of lentiviral gene therapy between these ten constructs.

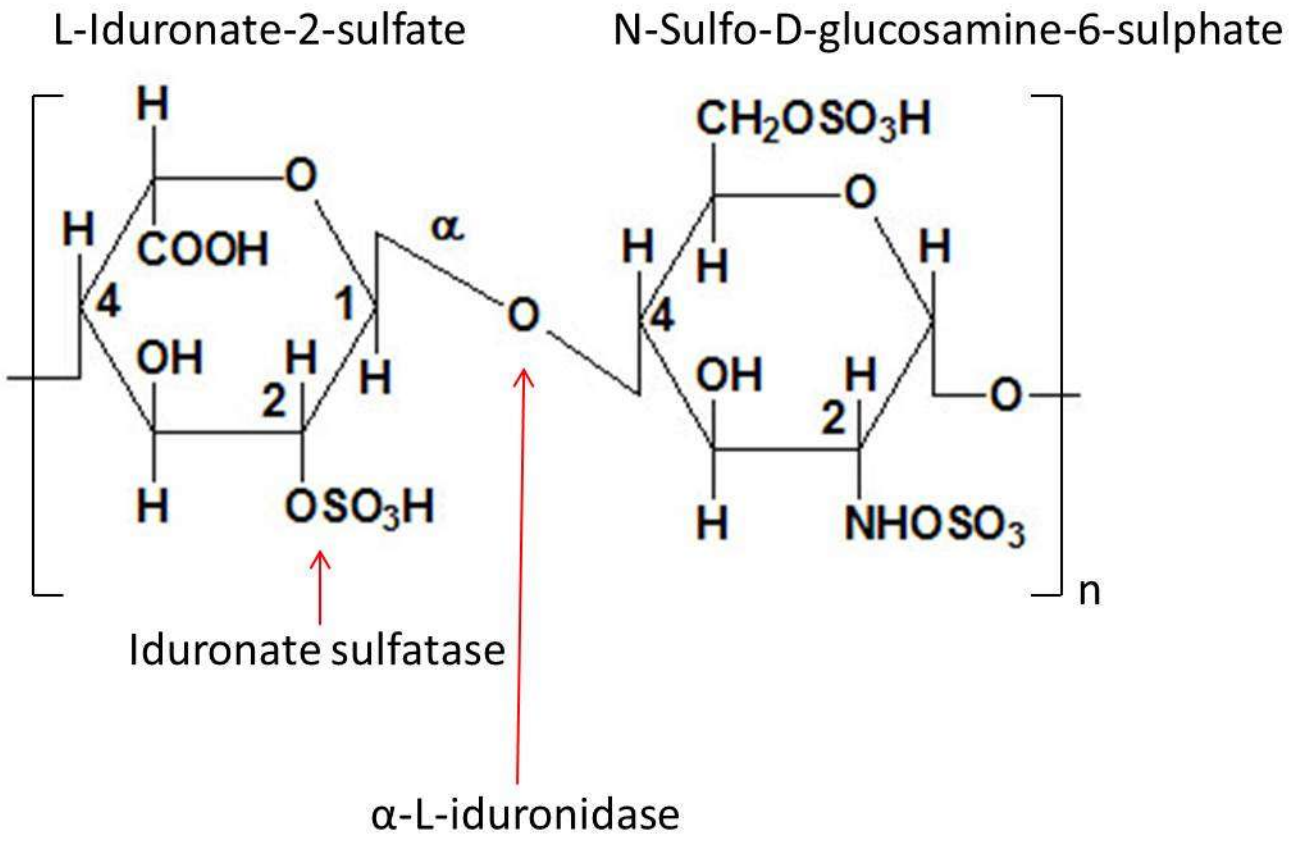

Figure 1. GAG degradation and MPS diseases. The structure of heparan sulfate is illustrated above: repeating units of L-iduronate-2-sulfate and N-Sulfo-D-glucosamine-6-sulphate. Iduronate sulfatase can catalyze breaking down the sulfate group, and its defiency leads to MPS II. After the sulfate group separating from the remaining part, α-L-iduronidase can break down α (1→4) linkeage between two components of each unit, and thus degrade heparan sulfate.

Chapter 2

Standardization of α-L-iduronidase Enzyme Assay with Michaelis-Menten Kinetics

Abstract

Mucopolysaccharidosis type I (MPS I) is a lysosomal disease that leads to progressive neurodegeneration, mental retardation and death before the age of 10 years. MPS I results from deficiency of the lysosomal enzyme α-L-iduronidase (laronidase, IDUA) and subsequent accumulation of glycosaminoglycans (GAG). Currently, an IDUA enzyme assay with the substrate 4-methylumbelliferyl-α-L-iduronide (4-MU iduronide) is employed to measure enzyme activity. However, final substrate concentrations used in the literature vary greatly, from 25 μM to 1425 μM (K_m≈180 μM). In this study, IDUA enzyme assays with 15 different substrate concentrations were conducted. The resulting enzyme values with each substrate concentration were significantly different from each other. Interestingly, these values obey Michaelis-Menten kinetics. Therefore, it is proposed that IDUA enzyme assays should be conducted (1) under substrate saturating conditions (>10 K_m); (2) or with concentrations significantly below substrate saturation, and the resulting values standardized through Michaelis-Menten kinetics.

1. Introduction

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disease, resulting from deficiency of α-L-iduronidase (IDUA, E.C.3.2.1.76) and subsequent accumulation of glycosaminoglycans (GAG). MPS I leads to systemic disease, including progressive neurodegeneration, mental retardation and death before the age of 10 years.

In the efforts to treat MPS I, measurement of IDUA enzyme activity is an essential parameter, and is widely used in the field for research and clinical diagnostic testing, as well as in the regulatory evaluation of new therapies reviewed by US Food and Drug Administration (FDA), European Medicine Agency (EMEA), and other regulating bodies. Currently, an IDUA enzyme assay with 4-methylumbelliferyl-α-L-iduronide (4-MU iduronide) as substrate is in use. The assay follows the release of the 4-MU fluorophore from the substrate as a result of IDUA turnover. In fact, lack of methodological uniformity in the lysosomal enzyme activity assay was recently cited as a concern by an FDA reviewer [1].

Specifically, substrate concentrations used in different labs vary greatly, from 25 μM to 1425 μM [2-15], while the K_m was found to be approximately 180 μM [16]. Accordingly, even the enzyme activity of the same sample within the same lab varies greatly (summarized in Table 1). Importantly, a kinetic reading method was employed in several studies [9-11]. They measured the fluorescence every 10 min for 2 hours and stopped the reaction following a further 4-6 hours. The main concern arises from the most suitable pH for 4-MU fluorescence being 10.4, provided by the stopping buffer (glycine carbonate buffer, pH 10.4). During the kinetic reading, the fluorescence measured may not be accurate without a standardized pH. Therefore, an IDUA enzyme assay protocol with low cost, high accuracy and methodological standardization is desperately needed.

In this study, IDUA enzyme activity of liver from heterozygous mice (*idua*-/+) was measured at different substrate concentrations. The resulting values significantly differ from each other, but fit Michaelis-Menten kinetics well. Therefore, to obtain an accurate velocity (V_{max}), and to make the results universally comparable, it is proposed that IDUA enzyme assays should be conducted either under substrate saturating conditions, or with concentrations significantly below substrate saturation, followed by standardization of the values using the Michaelis-Menten kinetic model.

2. Material and Methods

2.1 Collection of mouse samples

MPS I deficient mice (*idua*-/-) generated by insertion into exon 6 of the 14-exon *idua* gene on the C57BL/6 background, were offsprings of breeder originally from Dr. Elizabeth Neufeld, UCLA. Heterozygotes (*idua*-/+) were genotyped by PCR. All mouse care and handling procedures were in compliance with the rules of the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota. Harvested organs were homogenized using a Brinkman Polytron and permeabilized with 0.1% Trion X-100. Tissue homogenates were diluted with phosphate buffered saline (PBS, 0.01 M, pH 7.4).

2.2 IDUA enzyme assay

IDUA activity was determined by a fluorometric assay using 4-methylumbelliferyl α-L-iduronide (4-MU iduronide, Glycosynth #44076) as the substrate. 4-MU iduronide was diluted with sodium formate buffer (0.4 M, pH 3.5) to different concentrations. Then, 25 µL aliquots of substrate of different concentrations were mixed with 25 µL aliquots of tissue homogenates. The mixture was incubated at 37 °C for 30 min, and 200 µL glycine carbonate buffer (pH 10.4) was added to quench the reaction. IDUA catalyzed the cleavage of the non-fluorescent substrate (4-MU iduronide) into a fluorescent product (4-MU). 4-Methylumbelliferone (4-MU, Sigma #M1381) was used to make the standard

curve. The resulting fluorescence was measured using a Bio-Tek plate reader with excitation at 355 nm and emission at 460 nm. IDUA enzyme activity was expressed in units (nmol converted to product per hour) per mg protein as determined with a Pierce protein assay kit (Fisher # PI22662) . All reactions were run in triplicate.

2.3 Statistical analysis.

For evaluation of differences between samples, post hoc Tukey test was used for comparisons between paired samples, and one-way analysis of variance (ANOVA) for comparisons between three or more samples. For evaluation of the Lineweaver-Burk plot, linear regression analysis was conducted. The linearity of this plot was determined by an adjusted R squared value and the p value. All data analysis was conducted with SAS 9.3 (North Carolina, USA).

3. Results and discussion

According to Michaelis-Menten kinetics [17], below substrate saturation, velocity increases as substrate concentration increases. To test this in the context of the IDUA enzyme assay, liver was harvested from heterozygous mice (*idua*-/+, n=3) and IDUA enzyme activity was measured with 15 different final substrate concentrations (25, 40, 50, 60, 80, 100, 120, 140, 160, 180, 200, 250, 500, 2000 and 4000 μM). Interestingly, the resulting mean activity values varied from 0.5±0.02 to 4.1±0.37 nmol/h/mg. Significant differences were found between enzyme assays using different substrate concentrations. Furthermore, these calculated values obeyed Michaelis-Menten kinetics (Figure 2). After the substrate concentration increased to 2000 μM, the velocity plateaued. From the Michaelis-Menten plot, the V_{max} is approximately 4.5 nmol/h/mg, and K_m is approximately 174.2 μM ($V = V_{max}/2$). This value of K_m was similar to the numbers reported previously [16] ($K_m \approx 180$ μM).

To confirm these values gave a linear fit on a Lineweaver-Burk plot and to obtain accurate V_{max} and K_m values [18], liver was harvested from heterozygous mice (*idua*-/+, n=7) and IDUA enzyme activity was measured for 11 different final substrate concentrations (40, 50, 60, 80, 100, 120, 140, 160, 180, 200 and 250 µM). Significant differences were again found between enzyme assays with different substrate concentrations ($p<0.0001$). A Lineweaver-Burk plot was made by a linear regression analysis, with a slope of 43.313 ($p<0.0001$), intercept of 0.226 ($p<0.0001$) and adjusted R squared value of 0.9829 (Figure 3). Thereby, K_m and V_{max} were determined (K_m = 191 µM, V_{max} = 4.42 nmol/h/mg). This value of K_m was similar to the numbers reported previously [16] (K_m≈180 µM).

Importantly, Michaelis-Menten kinetics only applies to initial velocity. Therefore, one underlying assumption is that substrate concentration is sufficient to remain almost unchanged during the reaction ([S] ≈ [S_0]). Elsewhere, as substrate is consumed, the velocity will decrease. To determine the appropriate incubation time, 25 µL plasma from heterozygous mice (*idua-/+*) was mixed with 25 µL substrate (final [S] = 180 µM). All reactions were divided into 5 groups, and one group of reactions was quenched every 10 min. After the fifth group was quenched, the fluorescence was measured (Figure 4). The resulting curve was linear (R^2=0.9953), indicating that the velocity stays stable through 50 minutes at the substrate concentration of 180 µM.

Therefore, it is proposed to standardize the IDUA enzyme assay through two options. (a) IDUA enzyme assay under substrate saturating conditions to obtain V_{max} directly. (b) IDUA enzyme assay using a final substrate concentration of K_m. To determine V_{max} in this situation, the resulting values are multiplied by 2, as the Michaelis-Menten equation simplifies to $V = \frac{Vmax[S]}{Km+[S]} = \frac{Vmax}{2}$ when [S]=K_m. Alternatively, other concentrations significantly below substrate saturation can be used and adjusted accordingly with the Michaelis-Menten equation: $V = \frac{Vmax[S]}{Km+[S]}$.

Notably, when using non-saturating substrate concentrations, one must ensure that the velocity is relatively stable throughout the whole incubation time. Considering the cost of 4-MU iduronide, the latter option that utilizes lower substrate concentrations would be preferable.

4. Acknowledgements

The authors would like to thank Brenda Koniar for mouse breeding, Peng Liu for help with Figure 2. This work was supported by NIH grant P01HD032652 .

IDUA activity (nmol/h/mg)	Final [S] (μM)	Substrate source	Adjustment	Incubation time (hour)	Reaction temperature(℃)	Ref.
0.8	25	CalBiochem	N	1	22	[2]
2	25	CalBiochem	N	1	22	[3]
0.3	25	Glycosynth	N	1	37	[4]
0.6	25	Glycosynth	N	1	37	[5]
0.8	25	Glycosynth	N	1	37	[6]
2.5	25	Glycosynth	N	1	37	[7]
2.1	50	CalBiochem	N	1-6	37	[8]
0.8	50	TRC	N	6-8	37	[9]
1.6	50	TRC	N	6-8	37	[10]
6	50	CalBiochem	N	6-8	37	[11]
6.6	1000	CalBiochem	N	17	37	[12]
0.9	1425	CalBiochem	N	6	37	[13]
2.8	1425	Glycosynth	N	6	37	[14]
6	1425	Glycosynth	N	6	37	[15]
4.4	180	Glycosynth	Y	0.5	37	This study

Table 1. Comparison of IDUA enzyme activity in liver of heterozygous mice from different studies. Enzyme levels were divided by 2 if only values in wild type mice were provided in the original paper (IDUA enzyme activities in heterozygous mice are approximately half of that in wild-type individuals). N=NO, Y=YES.

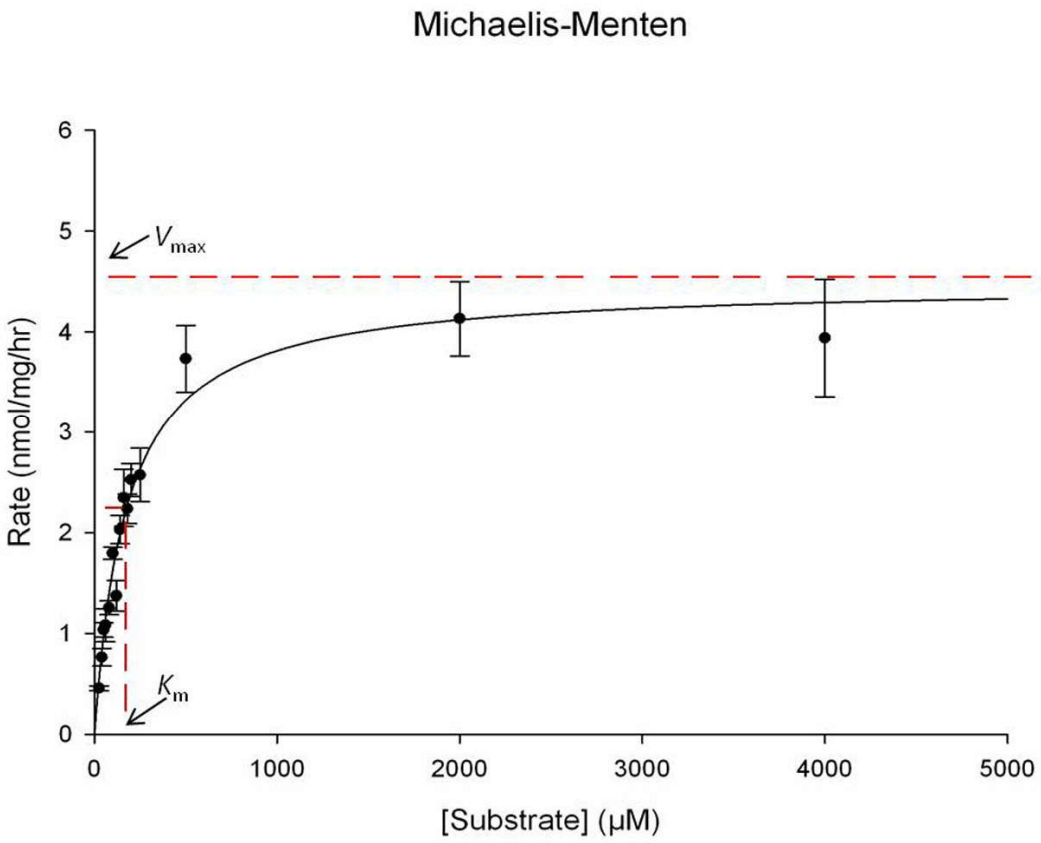

Figure 2. Michaelis-Menten analysis of IDUA enzyme activity in liver from normal heterozygous mice (idua-/+). Each dot is the average value from 3 mice. The line indicates the approximate plateau that defines Vmax. This figure is produced by fitting a hyperbolic curve through TriLookup software.

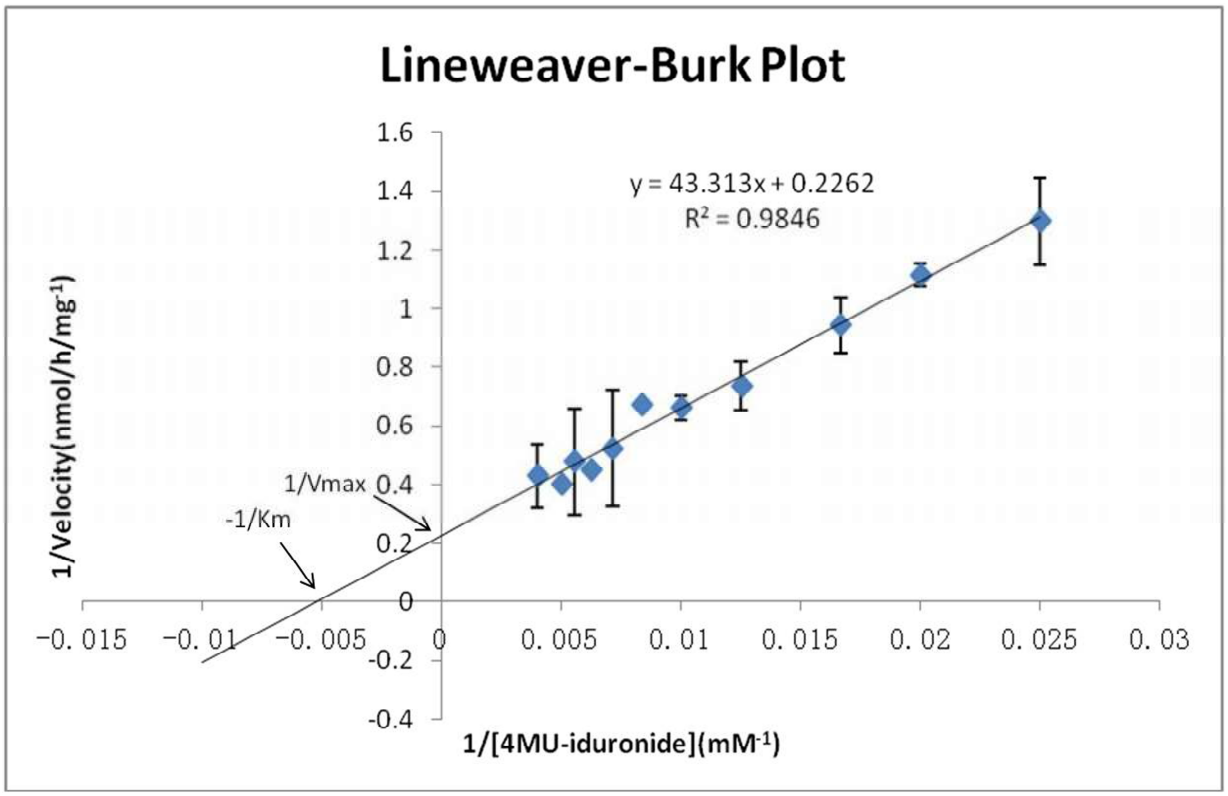

Figure 3. Lineweaver-Burk analysis of IDUA enzyme activity in liver from normal heterozygous mice (idua-/+). Each dot is the average value from 7 mice.

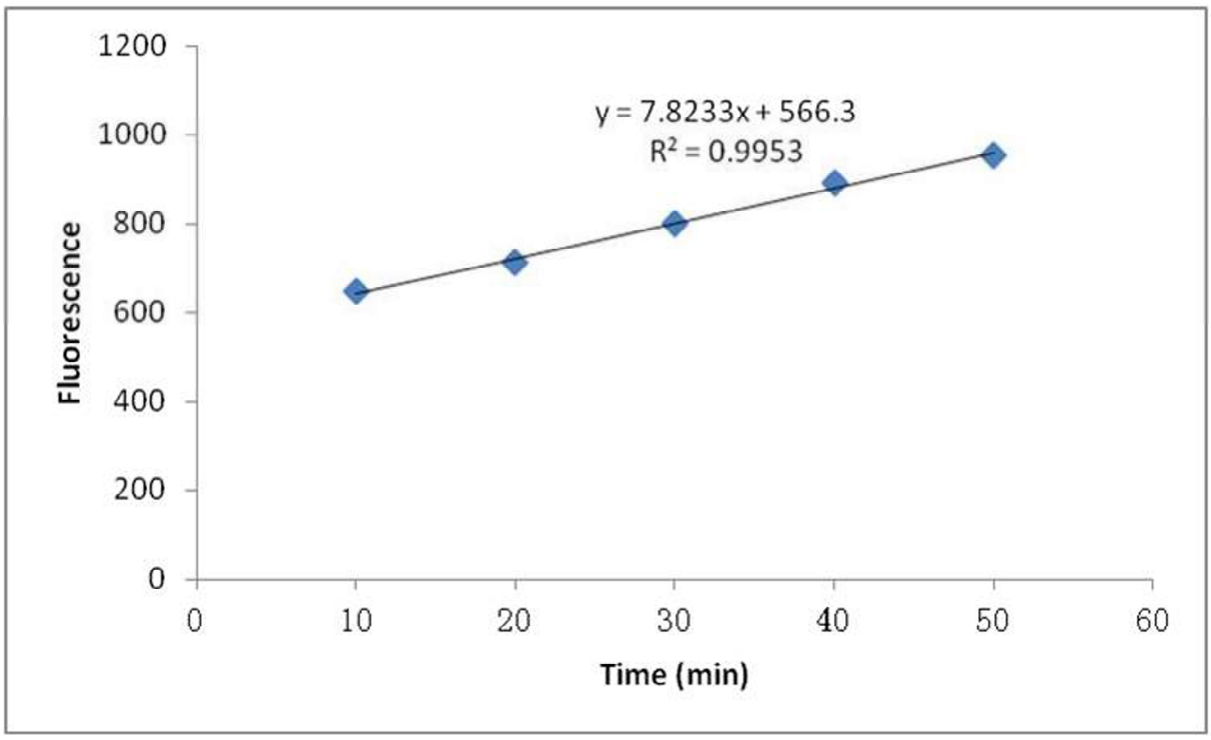

Figure 4. IDUA enzyme assay with a final substrate concentration of 180 μM is linear through 50 min. Five groups of reactions were quenched at different time-points (10, 20, 30, 40, and 50 min), and the fluorescence was measured.

Chapter 3

High-Dose Enzyme Replacement Therapy in Murine Hurler Syndrome

Abstract

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disease which leads to systemic disease, including progressive neurodegeneration, mental retardation and death before the age of 10 years. MPS I results from deficiency of α-L-iduronidase (laronidase, IDUA) in lysosomes and subsequent accumulation of glycosaminoglycans (GAG). Clinical enzyme replacement therapy (ERT) with intravenous laronidase reverses some aspects of MPS I disease (e.g., hepatomegaly, splenomegaly, glycosaminoglycanuria) and ameliorates others (e.g., pulmonary function, cardiac disease, arthropathy, exercise tolerance). However, neurologic benefits are thought to be negligible because the blood-brain barrier (BBB) blocks enzyme from reaching the central nervous system (CNS). We considered the possibility that a very high dose of intravenous laronidase might be able to transit across the BBB in small quantities, and provide some metabolic correction in the brain. To address this question, we administered high-dose laronidase (11.6 mg/kg, once per week, 4 weeks) to adult MPS I mice. IDUA enzyme activity in cortex of injected mice increased to 97% of that in wild type mice ($p<0.01$). GAG levels in cortex were reduced by 63% of that from untreated MPS I mice ($p<0.05$). Further, immunohistochemical analysis showed that the treatment reduced secondary storage material GM3-ganglioside in treated MPS I mice. Water T-maze tests showed that the learning abnormality in MPS I mice was surprisingly reduced ($p<0.0001$). In summary, these results indicated that repeated, high-dose ERT facilitated IDUA transit across the BBB, reduced GAG accumulation within the CNS, and rescued cognitive impairment.

1. Introduction

MPS I is an autosomal recessive inherited disease that leads to systemic, progressive neurodegeneration, mental retardation and death before the age of 10 years. Hurler syndrome (MPS IH, OMIM #67014) results from deficiency of α-L-iduronidase (IDUA, E.C.3.2.1.76), which degrades the GAG heparan sulfate and dermatan sulfate. The widespread accumulation of GAG leads to progressive cellular damage and organ dysfunction, with the central nervous system (CNS) being one of the primary sites of pathology. The CNS pathology in Hurler syndrome patients is severe, manifested by hydrocephalus, mental retardation, learning delays and dementia.

Clinical IDUA ERT ameliorates exclusively certain visceral defects [1]. The uptake of infused laronidase to affected tissues relies on the mannose 6-phosphate receptor (M6PR), which is down-regulated in the blood-brain barrier after the first two weeks of life [2]. Further, infused IDUA is rapidly cleared from the circulation by M6PR and mannose receptor (MR) [3]. As a result, neurological benefits of ERT with the conventional dose are thought to be negligible because the BBB blocks infused enzyme from reaching the CNS [4, 5]. Novel therapies leading to metabolic correction in the CNS and neurological benefits are desperately needed.

High-dose ERT has been shown in several lysosomal disease animal models to ameliorate neuropathology, including mice with aspartylglycosaminuria [6], Krabbe disease [7], MPS VII [8], α-mannosidosis [9,10], arylsulfatase A deficiency [11,12], MPS II [13], and guinea pigs with α-mannosidosis [14]. However, a high-dose ERT study [15] conducted in MPS IIIA mice showed no neurological benefits, indicating a potential difference in mechanisms of enzyme uptake or that the dose required in MPS IIIA is higher than other lysosomal diseases.

In this study, a single or multiple injections of high-dose IDUA was administered in an MPS I mouse model. For the first time, this study showed that repeated, high-dose ERT allows sufficient enzyme delivery to the CNS and improves cognitive function. These results may lead to improved treatment for MPS I patients with cognitive impairment.

2. Material and Methods

2.1 MPS I mice and injection

MPS I deficient mice (*idua*-/-), a kind gift from Dr. Elizabeth Neufeld, UCLA, were generated by insertion of neomycin resistance gene into exon 6 of the 14-exon IDUA gene on the C57BL/6 background. MPS I mice (*idua*-/-) and heterozygotes (*idua*-/+) were genotyped by PCR. Aldurazyme® (BioMarin Pharmaceutics) was concentrated by centrifugation in Amicon Ultra Centrifugal Filters (Millipore) to a final concentration of ~1.2 mg/mL or 11.6 mg/mL. The preparations were diluted in 120 µL phosphate buffered saline (PBS), and injected over 10-15 seconds by the tail vein at the dose regimens detailed in the Results section. All-mouse care and handling procedures were in compliance with the rules of the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

2.2 IDUA enzyme assay

IDUA activity was determined by a fluorometric assay using 4-methylumbelliferyl α-L-iduronide (4-MU iduronide, Glycosynth #44076) as the substrate. 4-MU iduronide was diluted with sodium formate buffer (0.4 M, pH 3.5) to different concentrations. Then, 25 µL aliquots of substrate of different concentrations were mixed with 25 µL aliquots of tissue homogenates. The mixture was incubated at 37 °C for 30 min, and 200 µL glycine carbonate buffer (pH 10.4) was added to quench the reaction. IDUA catalyzed the cleavage of the non-fluorescent substrate (4-MU iduronide) into a fluorescent product (4-MU). 4-Methylumbelliferone (4-MU, Sigma #M1381) was used to make the standard curve. The resulting fluorescence was measured using a Bio-Tek plate reader

with excitation at 355 nm and emission at 460 nm. IDUA enzyme activity was expressed in units (nmol converted to product per hour) per mg protein as determined with a Pierce protein assay kit (Fisher # PI22662) . All reactions were run in triplicate.

2.3 Tissue GAG assay

Tissue GAG assays were conducted as described previously [16]. The supernatants of tissue homogenates were treated by Proteinase K (NEB #CX27439) with the ratio of 3(Pro K):1(sample), incubated at 55°C for 24 hours, and boiled for 10 min to inactivate the enzyme. Then, samples were incubated with 250 U DNase (Sigma # D4527-10KU) and 2.5 µg RNase (Sigma # R6513-10MG) at room temperature for 24 hours. After boiling for 10 min to inactivate the enzymes, GAG concentration was determined by the Blyscan Sulfated Glycosaminoglycan Assay (Biocolor Inc). Results were expressed as µg GAG/mg protein.

2.4 Water T-maze test

Water T-maze test was conducted as described previously [17]. Four days after the third injection, mice from all three groups were subjected to water T-maze testing which assessed the learning ability of mice. A pool was filled approximately 10 cm deep with water (room temperature) mixed with non-toxic white acrylic paint in order to prevent visual detection of submerged objects. A "T" shape maze was set in the water, and a platform, at the end of the left (or right) arm, was submerged 0.5-1 cm below the surface of the water. In run #1, a barrier was placed across one arm. Mice were released at the base of the "T", forced to swim to the top, and choose either the left or right arm to locate the platform. Then, in run #2, the barrier was removed and the platform was placed at the end of the other arm. The correct choice was the alternate arm from where the platform was placed in run #1. Each mouse performed 8 trials per day for 8 days.

2.5 Immunofluorescence

Immunofluorescence was conducted as described previously [18]. Mice were perfused with 20 mL 0.01 M PBS and sacrificed. The brain was removed, fixed overnight in paraformaldehyde and cryopreserved in 30% sucrose. Then, the brain was frozen on dry ice and sectioned coronally. After washing, free-floating sections were blocked with PBS containing 5% normal goat serum and 0.3% Triton X-100. Primary antibody against GM3-ganglioside (1:50, Abcam) was added and the sections were incubated at 4°C overnight. After washing, sections were incubated with goat-anti mouse IgM (Antibodies-online) and DAPI (Sigma) at room temperature for 1 hour. After mounting on the slides, sections were examined with a microscope (Zeiss).

2.6 Enzyme-linked immunosorbent assay (ELISA)

ELISA was conducted as described previously [19]. Aldurazyme (BioMarin Pharmaceutics) was diluted with Tris-buffered saline (pH=7.4) to a final concentration of 4 μg/mL. In a 96-well microplate, 50 μL diluted Aldurazyme was incubated at 4°C overnight. After washing and blocking with Tris-buffered saline containing 1% BSA at room temperature for 2 hours, 100 μL serum (1:50 dilutions) was incubated at room temperature for 1 hour. After washing, the wells were incubated with 100 μL anti-mouse IgG alkaline phosphatase conjugate (Abcam #97027) at room temperature for 1 hour. After washing, the wells were incubated with 200 μL of 1 mg/ml p-nitrophenyl phosphate chromogenic substrate (Sigma # P7998-100ML) at room temperature for 1h. Then, 50 μL EDTA (0.1 M, pH 8.0) was added to stop the reaction. Absorbance at 405 nm was quantified by using a Bio-tek plate reader.

2.7 Statistical analysis

Data are represented as mean±standard error. For evaluation of differences between samples, Tukey test for comparisons between paired samples and one-

way or two-way analysis of variance (ANOVA) for comparisons between three or more samples were applied. Statistical significant level was set at $p<0.05$. Data analysis was conducted with SAS 9.3.

3. Results

3.1 Biodistribution of IDUA after single high-dose ERT

An initial experiment was designed to determine the potential effects of high-dose ERT on the transit of laronidase across the BBB. A single injection of 0.58 mg/kg (0.7×10^4 pmol/kg, clinical standard dose) or ~1.2 mg/kg (1.4×10^4 pmol/kg, approximately 2 fold the standard dose) laronidase (Aldurazyme) was intravenously administered to adult MPS I mice (*idua-/-*, 10 to 20 weeks old, n=6 for high-dose group, n=7 for normal-dose group) by the tail vein. Age-matched untreated MPS I mice (n=7) and heterozygotes (*idua-/+*, n=6) were included as controls. It has been shown that the plasma half-life of infused IDUA in dogs is approximately 20 min [20]. Although there might be a difference in plasma half-life between dogs and mice, we presumed that 2 hours would be sufficient for infused IDUA to enter the tissues. Two hours after the injection, all mice were sacrificed, and multiple organs including brain cortex, cerebellum, heart, liver, lung and spleen were collected. IDUA enzyme activity in tissue homogenates was assessed by IDUA enzyme assay. In liver and spleen, which acquired the highest enzyme concentration, the IDUA enzyme activity in the high-dose group increased to 98-fold and 24-fold of that in wild-type mice, respectively (Figure 5). In lung and heart, the IDUA enzyme activity in the high-dose group increased to 24-fold and 93% of that in wild-type activity, respectively. Moreover, IDUA enzyme activity in brain cortex of the high-dose group mice increased to 10.1% of that in wild-type mice. There was no significant increase in enzyme activity in cerebellum. In the normal-dose group, we also observed enzyme activity increases in heart, liver, lung and spleen. However, compared with untreated MPS I mice, no significant increase was observed in brain cortex or cerebellum

of the normal-dose group. Also, no statistically significant age or gender effects were found in IDUA enzyme activity.

Serum was collected prior to sacrifice, and high IDUA enzyme activity was observed in treated MPS I mice: 110.6±49.8 nmol/h/mg for high dose group and 78.4±25.3 nmol/h/mg for normal dose group. IDUA enzyme activity in heterozygous and untreated MPS I mice was almost non-detectable. Although perfusion was conducted, there is still a possibility that the enzyme activity increase in brain cortex can be attributed to serum within brain capillaries.

3.2 Biodistribution of IDUA after repeated, high-dose ERT

Based on the results of aforementioned single high-dose ERT and high-dose ERT studies on other lysosomal disease models, we designed a repeated ERT schedule with a 20-fold higher than the human therapeutic dose (~11.6 mg/kg body weight, 1.4×10^5 pmol/kg). Adult MPS I mice (11.7±2.7 week old, n=12) received 4 injections of 20-fold dose IDUA once a week. Age-matched untreated MPS I mice (*idua-/-*, n=11) and heterozygotes (*idua-/+*, n=11) were included as controls. All mice were sacrificed five days after the 4th injection, and multiple organs were collected.

An increase in enzyme activity was observed in heart (1.9-fold of wild-type activity), liver (95.1-fold of wild-type activity), lung (3.4-fold of wild-type activity) and spleen (21.5-fold of wild-type activity) (Figure 6A, B). IDUA enzyme activity in brain cortex and cerebellum of treated MPS I mice increased to 97% and 1.2-fold of that in wild-type mice, respectively (Figure 6A). Also, no statistically significant age or gender effects were found in IDUA enzyme activity. Serum was collected on D3, D10 and D26 (injection on D0, D7, D14, D21). Since IDUA enzyme levels in serum of treated mice were undetectable (data not shown), the enzyme activity increase in cortex and cerebellum cannot be simply explained by the presence of blood in the capillaries of the brain.

3.3 Reduced tissue GAG accumulation after repeated, high-dose ERT

To determine the effect of repeated, high-dose ERT on GAG accumulation, GAG was quantified in tissue homogenates. Liver and spleen acquired the highest enzyme levels after injection, correspondingly, GAG accumulation was reduced the most in these two organs: by 92%, and 83%, respectively (Figure 7B). Also, GAG accumulation in heart and lung was reduced by 76% and 87%, respectively (Figure 7A, B). GAG accumulation in cortex was reduced by 63% (Figure 7A). Also, no statistically significant age or gender effects were found in GAG accumulation.

To further validate the GAG accumulation reduction within the CNS, histochemical analysis was conducted. Secondary accumulation of GM3-ganglioside was apparent in the brains of untreated MPS I mice, visualized by positive immunofluorescence staining of punctate aggregates throughout most of the brain (including thalamus, hippocampus and nuclei septi). In contrast, only a few punctate aggregates were observed in the same regions of brains from treated MPS I mice (Figure 8).

3.4 Cognitive improvements after repeated, high-dose ERT

Water T-maze tests were employed to evaluate learning behavior. Five days after the third injection, mice from all three groups were subjected to 8 trials of water T-maze tests per day for a duration of 8 days. For each mouse, the percentage of finding the platform correctly was recorded. On D0, mice from all three groups had a similar correct percentage (36 to 42%). However, the heterozygote (*idua*-/+) group improved significantly over the testing period, reaching a final level of 75%. Treated MPS I mice also improved significantly during the trials, reaching a final level of 67%. In contrast, untreated MPS I mice only improved modestly from 36% to 45%. Since this test is mainly about decision-making, swimming speed was not recorded nor evaluated. Also, the

platform was underneath opaque water, eye disease of MPS I mice is unlikely to be a confounding factor. There did not appear to be any physical defects in the MPS I mice that could have affected the results. Water T maze tests showed that learning abnormalities observed in MPS I mice were reduced significantly by repeated, high-dose ERT (Figure 9, $p<0.0001$).These results showed that by repeated, high-dose ERT, the CNS pathology in MPS I mice was functionally rescued.

3.5 Anti-IDUA immune response after repeated, high-dose ERT

Several previous studies showed that infused IDUA elicited humoral immune response in patients and animal models [19, 21]. Serum was collected from 12 mice of the three groups at different time-points and processed for ELISA. In treated MPS I mice, a significant increase in anti-IDUA antibody concentration was observed after the second injection (Figure 10A). Also, right before sacrifice, serum was collected from 29 mice of the three groups and processed for ELISA. In treated MPS I mice, there were significant higher antibody levels compared with the other two groups (Figure 10B).

Strong adverse infusion reactions were found in some treated mice after the second injection but were alleviated by injection of 2 mg/kg dexamethasone 1 hour prior to enzyme infusion, prior to the subsequent injections. Based on ELISA data, treated mice can be divided into two groups: those with or without high anti-IDUA levels. However, no significant difference in enzyme activity, GAG accumulation or performance in behavior tests was observed between these two groups.

4. Discussion

4.1 Limitations of ERT

Progressive neurodegeneration in MPS I patients is a devastating feature leading to dramatic intellectual impairment. ERT has already been used in clinical

application for treating MPS I and other lysosomal diseases. Delivery of infused enzymes to lysosomes is mediated by MR on macrophages or ubiquitously expressed M6PR. A previous study in mice has shown that M6PR-mediated transcytosis in the BBB is down-regulated after two weeks of life [2]. As a result, although ERT can achieve significant visceral storage reduction, neurological improvements are thought to be negligible. It has been a long-held dogma that the BBB prevents the transit of lysosomal enzymes from the blood to the interstitial space surrounding neuronal and glial cells of the CNS. Any means of delivering enzyme across the BBB would be of major clinical significance.

In this study of high-dose ERT, the most corrective effect was achieved in liver and spleen, which took up the majority of infused IDUA, as shown by IDUA enzyme assays. The IDUA enzyme activity in brain cortex reached 50% of that in wild-type mice. Considering the fact that only a small amount of IDUA can make a great difference in patients, this significant increase in the CNS has promise of providing therapeutic benefits [22-24]. Consistent with this observation, total GAG accumulation in brain cortex was reduced after 4 injections. Further, behavior analysis showed that even when the CNS impairment is clearly manifested, high-dose ERT can be effective in preventing and possibly correcting this impairment.

4.2 Analysis of Mechanism

Less than 200 μL of laronidase was administered by the tail vein, ruling out the possibility of passage of enzyme through the blood-brain barrier as the result of a hydro-dynamic effect resulting from the rapid injection of a large volume of enzyme solution. Also, blood from each mouse was collected right before sacrifice, and plasma IDUA enzyme levels in treated mice were approximately 0 (data not shown). This result excludes the possibility of blood contamination of the brain. Furthermore, although we could not directly visualize IDUA in brain sections with antibody staining (data not shown), increased enzyme activity,

decreased GAG concentrations and decreased GM3-ganglioside suggest that infused IDUA reached the CNS tissue.

The mechanism by which intravenous IDUA transits across the BBB and reaches the brain remains an open question at present. One possibility may be fluid-phase pinocytosis. This type of uptake is strictly dependent on concentrations and is relatively slow. High-dose ERT can lead to a high enzyme level in blood, which makes it possible for fluid-phase pinocytosis to be effective. Another possibility is the extracellular pathway which has been shown to allow small amounts of molecules as large as albumin to cross the BBB [25]. Also, there exists the possibility that residual M6PR or other uncharacterized receptors more efficiently facilitate the transit of IDUA in the context of high plasma enzyme levels. However, we cannot rule out the possibility that the integrity of the BBB in MPS I mice might be impaired by disease-related factors. Identifying the mechanism of delivery of IDUA to brain may suggest strategies to enhance the process and make ERT for CNS disease reduction a more realistic goal.

4.3 Implications and Future Directions

The inability of normal-dose ERT to correct pathology within the CNS may be due to two obstacles: 1) other tissues, e.g. liver and spleen, internalizing most of the infused IDUA, preventing sufficient enzyme delivery to the CNS; 2) antibody binds the infused IDUA, further reducing the efficiency of delivery to the CNS. Several high-dose ERT studies have been conducted in other lysosomal disease animal models. Most studies showed a surprising enzyme delivery to the CNS (results summarized in Table 2), indicating that the BBB is not impermeable to infused enzymes. Therefore, it is a matter of 'inefficiency' rather than an insurmountable obstacle. The inefficiency of the CNS delivery in normal-dose ERT may be circumvented by high-dose ERT.

High humoral immune response against IDUA was identified in treated mice by ELISA. In spite of high anti-IDUA antibody levels, significant increase in IDUA enzyme activity was observed and GAG accumulation was reduced. In fact, a recent study showed that development of antibodies to IDUA negatively correlates with improvement in performance on behavioral tests in murine MPS 1 [26]. It will be interesting to see whether infused IDUA can efficiently cross the BBB in immunotolerant or immunodeficient animals. In addition, minimizing the immune response perhaps by modifying the epitopes of IDUA enzyme can be a promising future direction.

4.4 Significance

A recent murine study, using 1.2 mg/kg dose every 2 weeks (equivalent to clinical standard dose 0.58 mg/kg per week) achieved GAG reduction in brain and neurological improvements [26]. Similarly, a canine study showed that dogs treated from birth with ERT (0.58 to 1.57 mg/kg) also led to reduction of GAG accumulation in the brain [27]. This study, for the first time, assessed repeated high-dose ERT in MPS I mice. Using a 20-fold dose (11.6 mg/kg) compared to the generally accepted therapeutic dose in people, may not be applicable to patients considering the cost and immune response. However, it has been suggested that dose conversion using body surface area (BSA) instead of body weight is more appropriate [28]. According to a formula provided by the Food and Drug Administration [29], 11.6 mg/kg in this murine study would be approximately 0.94 mg/kg in adult patients, which is not a great increase from the standard dose (0.58 mg/kg). These facts make this study more meaningful in terms of potential clinical application: only a small increase in dose may make a great difference. These results provide the proof of principle: when the enzyme level is high enough, a small amount of IDUA can cross the BBB of adult animals, and provide neurological benefits. Although intraventricular [18], and intrathecal [31, 32] administration of enzyme or gene vector have produced neurological improvements, these strategies are limited by their invasive nature. The best

therapeutic strategy might be the one that delivers enzyme uniformly to the brain. Considering the fact that each neuron is estimated to be approximately 15 μm from blood vessels, the bloodstream would be an ideal conduit for enzyme delivery.

5. Acknowledgements

The authors would like to thank Brenda Diethelm-Okita for editing ELISA protocol. This work was supported by NIH grant P01HD032652 .

Animal model	Immuno-competent	Doses(n)	Enzyme activity achieved (% Normal)	Brain storage reduced (%Affected)	Reduced Neuropathology	Reference
α-mannosidosis mice	Y	250 U/kg(2)	ND	74%	ND	[9]
α-mannosidosis mice	Y	500 U/kg (2)	15%	50%	Y	[10]
α-mannosidosis pigs	Y	10 mg/kg (1)	ND	ND	Y	[14]
ASA mice	Y	20 mg/kg (4)	ND	30%	Y	[11]
ASA mice	N	50 mg/kg (52)	ND	34%	Y	[12]
AGU mice	Y	10 mg/kg (8)	10%	20%	Y	[6]
Krabbe mice	Y	6 mg/kg	7%	18%	Y	[7]
MPS II mice	Y	10 mg/kg (45)	5%	ND	Y	[13]
MPS IIIA mice	Y	20 mg/kg (4)	22%	0	N	[15]
MPS VII mice	N	20 mg/kg (4)	2.5%	ND	Y	[8]
MPS I mice	Y	11.6 mg/kg (4)	97%	63%	Y	This study

Table 2. Summary of outcomes from high-dose ERT studies conducted on animal models of lysosomal diseases with neurodegeneration. Y= yes; N=no; ND = not determined.

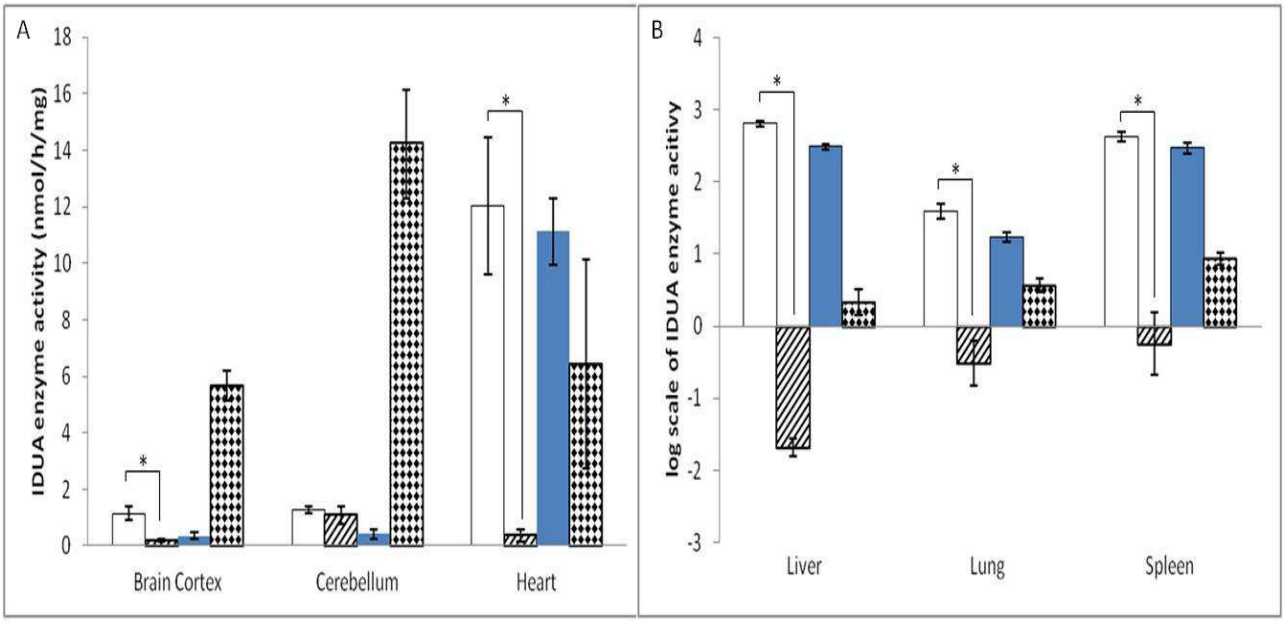

Figure 5. IDUA enzyme activity in multiple organs increased after a single injection of ~1.2 mg/kg laronidase. A total of 28 age-matched mice were assigned into four groups: 1) MPS I mice (idua-/-, n=6, white columns) injected with 2 fold (~1.2 mg/kg) laronidase; 2) MPS I mice (n=6, dark columns) injected with normal-dose (0.58 mg/kg) laronidase; 3) MPS I mice (n=6, hatched columns) with no injections; 4) heterozygote (idua+/-, n=5, diamond columns) with no injections. Data are mean ± standard errors. * $p<0.05$.

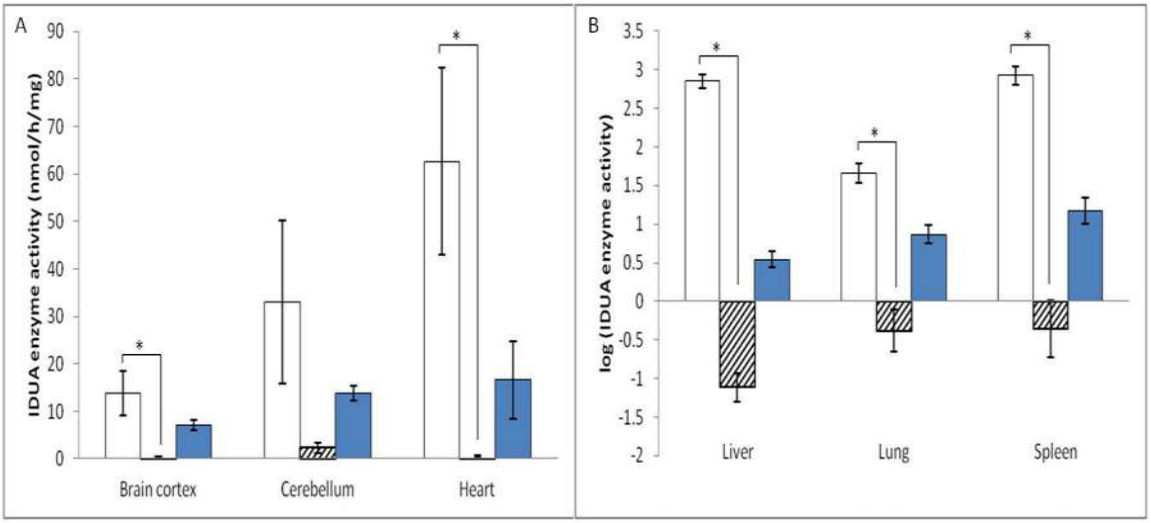

Figure 6. IDUA enzyme activity in multiple organs increased after 4 injections of ~11.6 mg/kg laronidase. A total of 34 age-matched adult mice were assigned into four groups: 1) MPS I mice (*idua*-/-, n=12, white columns) injected with 20 fold (~11.6 mg/kg) laronidase; 2) MPS I mice (n=11, hatched columns) with no injections; 3) heterozygote (*idua*+/-, n=11, dark columns) with no injections. Data are mean ± standard errors. * $p<0.05$.

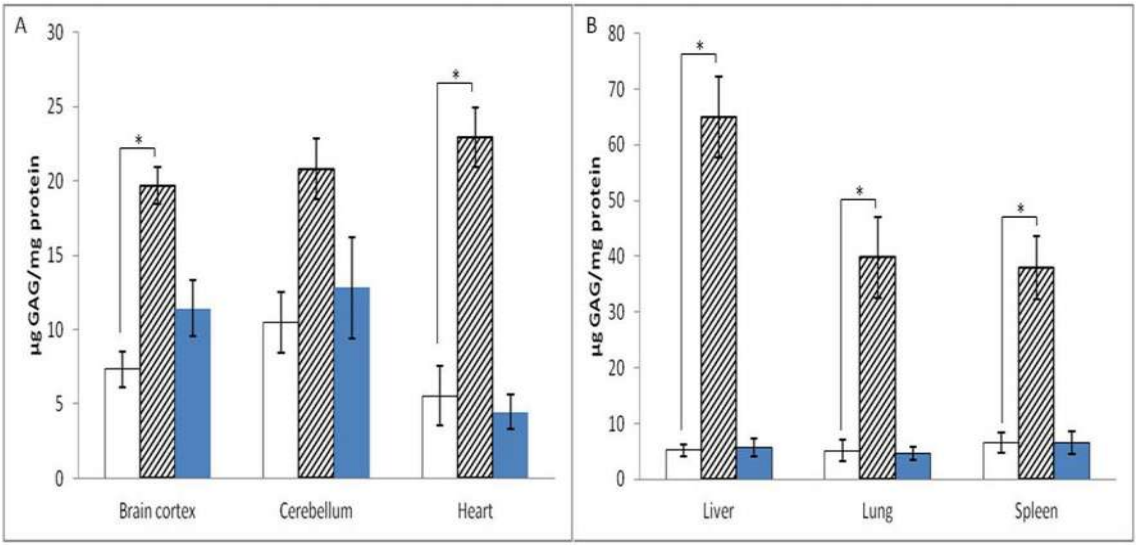

Figure 7. GAG accumulation reduced in multiple organs after 4 injections of ~11.6 mg/kg laronidase. Data are mean ± standard errors, p values are calculated by one-way ANOVA and Tukey test. * $p<0.05$. Treated MPS I: white columns; untreated MPS I: hatched columns; heterozygous mice: dark columns.

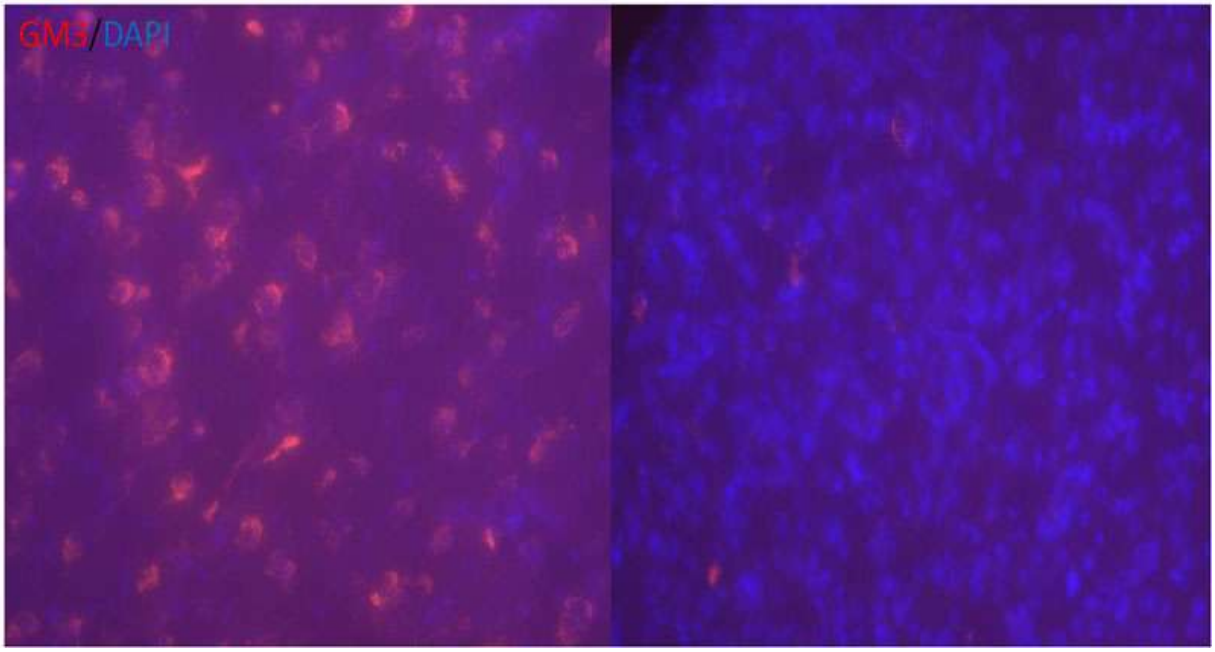

Figure 8. Secondary storage material GM3-ganglioside was reduced in the hippocampus region of treated MPS I mice. (Red: GM3; Blue: DAPI. Left: untreated MPS I; right: treated MPS I)

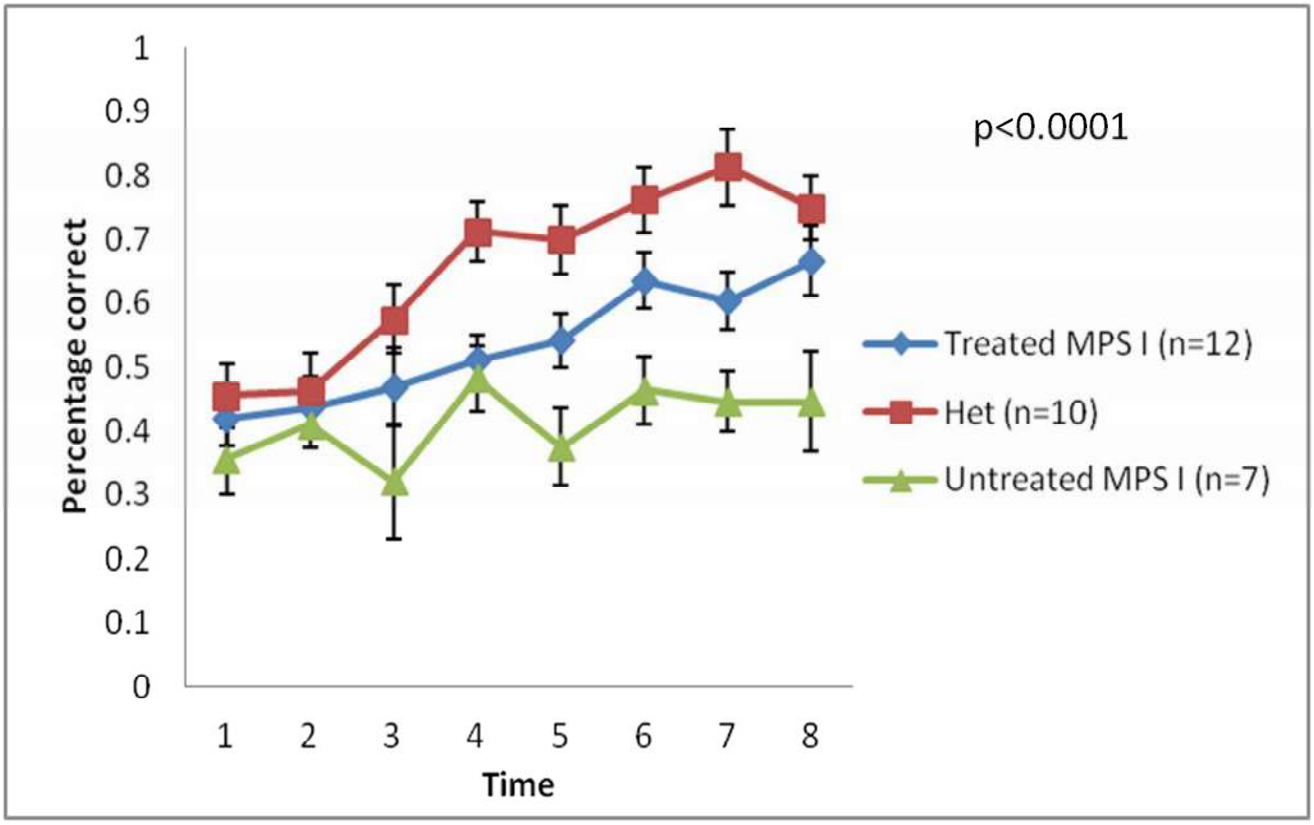

Figure 9. Learning abnormality in MPS I mice was surprisingly reduced by repeated high-dose ERT. Five days after the third injection, all three groups underwent 8-day water T maze test which investigate learning ability of mice. X axis values mean time in days. Data are mean ± standard errors, p value was calculated by two-way ANOVA.

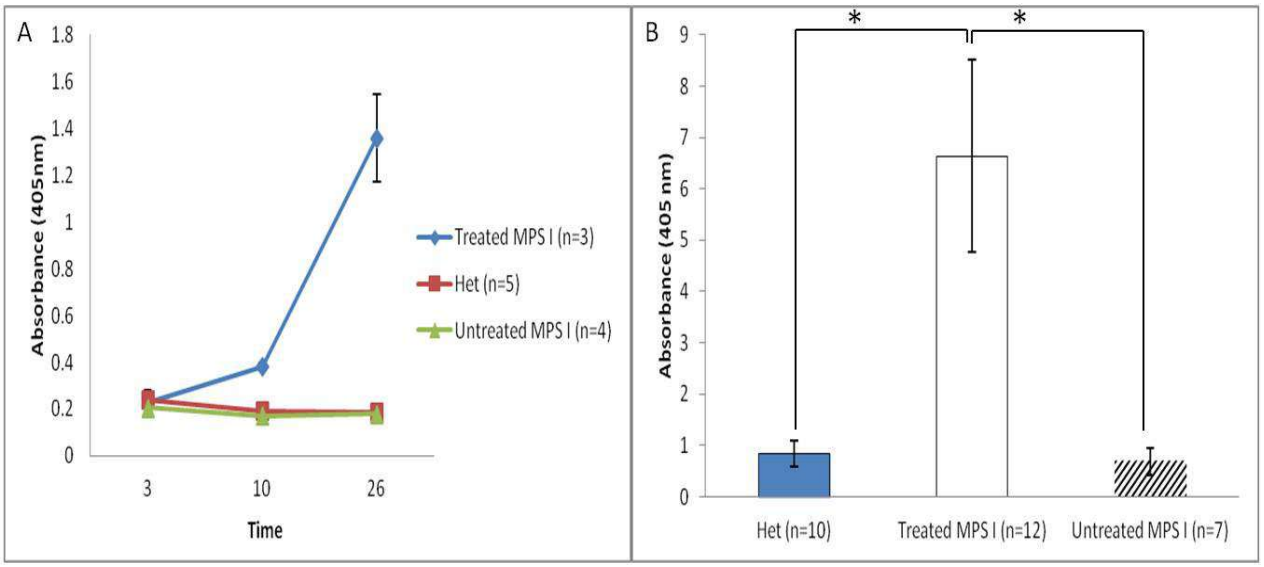

Figure 10. Anti-IDUA immune response detected after repeated high-dose ERT. (A) The IDUA antibody levels were measured by ELISA in serum collected at various time-points (D3, D10, D26). X axis values mean time in days. The serum was tested at 1:50 dilution. **(B)** Serum from a total of 29 mice were collected right before sacrifice. The IDUA antibody levels were measured by ELISA. The serum was tested at 1:50 dilution. Data are mean ± standard errors. * $p<0.05$.

Chapter 4

Lentiviral Gene Therapy for Hurler Syndrome

Abstract

Mucopolysaccharidosis type I (MPS I) is a lysosomal disease caused by α-L-iduronidase (IDUA) deficiency and subsequent accumulation of glycosaminoglycan (GAG) heparan sulfate and dermatan sulfate. Lentiviral vector encoding correct IDUA sequence could be used for gene therapy treating MPS I disease. Previous study in our lab showed that intravenous injection of a lentiviral vector (CSP1) into neonatal MPS I mice achieved metabolic correction and neurological improvements. To improve the efficacy of this lentiviral gene therapy, 9 more lentiviral constructs were designed by codon optimization, different combinations of promoters and enhancers. These ten plasmids were transfected into HEK 293FT cells, and IDUA enzyme activity in cell lysates and supernatants were compared. The transfection efficiencies of each plasmid were comparable (~60%), shown by co-transfection of a plasmid encoding GFP. A total of 5 plasmids showed higher enzyme activity in cells (from 6,482 ± 1,258 to 7,358 ± 956 nmol/h/mg) than CSP1 (5,613 ± 1,047 nmol/h/mg). These 5 candidates also had the highest enzyme activity in supernatants (514 ± 213 nmol/h/mL to 861 ± 151 nmol/h/mL). Therefore, these 5 plasmids and CSP1 were selected for lentiviral vector production and further *in vivo* comparison. A total of 6 lentiviral vectors will be injected into temporary facial vein in neonatal MPS I mice, and tissue IDUA levels on day 30 will be compared to select the optimal vector, which has the highest IDUA levels. These results will pave a way for developing a directly applicable lentiviral gene therapy clinical trial for MPS I patients, and provide proof-of-concept evidence for treating other lysosomal diseases.

1. Introduction

The CNS pathology in human Hurler syndrome patients is severe, manifested by hydrocephalus, mental retardation, learning abnormality, dementia and even death before the age of 10 years. Brain, bone, and heart valves are difficult-to-reach sites for enzyme due to the inefficient enzyme diffusion. Specifically, the CNS pathology of Hurler syndrome is extremely difficult to treat because the BBB blocks the entry of enzyme into the CNS (1, 2). HSCT can only achieve partial neurological improvements, which is manifested by low IQ and neurocognitive disability in patients (3). While ERT is thought to have negligible neurological improvements, it has been shown that a high dose infusion of IDUA can lead to significant increase of enzyme activity in the brain cortex (4). Considering the fact that a small amount of enzyme is sufficient to improve neurological pathology (5-7), this should be sufficient to treat neurological pathology. However, ERT is limited by the need for frequent, life-long, expensive treatments. Moreover, high dose treatment is not plausible for clinical application considering the immune response against infused enzyme (8).

Gene therapy has emerged as a promising strategy for treating MPS I diseases. In the past few years, gene therapy for MPS I disease with different vectors in animal models has been conducted, which include retrovirus (9, 10), lentivirus (8, 11), adeno-associated virus (AAV) (3, 12-14), Sleeping Beauty (SB) transposon (15) and minicircles (16).

Lentiviral vector can transfer genes into a variety of non-dividing cell types, including neurons, retinal cells, muscle cells and hematopoietic stem cells (HSCs). Neurological improvements have been shown in disease animal models after injection of lentiviral vector directly into the brain (17). Previous study in our lab has showed that a single intravenous administration of lentiviral vector (CSP1)

into neonatal MPS I mice can achieve metabolic correction and neurological improvements. These results demonstrated the efficacy of neonatal lentiviral vector administration for treating Hurler syndrome, with implications for treatment of other lysosomal diseases. However, it is difficult to apply the dose used in this study into clinical trials. In light of this, we designed 9 more constructs by codon optimization, different combinations of promoters and enhancers, which are expected to have better transgene expression. In this study, we compared the transgene expression of these 10 plasmids in cultured cells and identified 5 candidates which have the highest transgene expression. These 5 candidates, together with CSP1, have been produced into lentiviral vectors and will be tested in neonatal MPS I mice through intravenous administration.

2. Methods

2.1 Plasmid construction

The human IDUA cDNA generated by reverse transcription PCR (RT-PCR) amplification from total mRNA of an unaffected individual was inserted into the multi-cloning sites of pHIV-CS. The IDUA expression was under the control of PGK promoter and named as pSCP1. Then, codon optimization of IDUA cDNA sequence was performed, resulting in oIDUA sequence. By adding different promoters (CLP, PGK and EF-1α) and variants of WPRE, we constructed 9 more plasmids as followed: pCEFID, pCEFoID, pCPGKoID, pCCLPoID, pCEFoID-M1, pCEFoID-M2, pCEFoID-dWPRE, pCCLPID-dWPRE and pCCLPID. All plasmids were confirmed by sequencing. The plasmid construction was done previously in Gene Therapy and Diagnostics Laboratory (C.B. Whitley, personal communication).

2.2 *In vitro* plasmid transfection

For each transfection, 25 μg of candidate plasmid and 25 μg of HIV CMVeGFP plasmid were mixed with 133 μL 2.5 M $CaCl_2$ (25˚C) and 1.33 mL RNase/DNase free sterile H_2O. After adding 1.33 mL of 2x HEPES buffered saline (HBS, pH

7.1), 7 mL serum free medium was added to the mixture. Then, the HEK 293FT cells were incubated with this transfection solution for 4 to 6 hours (37˚C, 5% CO_2). After removing the transfection solution, cells were incubated with 9 mL of 10% fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM) for another 48 hours. Finally, cells and medium were collected by centrifuge and processed for IDUA enzyme assays. The plasmid transfection was conducted by Iowa Viral Vector Core Facility (500 Newton Road 221 Eckstein Medical Research Building Iowa City, IA 52242).

2.3 IDUA enzyme assay

IDUA enzyme assay was conducted as previously described (3, 18). IDUA activity was determined by a fluorometric assay using 4-methylumbelliferyl α-L-iduronide (4-MU iduronide, Glycosynth #44076) as the substrate. 4-MU iduronide was diluted with sodium formate buffer (0.4 M, pH 3.5). Then, 25 µL aliquots (360 µM) of substrate were mixed with 25 µL aliquots of tissue homogenates (diluted with 0.2% bovine serum albumin in phosphate buffered saline). The mixture was incubated at 37 °C for 30 min, and 200 µL glycine carbonate buffer (pH 10.4) was added to quench the reaction. IDUA catalyzed the cleavage of the non-fluorescent substrate (4-MU iduronide) into a fluorescent product (4-MU). 4-Methylumbelliferone (4-MU, Sigma # M1381) was used to draw the standard curve. The resulting fluorescence was measured with a Bio-Tek plate reader with excitation at 355 nm and emission at 460 nm. IDUA enzyme activity was expressed in units (nmol converted to product per hour) per mg protein as determined with a Pierce protein assay kit (Fisher # PI22662) or per mL plasma. Then, IDUA eznyme activity was adjusted by Michaelis-Menten equation as described previously (18). All reactions were performed in triplicate.

2.4 Statistical analysis

Data were represented as mean ± standard errors. For evaluation of differences between samples, Tukey test for comparisons between paired samples and one-

way or two-way analysis of variance (ANOVA) for comparisons between three or more samples were performed. Statistical significant level was set at $p<0.05$. Data analysis was conducted with SAS 9.3.

3. Results

3.1 Construct design for achieving higher transgene expression

The original construct (CSP1) we tested in MPS I mice used the PGK promoter to drive expression of IDUA. The sequence information of 9 more constructs is shown in Figure 11. Through codon optimization, optimized IDUA (oIDUA) sequence was obtained and used in 6 constructs. Also, CLP and EF-1α promoters were selected and engineered into 3 and 5 constructs, respectively. Further, Woodchuck Hepatitis Virus (WHV) Posttranscriptional Regulatory Element (WPRE) was introduced into 7 constructs. WPRE contains three subelements: alpha, beta and gamma. Subelement gamma and alpha contribute to most of the enhancing function, while beta contains WHV X protein. Due to safety concern about the oncogenic ability of WHV X sequence, CEFoID-M1 is designed by creating mutations in WHV X sequence, and CEFoID-M2 is designed by deleting alpha and beta subelements.

3.2 Comparison of plasmid transgene expression in cultured cells

The ten plasmids were co-transfected with plasmid encoding GFP into HEK 293FT cells. Cell lysates and supernatants were collected for IDUA enzyme assays (Figure 12, 13). There are 5 constructs (CEFoID, CEFoID-M1, CEFoID-M2, CEFoID-dWPRE and CCLPoID) yielding higher IDUA levels in cells than CSP1. Also, these 5 constructs had the highest IDUA levels in supernatants, which confirmed the results within cells. CEFoID yielded higher IDUA levels than CEFID (7,359 ± 956 vs 4,879 ± 947 nmol/h/mg, $p=0.045$), while CCLPoID achieved higher IDUA levels than CCLPID (7,334 ± 858 vs 3,784 ± 656 nmol/h/mg, $p=0.03$). These results show that codon optimization can achieve higher transgene expression. CEFoID and CCLPoID had IDUA levels at 7,359 ±

956 and 7,334 ± 858nmol/h/mg, respectively, which is higher than that of CPGKoID (3,732 ± 106 nmol/h/mg, $p<0.02$). These results show that EF-1α and CLP promoter can achieve higher transgene expression. Also, we compared constructs with or without WPRE (CEFoID vs CEFoID-M1 vs CEFoID-M2 vs CEFoID-dWPRE), although CEFoID-dWPRE had lower IDUA levels than the other three constructs, it is not statistically significant. This observation was confirmed when comparing CCLPID (3,784 ± 656 nmol/h/mg) and CCLPID-dWPRE (3,221 ± 302 nmol/h/mg). Collectively, through *in vitro* transfection studies, we identified 5 candidates (CEFoID, CEFoID-M1, CEFoID-M2, CEFoID-dWPRE and CCLPoID) for packaging into lentiviral vectors.

5. Discussion

Previous studies have showed that the effects of gene therapy ultimately depend on an interplay of the construct design, the types of target cells, the vector type and dose, and the administration method. Transcriptional regulatory elements, for instance, promoters and enhancers, are essential vector components for optimization of transgene expression. It has been shown that the PGK promoter leads to moderate transgene expression, both *in vitro* (19, 20) and *in vivo* (21). Lentiviral vector with EF-1α was shown to lead to higher transgene expression than that with PGK (22). However, the effects of different promoters on transgene expression are still not well elucidated, due to the variety of lentiviral vectors used in studies and lack of direct comparison of promoters and transgene expression. Moreover, the use of WPRE is often important for transgene expression and titers of therapeutic vectors (23-25). However, the enhancing ability of WPRE depends on the types of target cells, the type of viral vector context and its sequence (26-29). WHV X protein is a transcriptional activator implicated in the development of liver tumors (30), which raises the safety concern about usage of WPRE in vectors for gene therapy. Herein, we designed constructs with full-length WPRE, mutated WPRE, truncated WPRE and without WPRE for a side-by-side comparison.

Our results show that 1) codon optimization significantly increased IDUA transgene expression; 2) compared with PGK promoter, EF-1α and CLP can achieve significantly higher transgene expression; 3) WPRE did not significantly enhance transgene expression. Further, based on the *in vitro* results, CEFoID, among the 10 constructs tested in this study, may be the most suitable construct for a clinical trial of lentiviral gene therapy for MPS I. These observations are yet to be further confirmed in a MPS I mouse model.

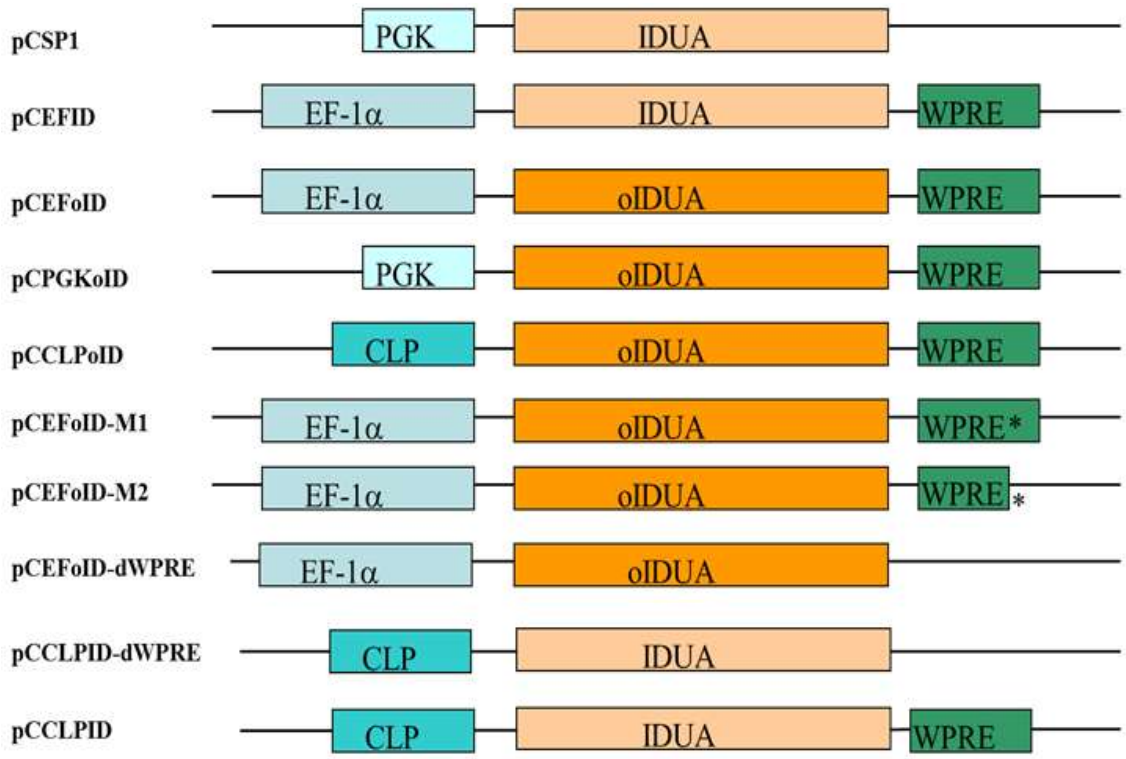

Figure 11. Construct information of 10 lentiviral vectors. oIDUA stands for codon optimized version of IDUA cDNA sequence. PGK: phosphoglycerate kinase 1 promoter; EF-1α: elongation factor 1-alpha promoter; IDUA: IDUA cDNA; oIDUA: codon optimized IDUA cDNA; WPRE: WHV posttranscriptional response element; WPRE*: mutated WPRE; short WPRE*: truncated WPRE by deleting alpha and beta subelements.

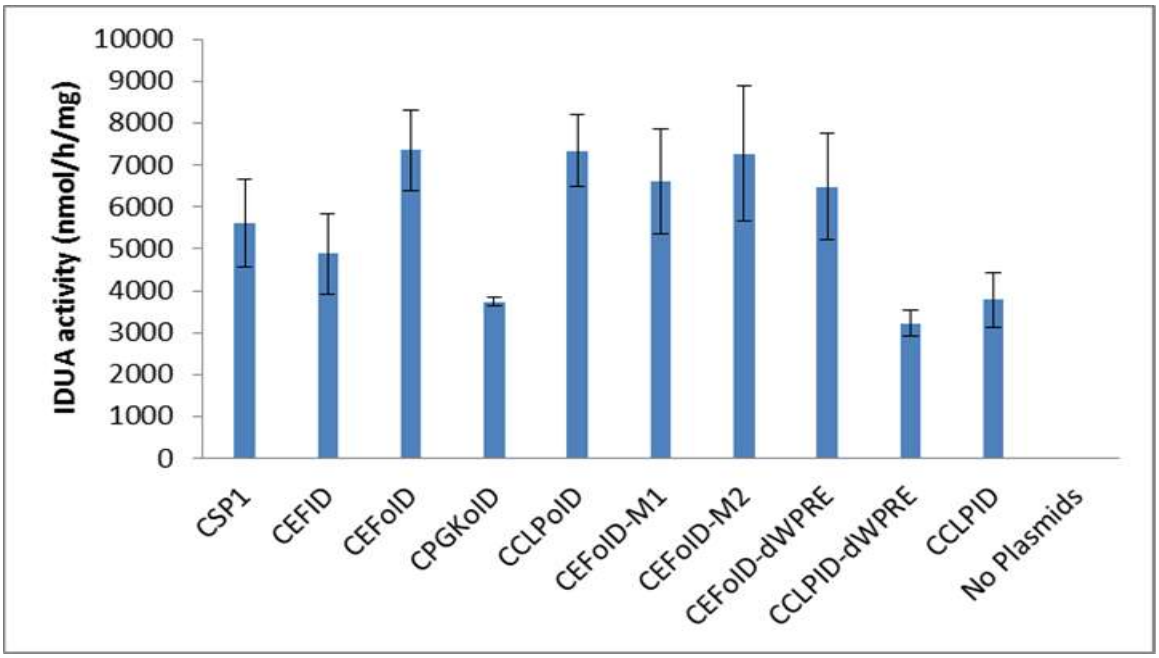

Figure 12. IDUA enzyme levels in cell lysates after transfection of lentiviral plasmids. HEK 293FT cells with no plasmids had IDUA enzyme activity of 0.97 ± 0.34 nmol/h/mg protein.

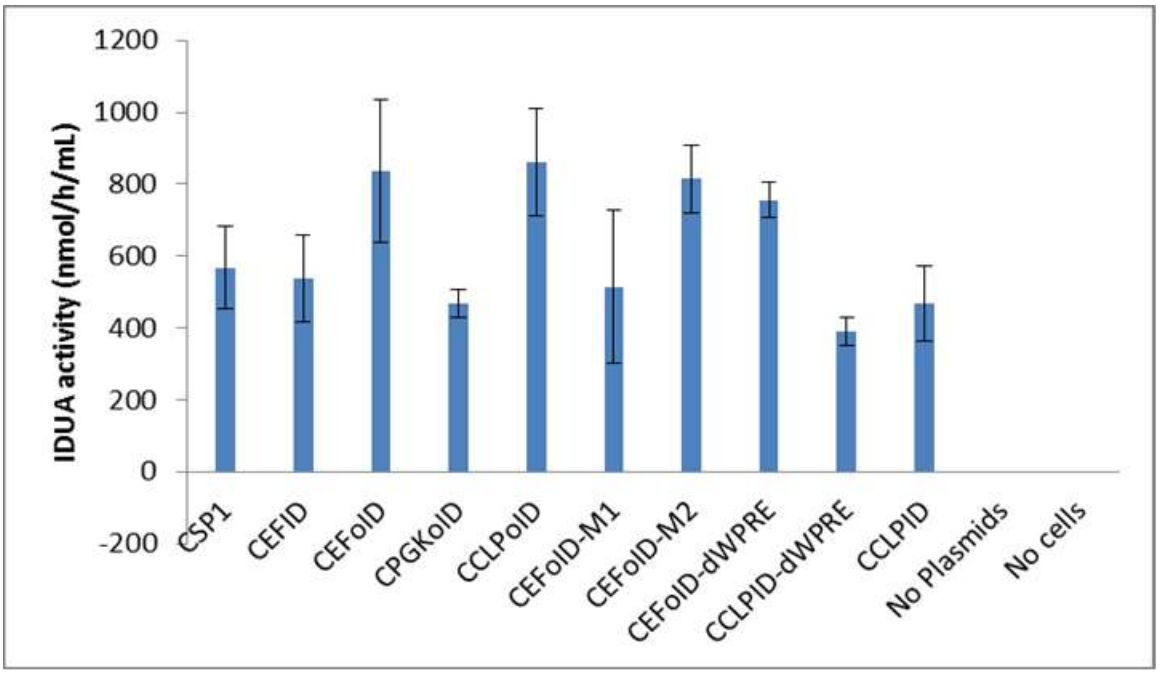

Figure 13. IDUA enzyme levels in supernatants after transfection of lentiviral plasmids. IDUA enzyme activity in HEK 293 FT cells with no plasmids is <0.01 nmol/h/mL, while IDUA enzyme activity in cultured medium without cells is 0.

Chapter 5

Conclusions

In the efforts to treat MPS I, measurement of IDUA enzyme levels is an essential assessment, and is widely used in research, clinical diagnostic testing, and the regulatory evaluation of new therapies. However, lack of methodological uniformity in the lysosomal enzyme activity assay has raised concern from FDA reviewers. Specifically, the reaction temperature ranges from 22 to 37 ˚C, with the reaction time ranging from 1 to 17 hours. More importantly, substrate concentrations used in different labs vary greatly, from 25 μM to 1425 μM. Consequently, even the enzyme level of the same sample within the same research group varies greatly. We successfully applied Michaelis-Menten kinetics into IDUA enzyme assays. By adjustment of Michaelis-Menten equation, IDUA enzyme activity with different substrate concentrations can be converted into V_{max}. Further, the optimal reaction temperature and time was identified for IDUA enzyme assays. In Chapter 2, an IDUA enzyme assay protocol with low cost, high accuracy and methodological standardization was proposed. Based on these results, a standard protocol for IDUA enzyme assay was established, which will not only benefit studies in MPS I disease, but also provides guidance for enzyme assays in other lysosomal diseases.

Study in Chapter 3 for the first time demonstrated that repeated, high-dose ERT can provide not only metabolic correction but also neurological improvements. With 4 weekly 20 fold dose injections, IDUA levels were increased and GAG storage was reduced in brain cortex and cerebellum. More importantly, learning abnormality in MPS I mice was significantly reduced. These results provide proof of principle that with sufficient plasma IDUA levels, a small amount of enzyme can provide neurological benefits in adult affected animals. Although intraventricular (1) and intrathecal (2, 3) administration of enzyme or gene vector have produced neurological improvements, these strategies are of limited use by their invasive nature. In light of this, the optimal therapeutic strategy might be delivering enzyme uniformly to the CNS.

Previous study in our lab has already demonstrated the efficacy of a lentivital vector (CSP1) in MPS I mouse model. To further improve the efficacy of this lentiviral gene therapy, we designed 9 more constructs by codon optimization and different combinations of promoters and enhancers. Achieving higher IDUA transgene expression in MPS I mice with the same dose will remarkably increase the ease of producing sufficient vector for human clinical trials, and improve safety by virtue of reducing the total exposure to lentiviral vector and insertional events. In Chapter 4, we comparatively evaluated 10 lentiviral constructs by measuring IDUA enzyme activity following transfection into HEK 293FT cells. Based on IDUA levels in cell lysates and supernatants, 6 constructs were selected for virus production and *in vivo* confirmation in MPS I mice. Also, these results provided evidence for future design of lentiviral and other vectors.

In theory, gene therapy is an ideal option for treating MPS diseases because a single injection can provide sustained IDUA expression and correction in both visceral organs and CNS. In spite of the high sustained enzyme expression, lentiviral and retroviral vectors are associated with safety concern about insertional mutagenesis. The main drawback of AAV and non-viral vectors may be that the episomal transgene expression cannot last for long. Recently, new genome editing methods, for instance, zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALENs) and CRISPRs (clustered regularly interspaced short palindromic repeats) emerged as promising strategies for gene therapy. These methods can create DNA double strand break (DSB) at specific sites. Through homology directed repair (HDR), a correct DNA sequence can be integrated and provide expression of therapeutic genes. The main advantage of these methods will be providing sustained transgene expression through integrated sequence and avoiding risk of insertional mutagenesis by specific sequence targeting. There are already some cutting-edge studies using these genome editing methods for gene therapy: ZFN (4, 5), TALEN (6) and CRIPR (7-

10). Currently, the main focus in this field is establishing successful delivery methods and eliminating off-target effects.

Closing remarks

Collectively, the results presented in this dissertation provide preclinical data supporting the design of a clinical trial to test intravenous administration of lentiviral vector in MPS I patients. This strategy could further be applied for the treatment of other lysosomal storage diseases and neurological disorders in which widespread distribution of a gene product is necessary.

Chapter 6

Biliography

Chapter 1

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Chapter 5

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