Enzyme replacement therapy treatment from birth increases therapeutic efficacy and generates tolerance to enzyme in canine mucopolysaccharidosis type I

Ashley Dawn Dierenfeld

Iowa State University

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Enzyme replacement therapy treatment from birth increases therapeutic efficacy and generates tolerance to enzyme in canine mucopolysaccharidosis type I

by

Ashley Dawn Dierenfeld

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Major: Genetics

Program of Study Committee:
N. Matthew Ellinwood, Major Professor
Joan Cunnick
Anumantha Kanthasamy
Susan Lamont

Iowa State University
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ABSTRACT

Mucopolysaccharidosis I is a lysosomal storage disease where the lack of α-L-iduronidase leads to lysosomal accumulation of heparan and dermatan sulfates. This storage results in varying degrees of cardiac, skeletal, respiratory, corneal, and neural disease. Few treatments are available to patients with limited results, especially in treatment of cardiac and skeletal complications. We treated neonatal mucopolysaccharidosis I dogs with intravenous recombinant enzyme replacement therapy at the conventional 0.58 mg/kg or a higher 1.57 mg/kg weekly dose. Higher dosage led to complete normalization of lysosomal storage in liver, spleen, lung, kidney, synovium and myocardium, as well as normalization of storage in the hard-to-treat mitral valve. There was normalization of all biochemical and functional cardiac disease and striking improvements in clinical and radiographic signs of skeletal disease. In addition to somatic improvements, high and intrathecal enzyme treatment led to decreased secondary storage burden of gangliosides in the cerebral gray matter. Importantly, all animals failed to mount an antibody response to enzyme therapy, consistent with neonatal tolerization. Taken together, these findings using the canine model advocate neonatal testing and early treatment of mucopolysaccharidosis I to more completely treat cardiac and skeletal manifestations of human mucopolysaccharidosis I disease.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

This thesis focuses on the treatment of a rare genetic disease, mucopolysaccharidosis type I (MPS I). Our study uses an animal model and a currently approved enzyme replacement therapy (ERT) and illustrates that early administration of treatment is superior to treatment initiated later in life and suggests an increased dosage may be more beneficial to patients.

Thesis organization

This thesis is organized in the journal format as outlined by the Iowa State University Graduate College. Chapter 1 reviews the current literature and outlines the significance of our study. Chapter 2 contains a journal submission of the bulk of the work, including all somatic implications of the treatment of the study. Chapter 3 investigates secondary ganglioside accumulation in the brain evident in this disease. Chapter 4 provides general conclusions on both research chapters and suggestions for future study. References for all chapters are located at the end of the thesis and are formatted according to guidelines for Science Translational Medicine.

Author contributions in chapter 2 of this work are as follows: Ashley Dierenfeld compiled and analyzed data, assisted in experiments, and spearheaded manuscript preparation; Michael McEntee conducted pathology studies; Merry Passage performed laboratory analyses and ELISAs; Steven Le conducted laboratory analyses; Jackie Jens was responsible for animal procedures and infusions; Elizabeth Snella conducted biochemistry
and molecular biology studies and necropsies; Karen Kline, Jennifer Parkes, and Jane Wengert performed IT infusions and neurological assessments; Wendy Ware and Lori Moran performed cardiology assessments; Elizabeth Riedesel and William Gross performed radiological assessments; N. Matthew Ellinwood was responsible for study design, coordination and oversight, animal procedures and manuscript preparation; and Patricia Dickson was responsible for study design, coordination, and oversight, and manuscript preparation.

In chapter 3, Ashley Dierenfeld conducted all experiments, analysis of data, and manuscript preparation. Elizabeth Snella and N. Matthew Ellinwood provided guidance on the technique used in ganglioside quantification. Additionally, N. Matthew Ellinwood and Patricia Dickson were responsible for study design, coordination, and oversight of the study. The results for the MPS I animals treated with intravenous and intrathecal ERT will be included in a future manuscript on the effects of treatment on the central nervous system.

**Literature review**

**Lysosomal storage diseases**

There are at least 41 different lysosomal storage disorders (LSDs), most caused by a deficiency in a single lysosomal enzyme. The resultant deficiency in enzyme activity leads to improper breakdown of cellular products and subsequent substrate storage in the lysosomes which distends these organelles from occupying 1% of the cellular volume up to 50% and reduces overall cellular function (1). The disease affects all cells, but lysosomal accumulation varies depending on the cell type (2). While these inborn errors of metabolism are individually rare (the most common LSD, Gaucher disease, affects 1 in 57,000 births (3)), as
a group the LSDs comprise 1 in 5,000 (1) to 1 in 7,700 live births (3). The LSDs range widely in phenotype and course of the disease, though severe cases cause mortality in the first decade of life. Typically, children develop normally for the first few years of life before losing developmental milestones, though severe cases can be diagnosed at birth while attenuated forms can remain undiagnosed for a decade or more. This variation within disease is attributed to the amount of residual enzyme activity (4). The most common clinical signs are central nervous system (CNS) involvement, skeletal disease, or a combination of the two (comprising 80%, 43%, and 35% of LSDs, respectively) (5). Clinical manifestations can also include enlarged liver and spleen, joint disease, short stature, coarse facial features, corneal clouding, cardiovascular disease, and respiratory disease (6). Depending on the substrate stored, some LSDs cause disease in multiple organ systems while others only exhibit clinical signs in the nervous system (7). Additionally, excess substrate can usually be seen excreted in the urine as a result of cell death or exocytosis (8).

Most genes causing LSDs are inherited in an autosomal recessive manner, though three are X-linked recessive. The genes of most of the LSDs have been identified and cloned, but definitive diagnosis is usually obtained through an enzymatic assay or analysis of stored substrates (7). Different mutations can lead to the same LSD leaving enzymatic methods of detection superior to genotyping an individual for diagnosis. Though genotype may help to provide families with genetic counseling (9) and may determine generalities of the course of disease (i.e. presence of neural involvement), most patients are compound heterozygotes, often with at least one private mutation, making genotype-phenotype correlation often difficult. Therefore, predicting the course of disease early on may be unreliable.
The LSDs can be grouped into broad categories based on the substrate stored and include neuronal ceroid lipofuscinoses, sialidoses, sphingolipidoses, oligosaccharidioses, mucolipidoses, type II glycogen storage disease, and mucopolysaccharidoses (10). The mucopolysaccharidoses (MPSs) are characterized by the storage of long-chain sugars called glycosaminoglycans (GAGs) and are divided into seven distinct diseases based on clinical manifestations, which reflect the pathologies associated with specific substrate storage.

**Lysosomal function**

Lysosomes have an acidic pH optimal for enzymatic digestion of molecules delivered by engulfment or fusion of digestive vacuoles. These enzymes are manufactured in the endosomal-Golgi compartment and undergo post-translational modifications including marking with a mannose 6-phosphate (M6P) moiety, which targets the enzyme for transport to the lysosome (6, 9). Not all enzyme reaches the lysosome; normal cells secrete significant amounts of M6P tagged enzyme into the extracellular space which may subsequently be taken up by other cells via M6P receptors on plasma membranes (11, 12).

Many different errors can occur while processing and trafficking the enzymes from the Golgi to the endosome: mRNA may be missing or code for an enzyme with a slightly modified primary amino acid sequence, post-translational modifications may be incorrect or absent, and recognition signals may not be appropriate for proper trafficking (13). Enzymes with abnormal primary sequence may not be folded properly, and may be targeted for degredation within the RER-Golgi apparatus. As a result of any one of these errors, metabolites build up in the lysosomes inhibiting intracellular trafficking to and from the
organelle, though it is unclear how the lack of degradation ultimately leads to cell and tissue dysfunction (14).

**Treatment**

Currently there are no complete cures for most of the LSDs, but there are several treatments available for patients including enzyme replacement therapy (ERT), gene therapy, hematopoietic stem cell transplantation (formerly known as bone marrow transplantation), pharmacoperone therapy, substrate deprivation therapy (SDT), and combination therapies. Enzyme replacement, hematopoietic stem cell transplantation (HSCT) and gene therapy help to ameliorate the disease by supplying individuals with the missing or inadequate enzyme activity while substrate deprivation therapy works to prevent anabolism of macromolecules that are stored in LSDs.

ERT was developed in 1991 for Gaucher disease (10), the most common LSD. Currently, ERT is available for nine different LSDs (7, 15) but costs $10,000 per kilogram per patient per year (16) leading to a typical annual cost of $100,000 to $750,000 (17). These therapies are typically administered as a weekly intravenous infusion and are generally well-tolerated (18, 19). Long-term ERT has led to increased range of motion, decreased urinary GAG excretion in MPSs, decreased liver size, increased height and weight, and reduced sleep apnea in MPS I patients; however, corneal clouding and cardiac disease were not corrected (20, 21) and significant heart valve disease progressed (21). Currently, no ERTs are approved to reduce the neuropathic signs of the disease and only the MPS VII mouse has shown significant enzyme levels to cross the blood-brain barrier (BBB) when administered 20 mg/kg intravenously, a level that is nearly 350 times the current approved dosage for ERT in
similar diseases \((22)\). Recently the enzyme was chemically modified and crossed the BBB, also in the MPS VII mouse \((23)\). Alternatively, intrathecal injection of the ERT engineered enzyme is showing promise for enzymatic activity in the CNS in the MPS I dog \((24, 25)\).

To date, most manufactured enzymes have a mannose 6-phosphate (M6P) modification to enable them to be taken up by the M6P receptors found on most cells \((26)\). Many enzymes are produced in Chinese hamster ovary cells as they have similar post-translational modifications as human cells, and also secrete excess enzyme into the culture media facilitating large scale production \((18)\). Large scale production is necessary as ERT is a life-long therapy for affected individuals. The enzyme for MPS I, as an example, has a five day half-life in tissues \((27)\), requiring weekly administration.

Gene therapies have shown varying degrees of success in animal models of LSDs. Major hurdles for gene therapies include delivering therapeutic amounts of gene product into specific tissues, maintaining in vivo expression, and regulating gene expression \((28)\). Long term effects of high levels of enzyme is unknown \((2)\) and vectors used in gene therapy have the potential to cause tumors \((29)\), and though they have not been seen in over 100 murine injections \((16)\), some patients and non-human primates have developed leukemia or lymphoma in HSCT directed gene therapy \((30)\). Adeno-associated virus and lentiviral vectors can infect non-dividing cells very efficiently but raise safety concerns due to oncogenic, immunogenic, and infectious potential that have not been evaluated \((31)\). Transposon-mediated gene therapy has also been investigated in the LSDs \((32)\), and immune responses are frequently seen in individuals with null mutations \((2)\). Although safety concerns are warranted, gene therapy has drastically improved skeletal symptoms in MPS VII dogs \((33,\)
Until these safety concerns can be addressed, however, gene therapy may not be widely used in patients.

Bone marrow transplantation (BMT) was the first attempted treatment for LSDs in 1981 (10, 35, 36) and remains the best treatment option in some cases. High morbidity and mortality (15-50% (37)), high cost, incomplete response to therapy, and limited positive outcomes are major downfalls to this therapy option (12, 28). Additionally, finding donor matches for HSCT to circumvent host rejection is difficult (15) and autologous HSCT elicits an immune response to cells expressing the therapeutic enzyme (38), making this therapy less appealing. Although sibling donors may provide a good genetic match, transplantation from a carrier donor is suboptimal to an individual with two functional genes (7). Recent studies have begun to evaluate the transplantation of umbilical cord blood as it has low pathogenicity and is immunologically immature (39, 40).

A benefit of HSCT is the ability for donor blood macrophages to cross the BBB and replace microglial cells in the brain of the recipient, however this process may take a very long time to accomplish (7). Children with MPS I and MPS II receiving BMT before 24 months of age have a much higher chance of normal neural development (41, 42). In MPS I murine and canine studies, enzymatic activity has been seen in the central nervous system (CNS) and lysosomal substrates were reduced with HSCT, though improvements in other body systems were inconsistent (43, 44).

In a 1999 study, BMT costs averaged $29,000 USD while annual medical costs for a severe MPS I patient without a BMT cost $56,000 USD (3). Though improvement is often seen in somatic and neurological symptoms of LSDs with HSCT, skeletal problems are
generally considered impossible to correct (7), though prevention of skeletal disease onset or progression is attainable.

Combination HSCT and ERT therapies have been introduced to try to combine the benefits of the two treatments. Mixed results pertaining to mortality and morbidity after HSCT were reported (37, 45), and one study recommended ERT for patients in poor clinical condition (37). ERT did not alter HSCT engraftment (45), and in one patient, ERT reversed airway obstruction and cardio complications before the HSCT (46). More studies need to be conducted with this combination therapy to determine its long-term efficacy and safety as these few studies had conflicting results.

Many aberrant protein transcripts in LSDs are not properly folded and, therefore, are inactive and degraded. Pharmacoperones are specific, small chemical chaperones that enhance proper folding. This therapy, dubbed enzyme enhancement therapy (18), has perhaps the most promising future as it has the ability to cross the BBB while being specific and easily administered by oral treatment (15). Cell lines with the most prevalent mutation in Gaucher disease, the most common LSD, have shown to double the enzymatic activity with pharmacoperone therapy (17), though murine trials yielded poor results in vivo (47). Not all mutations are improved by the same chaperones (15) because different mutant enzymes may be folded differently and have different epitopes for binding. Small amounts of residual activity may be enough to overcome pathology of the disease (15) and even activity levels as low as 0.4% of normal activity leads to an attenuated form of MPS I (4).

Substrate deprivation therapy (SDT) works to reduce the amount of substrate produced, theoretically leading to less storage of these molecules. Importantly, SDT will not reduce lysosomal storage in individuals without residual activity (often the most severely
affected patients), only slow progression of the disease. This is considered a viable therapy for individuals with attenuated forms of disease as residual enzymatic activity can effectively degrade the substrate and lessen the disease. It is important to note, however, that the lysosomal substrates that are being targeted have other important cellular metabolism functions and reduction of their production may severely alter other biological processes (7). Perhaps most encouragingly, SDT molecules such as miglustat have the ability to cross the BBB (48), though it is not currently approved for treatment of CNS associated complications of LSDs. Currently, SDT clinical trials are available for three different LSDs (15).

Although these treatments are currently available and help to lessen the severity of disease, in most cases they are incomplete cures. An ideal therapy would be cost-effective, safe, able to cross the blood-brain barrier when necessary, easily administered, and, above all, beneficial to the patient (1). Current therapies are approaching these goals, however certain symptoms are not relieved and the most devastating symptoms are the hardest to treat. Additionally, immune responses to any of these treatments can also hinder treatment efficacy and may damage organ systems.

**Mucopolysaccharidosis I**

MPS I was first characterized nearly 100 years ago by German pediatrician Gertrud Hurler (49) and was the first MPS described, probably because of its severity and relative prevalence. American ophthalmologist Harold Scheie and colleagues later described an attenuated form of MPS I characterized by the corneal clouding seen in patients (50). Because of its incidence, Hurler syndrome was coined MPS I and Scheie syndrome MPS V in the 1960s (51, 52), though later biochemical analysis revealed these syndromes were
variants of the same disease. In the 1970s the “Hurler corrective factor” was first described by Barton and Neufeld using cross-correction experiments (53) and was later recognized by Shapiro and Neufeld as α-L-iduronidase (IDU), the enzyme responsible for the disease (54).

MPS I is caused by loss of activity of α-L-iduronidase (EC 3.2.1.76) and attendant accumulation of the glycosaminoglycans (GAGs) dermatan and heparan sulfate. Symptoms include organomegaly, corneal clouding, skeletal deformities, cardiovascular disease, respiratory inadequacies, and varying degrees of central nervous system (CNS) involvement. MPS I can be further categorized into the severe Hurler (MPS IH), attenuated Scheie (MPS IS) and the intermediary Hurler-Scheie (MPS HI/S) based on severity and degree of CNS involvement. Hurler syndrome is the most severe of the MPSs as it produces death at the earliest age usually due to cardiac failure or respiratory complications (55). Although this continuum is helpful in categorizing clinical signs of disease, they are all syndromes of the same underlining disease and delineation between the groups is not clear-cut. Severely affected individuals often succumb to complications or respiratory or cardiac failure from the disease in the first decade of life while individuals with an attenuated form may live normal or near normal lifespans (11).

The human IDU gene is located on chromosome HSA 4p16.3 and spans 14 exons and approximately 19 kb and encompasses a sodium-independent sulphate transporter (56, 57) which is wholly found in exon II of IDU. More than 70 mutations in human MPS I have been identified (56, 58-61) and evaluated for genotype-phenotype correlations of clinical disease (4, 61). Generally, the phenotype will be associated with the least severe allele, but compound heterozygosity present in many patients makes direct correlation difficult. Null mutations lead to more severe disease while residual activity is usually associated with
attenuated forms (62). The most common two Hurler mutations, W402X and Q70X, together account for about 60% of Caucasian patients (4) while they are not seen in Israeli Arab (63) or Japanese (64) patients as these populations have their own mutations.

MPS I clinicians and researchers have developed tools for understanding this disease. A homology model of IDU was recently constructed (65), providing a structure for understanding mutations and prediction of phenotype-genotype correlations. Additionally, a patient registry was developed to help patients and clinicians develop a more complete knowledge of progression of the disease and to establish a trajectory of disease progression against which treatments can be assessed (66).

**Secondary Storage**

Gangliosides, specifically GM2 and GM3, are found elevated in MPS I neurons while virtually undetectable in normal neurons. Although it is not entirely clear why gangliosides are stored, as IDU does not function in the degradation of these glycosphingolipids (11), their storage may lead to neurodegeneration and serve as a secondary marker of disease. Gangliosides are expressed most abundantly in the brain and though GM2 has a clear role in dendritogenesis (67), the functions of other gangliosides are not clearly understood. Accumulation of gangliosides in the cell leads to severe consequences for neuronal health in the primary gangliosidoses. Ganglioside function and storage are further discussed in Chapter 3.
**Major problems in treating MPS I**

**Some tissues are less amenable to therapy.**

Although some tissues can be improved with current therapies, some tissues are considered hard-to-treat, including the heart, corneas (18, 21), and skeletal system (18). Previous studies in both animal models and human patients have seen mixed results with these tissues, probably because of the difficulty of enzyme diffusion into these compartments.

Cardiac manifestations usually include thickening of cardiac valves and large vessels (68) as accumulation of dermatan sulfate impairs elastic fiber assembly (69). Children receiving long-term ERT resolved left ventricular hypertrophy, however mitral and aortic valve thickness did not improve, and, in some cases, disease progressed. Additionally, regurgitation was not improved (70). Similarly, myocardium changes improved with HSCT (71-75), but mitral and aortic valve problems persisted and progressed (75).

Corneal clouding remains generally unresolved with treatment (76, 77). Corneal clouding has only been improved using HSCT, though improvement varied from individual to individual and was not usually completely cleared. Occasionally the clouding progressed (42).

Different strategies have shown varying degrees of success in treating skeletal disease in MPS I. Neonatal gene therapy in MPS I dogs reduced but did not completely correct skeletal problems (78), but bones were largely uncorrected by HSCT (42, 71, 79-81) and enzyme supplied via ERT is hard to take up in bone and cartilage (18).
Because of the difficulty of treating these tissues, therapy needs to be instituted before irreversible damage occurs and may require specific levels and targeting strategies to effect a response in these hard to treat tissues.

**Overcoming the blood brain barrier.**

The blood brain barrier (BBB) prevents free diffusion from the circulatory system into the central nervous system (CNS) of large charged molecules; however monocytes transplanted via HSCT or transformed via gene therapy can cross and form microglia (6) which may be beneficial to CNS disease. ERT (22, 23) and gene therapy (82-84) in mouse models and HSCT in cell lines and mouse and Rhesus monkey models have all resulted in very small amounts of enzyme present in the brain, but these strategies have not been reliably consistent in displaying this enzymatic activity.

In an effort to circumvent the blood brain barrier, studies have been conducted injecting the missing enzyme directly into the brain of MPS I rats and found enzyme could diffuse through the brain tissue and reduce lysosomal storage (85). Researchers have used the MPS IIIA Huntaway dogs to test this strategy and found high activity in the CNS but also encountered meningitis (86). Similar studies with MPS I canines have also found improved GAG clearance in the brain and meninges by injecting the enzyme through the cisterna magna, though dose-dependent immune responses have occurred (24, 25). Additionally, apolipoprotein B fusion proteins have been engineered to cross the BBB via an intravenous injection in Gaucher’s disease (83, 87). Clinical trials for intrathecal administration of recombinant human IDU (rhIDU) in MPS I human patients are underway (88, 89).
Immune response hinders treatment.

Because patients are deficient in a particular enzyme activity, it is not surprising they often mount an immune response to the supplemented recombinant enzyme. Although this would be expected in individuals that produce no residual enzyme due to a null mutation, individuals with low residual enzyme levels often have immune reactions, ostensibly because the supplemented therapeutic enzyme is different from the ineffective mutant enzyme the individual produces (90). Those with more residual activity have a reduced immune response to treatment while those with negligible enzymatic activity mount the greatest antibody response (61). The immune response ranges from complement activation (91) to specific IgGs to the enzyme (92, 93) to cytotoxic T lymphocyte activation (94) and have been seen in virtually all treatments of LSDs. Although these responses often decrease with time (20, 90), they have been shown to reduce the efficacy of treatment and have become one of the major hurdles facing LSD therapies. Transient immunosuppression may enhance ERT for LSDs, and the anti-IDU antibody binding may sterically prevent M6P binding and enzyme uptake into cell (26). This steric hindrance renders some treatments less than fully effective.

Because tolerance can occur naturally to proteins expressed in the neonatal animal, neonatal administration of therapeutic enzyme could possibly be a solution to detrimental immune responses. It is believed that the early administration of rhIDU to MPS I puppies led the animals to recognize the enzyme as self as the immune system developed, leading to a life-long tolerance of the enzyme administration as these animals never received immunosuppressive drugs (95).

Perhaps most interesting is the recent study illustrating immune tolerance improves the efficacy of ERT in MPS I canines (96). The animals tolerized via immunomodulation had
higher enzyme levels in most non-recticular organs, and lower GAG levels, lysosomal distress, and urinary GAG excretion (96). This was the first concrete evidence that immune responses reduce therapeutic attempts, speaking to the importance of inducing tolerance early to maximize therapeutic effects while pathology is progressing. Additionally, Dickson and Kakkis introduced a higher dosage of ERT and showed that tolerization in conjugation with higher dosage was more effective in correcting the heart valve defects and reducing GAGs in tissues and urine than the tolerized animals at the approved current dosage (96). Conversely, higher ERT dosage studies in human patients have been executed where GAG storage has not been significantly reduced, perhaps because of antibody production (93) not seen in this tolerization study (96).

In human ERT clinical trials, the immune responses are typically not considered harmful to the individual and seem to lessen after time (21, 90, 93). However, the new evidence linking immune tolerance to improved efficacy of treatment (96) may change this attitude as long-term treatment to the point of tolerance is a much longer time period than a 60-day immunosuppressive administration. The time lost in natural tolerization could equate to more time for GAG accumulation and consequently less time under beneficial treatment.

Animal models

There are many naturally occurring animal models for LSDs including mouse, rat, dog, cat, guinea pig, emu, quail, goat, cattle sheep, pig (12, 28, 97) flamingo and black bear (28). Mouse models provide a homogeneous genetic background, and although there are two spontaneous mouse mutations for LSDs, several knockouts have been developed for research. It is important to note that animal models are homologous to deficiencies causing
diseases in humans, and are not a direct correlation. Additionally, double knockout mice can help researchers understand lysosome function and predict human diseases (98).

Large animal models are crucial to the development of effective treatments for human disorders (99). While mice are a good starting point for many studies (so called proof of principle studies), large animal models are often preferred as pre-clinical models because they are more similar to humans based on size and heterogeneous genetic background, are long-lived, easy to image and conduct clinical assessments, and have clinical signs of disease (12, 28, 99) which may not be the case in some mouse models. Currently, there are 18 LSD dog and cat models with 13 colonies available for research (12, 28). Additionally, the nature of mutations may reveal cross-reacting immunological material (CRIM- or CRIM+) (99).

The canine model of MPS I was described at the University of Tennessee more than 25 years ago when three Plott hound littermates displayed stunted growth, progressive lameness, and visual difficulties. Biochemical analysis revealed IDU deficiencies (100) and this canine IDU deficiency was fully characterized and found to be an appropriate model for human MPS I (101). The gene and mutation have also been published (102) and affected animals are homozygous for the mutation, as all MPS I canines have arisen from the original 3 littermates (100). The canine IDU gene contains 14 exons over 13 kb and the mutation occurs in a base pair change between the 1 exon-1 intron junction resulting in a premature stop (102). The disease is inherited in an autosomal recessive manner in both the dog and human. Much like human MPS I, canines exhibit stunted growth, skeletal deformities, joint disease, corneal clouding, enlarged tongue, and heart disease (100), though skeletal disease is more severe in MPS I Hurler human patients than in dogs (78). Although it is difficult to assess mental acuity in dogs, it is believed that the canine MPS I most closely resembles
Hurler-Scheie syndrome because of the dysostosis multiplex and survivability to reproductive age \((100)\). The MPS dog model has been used to develop ERT for human patients \((92)\) and has been used in BMT \((103-106)\) and gene therapy \((76, 78)\) studies.

**Description of study**

Our study looked to evaluate the effect of treating MPS I canines from birth with conventional and a higher dose of recombinant human IDU (rhIDU) via weekly intravenous (IV) ERT. Previous neonatal studies have revealed improved outcomes \((76, 78, 107, 108)\) and it was hypothesized that immune response could be circumvented by introducing the enzyme at a young age. In an effort to treat neural disease in these animals, one group received intrathecal injections of the rhIDU once every three months in addition to their weekly IV infusion. The higher dosage of enzyme was hypothesized to reduce disease in a dose dependent fashion and perhaps cross the BBB as seen in a previous study using β-glucuronidase and the MPS VII mouse model \((22)\). Animals were enrolled in the study 12-18 months and sacrificed 48 hours after their last infusion.

Clinical and biochemical analysis were conducted for each animal. Clinical assessments include cardiology, radiology, ophthalmology, and neurology exams and analysis. Biochemical assays measuring tissue levels of soluble GAG, gangliosides, and IDU activity were conducted. Immune response to the rhIDU was assessed weekly.

**Significance**

Findings drawn from this study underscore the need to implement neonatal screening for LSDs. There are dried blood spot tests available for MPS I at this time \((109-111)\), though
they are not standard practice in neonatal testing. Our improved outcomes seen in animals treated from birth help to support the need for early screening and initiation of treatment.

Additionally, we saw improved treatment response in our higher dose animals, a finding not seen consistently in previous studies. Skeletal and cardiac disease were significantly reduced, a feat not accomplished before in MPS I ERT. This difference is believed to be attributed to the lack of an immune response seen in our animals in conjunction with the early initiation of therapy at the higher dose. The combination of neonatal administration of enzyme leading to natural tolerance and the increased dosage resulting in a dose-dependent response to therapy will be influential in the MPS I and LSD field. Children diagnosed and treated earlier with higher dosages of ERT may be able to significantly reduce the severity of disease in many different body systems and lead to a higher quality of life.

Limitations and assumptions

Although the canine model of MPS I is a valuable tool for therapeutic research of the human form of MPS I, the two diseases are not the same and therefore successful treatment in the canine model does not equate to a cure in the human disease. It has been generally accepted in the MPS community that the canine disease most closely translates to the Hurler-Scheie phenotype (100), though it is difficult to assess the mental capacities of dogs. Additionally, symptoms are not exactly the same in both organisms as discussed in the previous animal models section and immune tolerance to the rhIDU achieved in our study may not translate to human tolerance to the enzyme. These assumptions aside, the MPS I canine model has historically been a very effective model for the human form of the diseases
many clinical trials have arisen from canine studies. The results discussed in this thesis, though one of the longest-term studies on MPS I ERT in the canine model, does not follow the entire lifetime of an individual and therefore conclusions regarding long-term impacts cannot be definitively drawn. The results discussed in this thesis, though one of the longest-term studies on MPS I ERT in the canine model, does not follow the entire lifetime of an individual and therefore conclusions regarding long-term impacts cannot be definitively drawn.

Summary

LSDs are devastating diseases to young children who suffer from a deficiency in lysosomal enzyme activity. Though there are many treatments available for these diseases, there are no cures and many tissues are effectively untreatable or suboptimally treated with the current regimes. Several barriers to therapy including early diagnosis, treatment, and immune response have made current treatments inefficient. Our study evaluates altering a current treatment regime in the canine model and shows a promising future for addressing disease in human patients.
CHAPTER 2. HIGH DOSE NEONATAL INITIATED ENZYME REPLACEMENT THERAPY IN CANINE MUCOPOLYSACCHARIDOSIS TYPE I

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A.D. Dierenfeld¹, M.F. McEntee², M. Passage³, S. Le³, J.K. Jens¹, E.M. Snella¹, K.L. Kline⁴, J.D. Parkes⁴, W.A. Ware⁴, L.E. Moran⁴, J.A. Wengert⁴, R.D. Whitley⁴, D.M. Betts⁴, A.M. Boal⁴, E.A. Riedesel⁴, W. Gross⁴, N.M. Ellinwood¹,⁴, and P.I. Dickson³

¹ Department of Animal Science and the Center for Integrated Animal Genomics, Iowa State University, Ames, IA, ² Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, ³ Division of Medical Genetics, Department of Pediatrics, LA Biomed at Harbor-UCLA, Torrance CA, ⁴ Veterinary Clinical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA

Abstract

We treated neonatal mucopolysaccharidosis I (Hurler syndrome) dogs with intravenous recombinant enzyme replacement therapy at the conventional 0.58 mg/kg or a higher 1.57 mg/kg weekly dose. All animals failed to mount an antibody response to enzyme therapy, consistent with neonatal tolerization, in contrast to previous results in animals and patients treated at a later age. Higher dosage led to complete normalization of lysosomal storage in liver, spleen, lung, kidney, synovium and myocardium, as well as normalization of storage in the hard-to-treat mitral valve. There was normalization of all biochemical and functional cardiac disease phenotypes and striking improvements in clinical and radiographic
signs of skeletal disease. These findings argue for neonatal testing and early treatment of mucopolysaccharidosis I to more completely treat cardiac and skeletal disease.

**Introduction**

Mucopolysaccharidosis type I (MPS I, OMIM 607014-16) is a lysosomal storage disease caused by loss of activity of alpha-L-iduronidase (EC 3.2.1.76) and attendant accumulation of the glycosaminoglycans (GAG) dermatan and heparan sulfates. Signs include organomegaly, corneal clouding, skeletal deformities, cardiovascular disease, respiratory inadequacies, and varying degrees of central nervous system involvement. Phenotypes range from severe (Hurler syndrome) to attenuated (Scheie syndrome), and depend on the degree of residual enzyme activity associated with a given mutation (4). Left untreated, severely affected individuals often succumb to disease in the first decade of life while individuals with an attenuated form may live well into adulthood (11). Current treatment options include enzyme replacement therapy (ERT) and hematopoietic stem cell transplantation, but both approaches can be suboptimal and have attendant risks. Age at treatment varies, but for the severe (Hurler) form, the median age at diagnosis is 9.6 months, 3 months on average after the onset of symptoms (66, 112).

Recombinant human alpha-L-iduronidase (rhIDU) is used as ERT for MPS I. Current practice involves ERT administered intravenously (IV) at 0.58 mg/kg weekly (92). While this approved regime of ERT improves joint mobility and reduces urinary GAG levels and liver size, clinical studies have documented that it does not completely correct cardiac or skeletal abnormalities (21). Although cardiac ventricular hypertrophy has been resolved in children,
thickness of the mitral and aortic valves has remained unresolved (70). These are among the hardest tissues to treat and cardiac manifestations increase in severity with age.

We treated 12 MPS I dogs intravenously with rhIDU from birth. Consistent with immune tolerance to the recombinant human protein, no animals developed anti-rhIDU antibodies at any time over the 56 to 81 weeks of treatment. Tolerance, coupled with weekly administration of increased enzyme dosage, led to improved cardiac and skeletal manifestations. Regarding cardiac disease, therapy led to normal glycosaminoglycan levels in heart valve and myocardium of dogs treated with 1.57 mg/kg weekly IV ERT. These findings hold important implications for the efficacy of early ERT in MPS I patients, and support the beginning of treatment early in life.

Methods

Dogs were maintained at Iowa State University (ISU) according to IACUC protocols, and NIH and USDA requirements. The colony was founded by MPS I Plott hounds, beagles, and cross-bred dogs (113). Breeding stock originated from Harbor-UCLA, with additional breeding animals provided by Drs. Kathy P. Ponder and Mark E. Haskins. Diagnosis was by plasma iduronidase assay and PCR of the mutation in the first intron splice donor site (96, 102). Dogs began rhIDU at 3 to 23 days of age (Figure 2.1a-b). Fourteen dogs were enrolled. Two died within 7 days of birth, leaving 12 dogs which completed the study: 4 dogs received 0.58 mg/kg weekly IV rhIDU plus quarterly 0.058 mg/kg (maximum 1 mg) IT injections, 4 dogs received 0.58 mg/kg rhIDU weekly IV injections, and 4 dogs received 1.57 mg/kg rhIDU weekly IV injections. Controls included untreated MPS I affected and carrier dogs.
(termed “normal” in this manuscript), including 3 untreated MPS I and 5 unaffected dogs previously published (96). Affected controls were age-matched unless no significant age-dependent differences were observed. Normal animals (n = 12) ranged from 8-78 (23.9 ± 18.9) months and untreated affected animals (n = 12) ranged from 13-28 (19.0 ± 6.52) months.

**Administration of enzyme replacement therapy**

Lots 295292, 4986252, 5930176, and P10502 of rhIDU in buffer (100 mM sodium phosphate, 150 mM sodium chloride, 0.001% polysorbate 80, pH 5.5 – 5.6, donated by BioMarin Pharmaceutical, Novato, CA) were stored at 4°C. Enzyme was prepared within an hour of infusion by dilution in 0.9% saline (96). Dogs received 10 ml/kg weight. Conventional dosage (0.58 mg/kg) dogs received 2.5% dose during the first hour, and 97.5% of the enzyme over the second and third hours. For 1.57 mg/kg dosage, 2.5% dose was administered during the first hour, 12.5% in the second hour, and 85% in the third hour. No immunosuppressive drugs were administered. Dogs received 2.2 mg/kg diphenhydramine before each treatment to prevent allergic reactions.

**Biochemical measurements**

Euthanasia was by IV barbiturate overdose 48 hours after the last rhIDU IV infusion. Tissues were harvested immediately, frozen and shipped on dry ice to LA BioMed at Harbor-UCLA. Tissue homogenates were evaluated using 4-methylumbelliferyl-alpha-L-iduronide substrate as previously described (92, 96), with incubation at 37°C for 1 hour. Units of IDU activity (nmoles 4MU released/h) were normalized to mg protein. The Björnsson dye binding
method (114) was used with modifications (92, 96) for GAG measurements (µg GAG/mg dry weight).

**Histopathology**

Immediately after euthanasia, tissues were fixed in 10% (vol/vol) neutral buffered formalin, then paraffin embedded, sectioned, and stained with hematoxalin and eosin. Sections (4 µm) were scored blindly from 0-4 as previously published (96).

**Clinical Studies**

Mobility and posture were documented at the end of the study via video and photography. Posture, gait, joint mobility and neurological findings were assessed. Standard radiographs (lateral and ventral-dorsal (craniocaudal) views of skull, cervical spine, pelvis, forelimbs and hindlimbs) were scored blindly for abnormalities. Physeal abnormalities were scored: 0 = absent; 1 = present at any long bone site; 2 = bilateral symmetry of long bone sites; 3 = long bone and spine (endplate). Spinal changes were scored: 0 = no abnormalities; 1 = 1 disc space narrowed; 2 = >1 disc space narrowed; 3 = 1 space fused and; 4 = >1 disc space fused. Conditions in breeding stock included hip dysplasia, luxating patellae, and chondrodystrophism, which were not associated with affected status and were not included in final analyses. Carpal lucency, hip dysplasia, and stifle effusion seen in an earlier study (78) were not noted. For comparisons, IT-treated dogs were grouped with 0.58 mg/kg IV-treated dogs. Cardiac evaluations entailed echocardiograms (standard 2-dimensional, M-Mode and color flow Doppler views; spectral Doppler transvalvular velocities; and pulse wave tissue Doppler velocities at the lateral mitral annulus) and quantitative measures of cardiac size and
function. At least three individual readings were averaged per animal. Measurements of anterior mitral valve leaflet thickness used the right parasternal long axis view in diastolic frames in which the leaflet tip was clearly resolved and relatively still and measured the subjectively thickest portion. Aortic diameter was measured at the root using standard M-mode measurement from right parasternal short axis view at end-diastole using leading edge technique. Ophthalmology examinations included dazzle reflex, menace response, a papillary light and palperbral reflexes, intraocular pressure (Tonopen XL), examination of cornea and anterior chamber using hand-held slit-lamp biomicroscopy (SL-14 slit lamp, Kowa) and binocular indirect ophthalmoscopy. Pre-mortem complete blood counts, serum biochemical concentrations, and cerebral spinal fluid protein concentration, color, and cell counts were performed at ISU’s Clinical Pathology Laboratory. Serum anti-iduronidase IgG antibodies were measured by ELISA as previously published (96, 115). The OD value was calculated using dilutions in the linear signal range. Urinalysis was performed with reagent strips (Bayer Multistix 10 SG). Body weights were obtained weekly.

Statistics

Means and standard deviations were calculated in standard fashion. Multiple comparisons used ANOVA and Tukey-Kramer post-hoc adjustment with p<0.05 considered significant, except total radiograph scores which were assessed as described (77), using a general linear mixed model with treatment status and sex as fixed class variables, and age at radiographing as a random variable. Comparative analysis used 2-tailed Student’s t-test when ANOVA was not appropriate. Analyses were performed using SAS statistical software.
Results

MPS I dogs treated from birth are tolerant to enzyme replacement therapy

Affected dogs, diagnosed at birth, started ERT with rhIDU at 3 to 23 days of age (Figure 2.1). Eight dogs received 0.58 mg/kg as a once weekly intravenous infusion and four received 1.57 mg/kg as a once weekly intravenous infusion (Figure 2.1a and b). Four of the dogs receiving 0.58 mg/kg IV also received intrathecal rhIDU as part of a separate study (0.058 mg/kg every three months). Mean treatment duration was 69 weeks. All but one animal began treatment by 9 days of age or earlier; animal I-141 began at 23 days in the 0.58 mg/kg group.

Figure 2.1 Enrollment and induction of immune tolerance

(a) Study enrollment timeline for animals receiving 0.58 mg/kg weekly rhIDU (n=8), indicating age at first rhIDU dose (days) and age at sacrifice (weeks). Animals receiving concomitant IT ERT are indicated with *. IT ERT was administered at a dose of 0.058 mg/kg (maximum 1 mg) at three month intervals. (b) Enrollment timeline for dogs treated with 1.57 mg/kg weekly IV rhIDU (n=4). (c) A bar graph on a semi-log scale indicating serum anti-iduronidase IgG antibodies by ELISA depicting pretreatment and final levels expressed as OD units per µl undiluted serum. Tolerance is defined as a titer of less than 20 OD units/µl. One positive non-tolerant control animal shown for comparison (24, 96, 115).
Baseline serum anti-iduronidase antibody titers measured by ELISA ranged from 0.031 to 0.647 OD units (405 nm) per µL serum (tolerance < 20 OD units (96, 115)). Final titers ranged from 0.249 to 0.608 OD units (Figure 2.1c). Results are consistent with immunological tolerance to rhIDU in all animals.

Table 2.1 Iduronidase enzyme activity (U/hr/mg protein)

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<th>TISSUE</th>
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<th>Mean</th>
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<td></td>
<td>0.58 mg/kg (n=8)</td>
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<td>50.4</td>
<td>21.4</td>
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<td>2.51</td>
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<td>1.11</td>
<td>1.80</td>
<td>6.04**</td>
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<td>0.126</td>
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* heterozygous levels, from previously published studies (96)
**n=3

Enzyme replacement therapy from birth yields high enzyme levels and normalized GAG storage

48 hours after the last dose of IV rhIDU, animals were euthanized and tissues evaluated for enzyme activity, histopathology, and GAG storage. Iduronidase activity was increased in treated animals in liver, spleen, kidney cortex and medulla, and lung in a dose-dependent fashion (Table 2.1). Tissue GAG levels in liver, spleen, lung, myocardium, renal cortex, and renal medulla were at or below the normal range in all dogs treated from birth, including both dosing groups (Figure 2.2a-f). There was no overlap between untreated MPS I
and normal dogs, and no significant difference between dosage groups in these tissues (p≥0.60). Treated liver size (percent of body weight), was lower than affected dogs (p<0.0001) and was indistinguishable from normal levels (p≥0.54) in both dosage groups (6.50 ± 0.81% in untreated MPS I dogs, n = 5; 3.10 ± 0.35% in 0.58 mg/kg treated dogs, n = 8; 3.52 ± 0.71% in 1.57 mg/kg treated dogs, n = 4; and 3.53 ± 0.46% in normal dogs, n = 5).

**Figure 2.2 Glycoasminoglycan storage in tissues in dogs treated with IV rhIDU from birth**

(a-f) Glycosaminoglycan levels measured by Alcian blue dye-binding assay in kidney medulla, kidney cortex, liver, spleen, lung and myocardium of normal dogs, MPS I dogs treated neonatally with 1.57 or 0.58 mg/kg weekly IV rhIDU, and untreated MPS I dogs. Each dot represents one animal. Means and standard deviations were compared by ANOVA. Pairwise post-hoc analysis involved the Tukey-Kramer adjustment and p values are indicated at the top of each dot plot. Dotted lines indicate average of normal (unaffected) values.
Figure 2.3 Effects of IV rhIDU from birth on MPS I cardiac disease

(a) The GAG storage in the canine mitral valve in normal dogs, MPS I dogs receiving 1.57 mg/kg or 0.58mg/kg weekly IV rhIDU, and untreated MPS I dogs. GAG is expressed as µg per mg dry tissue. P-values show comparison of treatment group means to the mean of untreated MPS I dogs (ANOVA with Tukey-Kramer post-hoc analysis). (b) Average thickness (mm) of anterior mitral leaflet measured by 2-D echocardiography in diastole. P-values show comparison of treatment group means to the mean of untreated MPS I dogs. Each reading is the average of 3-15 measurements/dog. (c-f) Images of mitral valve by echocardiography in, (c) a normal dog, (d) a 0.58 mg/kg treated affected dog, and (e) an untreated MPS I dog. (f-i) Histopathology of the heart valve (right atrioventricular valve, black bar indicates 10 µm). (f) Normal, (g) MPS I affected treated with 1.57 mg/kg weekly IV, (h) MPS I affected treated with 0.58 mg/kg weekly IV, with mild residual lysosomal storage indicated by arrowheads, and (i) an untreated affected MPS I dog (lysosomal storage indicated by arrowheads).
MPS I dogs treated from birth have normal heart valves by biochemistry, echocardiogram, and histopathology

With IV rhIDU treatment from birth, cardiac valvular GAG levels were significantly reduced (p < 0.0001) to normal levels with an improved response in the higher dose treated animals (Figure 2.3a). Mitral valve GAG levels in treated dogs in this study were far lower than those seen in a previous study in which tolerant adult animals treated with 2.0 mg/kg weekly IV rhIDU averaged 68.9 ± 31.3 µg GAG per mg dry tissue (n = 3) (96).

By echocardiography the average thickness of anterior mitral leaflet in all treated animals was strikingly similar to normal animals (p ≥0.90) and significantly less (p ≤ 0.0009) than untreated animals (Figure 2.3b-e). Additional parameters evaluated included ejection fraction, left ventricular thickness, and aortic diameter among others, and were no different from normal in untreated MPS I dogs. Histopathology of the tricuspid valve also showed decreased lysosomal storage in treated animals (Figure 2.3f-i). The valve in the high dose treated animals was indistinguishable from those in normal animals, while trace storage was evident in the low dose treated dogs.

MPS I dogs treated from birth have more normal skeletons and joints

Radiographs were taken at ages 12.5-21.5 months for IV-treated, normal and untreated MPS I dogs (Figure 2.4), and clinical assessments, including photographs and video, were conducted pre-euthanasia. Radiographs were assessed by qualitative scoring for disc/spinal disease (scored 0-4), and physeal abnormalities (0-3), as part of a blinded post-
Figure 2.4 Effects of IV rhIDU from birth on skeletal radiographs

(a-i). Treated dogs averaged 12.8 ± 0.2 months (1.57 mg/kg, n=4), or 17.1 ± 1.0 months (0.58 mg/kg, n=8) age, affected dogs 18.1 ± 1.0 months (n=2), and normal dogs 18.4 ± 3.2 months (n=5). (a-c) Radiographs of normal, (d-f) 1.57 mg/kg treated and (g-i) 0.58 mg/kg treated MPS I affected canines, and (j-l) untreated MPS I affected canines. C3 on (b, e, h, k) indicates the third cervical vertebra. Note (j) the physeal remnant/open physis (circled) in an untreated 17 month old animal, abnormal for this age, not seen in (a) normal or (d, g) treated MPS I animals. (k) Severe disc disease seen in an untreated affected dog and (h) moderate intervertebral space narrowing in a 0.58 mg/kg weekly treated dog, not seen in (b), a normal dog, or (e), a 1.57 mg/kg/wk treated dog. (k) The circled area indicates a collapsed disc and resultant fusion. (k) also evident is C2-C3 narrowing of the intervertebral space and tipping of C3. (l) Toe splaying seen in untreated animal, but not seen in normal or treated dogs (c, f, and i). (m-o) Bar graphs indicating scoring of skeletal disease indicating average group (m) physis, (n) vertebra, and (o) total scores. Pairwise P values are indicated in the total score bar graph.
hoc analysis. Review of clinical findings and photographic documentation was also performed. Previous studies have documented the following orthopedic conditions; collapsed intervertebral spaces and vertebral disc herniation, vertebral ankylosis, osteopenia, focal articular erosions, degenerative joint disease, and joint effusions (103, 106). More recent studies have echoed these findings (78). In agreement with these earlier studies we found significant signs of spinal/disc disease in untreated dogs, including fusions of vertebral bodies in cervical, thoracic, and lumbar spine segments. In contrast to previous studies, our MPS I affected animals did not show significant signs of appendicular effusions, or dysplasia.

Comparison of treatment groups revealed clear and statistically significant (p = 0.018) radiographic differences. There was a clear significant trend of improved outcome due to enzyme dose (Figure 2.4o). Treated animals had reduced skeletal disease with reduced physeal abnormalities, less disc compression, and normal intervertebral spaces. Animals treated from birth with 1.57 mg/kg IV rhIDU displayed no spinal disc disease. In dogs receiving 0.58 mg/kg weekly IV rhIDU (with or without IT rhIDU) intervertebral disc space narrowing was seen commonly (7 of 8 dogs) in the cervical region and in other areas. Radiolucencies, consistent with persistent physeal or apophyseal endochondral remnants appeared commonly at the tibial tuberosity or humeral tubercle or head and less frequently at vertebral body endplates. These putative remnants were a consistent finding in our untreated MPS I dogs, all eight 0.58 mg/kg treated dogs and one 1.57 mg/kg treated dog.

Iduronidase activity was detected at therapeutic levels in rib cartilage in both dosing groups (Table 2.1). Histopathology showed treated animals had decreased lysosomal storage
in both rib periosteum (Figure 2.4b-d) and in synovium around the patella (Figure 2.4e-g) compared to untreated MPS I animals. The most striking clinical finding of orthopedic disease in our untreated dogs were extreme joint laxity, most notable in the carpus (Figure 2.5l), and evident splayed toes (Figure 2.4f), a stiff gait, an arched back, a characteristically upturned nose (Figure 2.5i), and limited voluntary cervical range of motion. By clinical assessment, treated dogs were clearly improved. The 1.57 mg/kg treated dogs had no signs of clinical orthopedic disease, while clinical signs were often present in the 0.58 mg/kg group, albeit improved relative to untreated affected dogs (Figure 2.5g-5l). Treated animals had normal appearing cranums with a normal mild stop as opposed to the pronounced stop (the canine equivalent of a depressed nasal bridge) and shortened and upturned muzzle seen in affected animals (Figure 2.5g-i). Additionally, they were more structurally sound, lacking the overextended carpal, metacarpal, tarsal and metatarsal joints of affected dogs (100) (Figure 2.4c, f, i, and l, and 2.5j-l).

In addition to skeletal differences, affected animals had large, winged tongues that were not seen in treated dogs. Based on blinded ophthalmologic examination, corneal clouding was present in treated animals but may have shown mild improvement, similar to previous studies (77). Histopathology analysis of corneal tissue exhibited some improvement in lysosomal storage in dogs receiving 1.57 mg/kg (Figure 2.5m-o). In dogs treated at the conventional 0.58 mg/kg dose, corneal lysosomal storage was similar to the untreated group (not shown). Anterior chamber flares were seen in untreated individuals indicating increased protein in aqueous humor, but was seen in only one of 12 treated animals.
Figure 2.5. Effects of IV rhIDU from birth on rib periosteum, joints, skull morphology, joint laxity, and cornea

(a-c) Histopathology of rib periosteum. (d-f) Histopathology of synovium around patella. Untreated affected MPS I (c and f) shows storage in both of these tissues, evidenced by the distended cells. Reduced storage in treated animals. (a-b, d-e), with no storage evident in the 1.57 mg/kg treated animals (a, d). (g-i) Skull morphology and (j-l) carpal laxity of normal dogs (g and j), MPS I affected animals receiving 1.57 mg/kg (h and k), and untreated MPS I affected dogs (i and l). Signs of skull abnormalities, including a dome-shaped head, a pronounced stop (depressed nasal bridge) and an upturned muzzle, are not evident in the normal (g), or high dose treated dog (h). (l) Severe carpal laxity in an untreated MPS I affected dog, not seen in the high dose treated (k) or normal (j) dogs. (m-o) Hematoxylin and eosin staining of the cornea from a normal dog (m), a dog treated with 1.57 mg/kg (n), and an untreated MPS I dog (o). Foamy intracellular storage is seen within the stroma in the untreated dog (o), and lessened in the treated animal (n).
MPS I dogs tolerate ERT when begun as a neonatal therapy

Animals received 831 IV rhIDU infusions without serious adverse reactions. The first two pups treated were treated at 7-8 hours post-natal, and died 2 and 6 days after the initial treatment. No obvious cause of death was found on gross post-mortem. Subsequent IV rhIDU treatments were not begun until days 3-23 days post-natal. Mild reactions to the infusions included restlessness and diarrhea. There were no fevers or changes in blood counts or serum biochemistries. Two dogs in the study, a 1.57 mg/kg IV-treated and an untreated MPS I dog developed clinically stable mild to severe cerebellar ataxia, possibly due to an earlier infection with canine distemper virus. Infection could not be confirmed in these dogs due to the chronicity of signs; however, infection was confirmed in other dogs displaying CNS signs during this period.

Discussion

Our findings in dogs treated from birth suggest that the combination of early and higher-dose treatment will increase effectiveness of ERT for MPS I. The MPS I dogs that began treatment between 3 to 23 days of age did not develop an immune response to ERT. The high dose treated dogs were indistinguishable from normal control animals in outward appearance. Skeletal radiographs and echocardiograms showed a normal to near-normal appearance of joints and heart valves, both systems which are resistant to treatment in adult animals. Radiographically, the high dose animals were not statistically distinguished from normals, and there was a clear trend favoring improved outcome relative to the conventionally dosed animals. Biochemical analysis showed normal GAG levels in the heart
valve of high dose treated dogs, which was not achieved previously with ERT. Histopathology confirmed a normal appearance of the valves. All other systemic internal organs and tissues evaluated for GAG storage showed normal GAG levels and normal histopathology in all from birth treated dogs – an outcome not seen in prior IV rhIDU studies in which treatment was initiated in older animals (92, 96, 116). The only non-CNS pathology evaluated that did not show complete or significant resolution or improvement was corneal disease. Although there was histological improvement in the corneas of high dosed dogs, clinical corneal clouding was not prevented, possibly reflecting the relative inaccessibility of this tissue. Signs consistent with uveitis were prevented, however, further study needs to better characterize ocular disease in MPS I canines to appreciate the importance of these findings.

Increased doses of ERT have previously been tested in MPS I patients. Patients ranging from 6 months to 5 years of age receiving 1.0 mg/kg weekly ERT did not show improved efficacy versus those receiving 0.58 mg/kg weekly (117). Similarly, older children and adults receiving 1.2 mg/kg weekly or 1.2 mg/kg every other week dose had a small, non-significant decrease in urinary GAG levels (93). No subjects were treated from birth, and 97-100% developed antibodies to ERT. In our previous study of a 2.0 mg/kg weekly dose in MPS I dogs, improvement in the histopathology of the heart valve was observed only when immune tolerance was present (96). Even in tolerant dogs, however, GAG levels in the heart valve did not decrease (mean 68.9 ± 31.3 µg/ mg dry weight in tolerant dogs receiving 2.0 mg/kg weekly, versus 77.5 ± 11.3 µg/ mg dry weight in non-tolerant dogs receiving the usual, 0.58 mg/kg weekly dose). In the current study, the improvement in GAG storage and
thickness of the valve in MPS I dogs treated with 1.57 mg/kg weekly from birth suggests that GAG deposition can be prevented. The absence of the immune response in these dogs also may have played a role in the increased effectiveness of the ERT.

Our study did not address how soon after birth therapy must begin in order to have the greatest effect. Dogs began treatment at 3-23 days of age. Treatment was initiated at day 23 in one animal (I-141), which had the worst score/evaluation in a number of criteria, including mitral valve thickness, corneal clouding, carpal laxity, mitral GAG, and disc disease. This finding suggests that delaying therapy 2-3 weeks, when puppy weight increases more than 2.5-fold, may adversely affect canine disease. Further study is required to address this. While our results show normal valvular GAG storage at one year of age with 1.57 mg/kg weekly ERT, we did not investigate longer term outcomes. The orthopedic response to therapy seen in the 1.57 mg/kg IV treatment group is in good agreement with the response seen in MPS I dogs treated at birth with retroviral-based neonatal gene therapy (78). Similar to our work, this study extended out to 12 months. It is unclear if improvements will persist and whether maintenance will require increased doses. The radiographic assessment of our high dose group at 12.8 months, and the low dose at an average of 16.5 months may have skewed our findings in favor of finding less degenerative changes in the marginally younger high dose treated animals. However, previous orthopedic studies of canine MPS I have found significant disease present already at or by 12 months of age (78, 106).

Two pups treated at less than 1 day of age died within a week. Necropsies did not reveal a cause of death, but neonate mortality in the first two weeks is high (17%) in this colony. There were no deaths in pups infused after 1 day of age. GAG storage levels in the
renal medulla and spleen in dogs treated with 1.57 mg/kg weekly ERT were below those of normal dogs. We saw no abnormalities in serum chemistries, nor did we see hematuria or proteinuria suggestive of renal damage or dysfunction. Enzyme infused during ERT localizes to lysosomes and has a low pH optimum, and hence presumably degrades only lysosomal GAGs. The reason for the low GAG levels in the renal medulla and spleen is unknown. In our radiographs we noted putative physeal chondral remnants in MPS I affected dogs; these were less evident in treated animals, and signs correlated with enzyme dose. These have been clear findings associated with canine MPS I affected status, albeit unremarked upon (Dr. Kathy P. Ponder, personal communication and (see (78), fig. 4K vs 4L, and supplemental materials)).

Early treatment of lysosomal storage diseases previously has been shown to improve outcomes, including some cardiac manifestations, both in animal models and human patients (41, 78, 105, 118-127). The majority of MPS I patients under 5 years old at the start of IV ERT who showed left ventricular hypertrophy at baseline normalized their left ventricular mass at 1 year (117). MPS VI cats younger than 46 hours at the start of intravenous ERT had improved clearance of lysosomal storage in hard-to-treat areas including heart valve and aortic smooth muscle; however normalization of these tissues was not achieved (119). Antibodies to ERT did not develop in the MPS VI cats treated from birth with IV ERT. MPS VII dogs treated at age 2-3 days with retrovirally-expressed canine beta-glucuronidase showed ~50% reduction in aorta and myocardial GAG; with levels 178% of normal (120). Similarly gene therapy treated neonatal MPS I dogs showed improved radiographic appearance of the skeleton at 1 or more years post-therapy, though some abnormalities still
persisted (78). In MPS I mice and dogs receiving gene therapy at birth, high resultant iduronidase serum levels prevented development of cardiac disease. However complete resolution was not seen in animals with enzyme levels lower than ~500 U/ml (76, 124). These relatively successful gene therapy studies still yielded results less compelling than those reported herein regarding the degree and consistency of responses seen in the cardiac tissue. Differences in syndromes (MPS I, VI, and VII), enzymes, levels, and kinetics of delivery (relatively steady state versus bolus delivery), either singly or in combination could explain this. Hematopoietic stem cell transplantation likewise ameliorated but did not prevent cardiac, skeletal or corneal disease in large animal models; however, animals were transplanted beyond the neonatal period (77, 103, 104, 106).

A humoral immune response to exogenous enzyme administration typically occurs in both patients and animal models, but was absent in our MPS I dogs treated soon after birth. The mutation in the canine MPS I model is reported to be a null (102), cross reactive immunological material negative (101) mutation, and immune naive adult MPS I dogs have a vigorous immune response to recombinant enzyme (92). Previous studies in canines have demonstrated that induction of immune tolerance to rhIDU increased enzyme levels and reduced GAG storage in many tissues (96). Administration of higher ERT dosage starting at a young age may be the key to immune tolerance and its impact on enzyme delivery. It may help the deficient individual accept the foreign enzyme as self, prevent an immune response to the enzyme, and thus make it more available to effect GAG clearance. Early administration also benefits the individual by reducing the initial burden sustained by tissues prior to treatment.
The improved outcomes resulting from early intervention in lysosomal storage diseases increase the urgency for the implementation of newborn screening for these disorders. A newborn screening method using dried blood spots can reliably distinguish MPS I affected individuals, carriers and controls (109-111). A pilot study of newborn screening for Pompe disease in Taiwan resulted in patients diagnosed between 9 and 22 days of age, suggesting that early initiation of therapy for lysosomal storage disease patients identified via newborn screening may be feasible (128). In addition to the implementation of an early testing program, long-term studies are also needed to verify the safety and efficacy of higher-dose ERT from birth in MPS I dogs.

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CHAPTER 3. QUANTIFICATION OF GM2, GM3, AND GD3
GANGLIOSIDE STORAGE IN MPS I AND IIIB CANINE BRAINS

To be included in a future manuscript on the effects of treatment on the central nervous system in MPS I canines

A.D. Dierenfeld¹, E.M. Snella¹, J.K. Jens¹, P.I. Dickson², K.L. Kline³, J.D. Parkes³, and N.M. Ellinwood¹,³

¹Department of Animal Science and the Center for Integrated Animal Genomics, Iowa State University, Ames, IA, ²Division of Medical Genetics, Department of Pediatrics, LA Biomed at Harbor-UCLA, Torrance CA, ³Veterinary Clinical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA

Abstract

The Mucopolysaccharidoses (MPSs) are lysosomal storage diseases where the lack of a specific lysosomal enzyme results in the accumulation of undegraded glycosaminoglycans (GAGs). In addition to primary storage of GAGs, neuropathic MPSs also show lysosomal storage of GM2, GM3, and GD3 gangliosides in the brain. Our study looked to quantify the amounts of GM2, GM3, and GD3 gangliosides in normal and affected MPS I and IIIB animals, as well as MPS I affected canines treated with enzyme replacement therapy (ERT) via intravenous (IV) and intrathecal (IT) administration. Levels of GM2, GM3, and GD3 were elevated in both MPS disease groups when compared to normal animals. Treatment of MPS I animals from birth with rhIDU decreased the secondary storage burden of gangliosides, especially GM3. Conventional dosage did not lead to statistical reduction in ganglioside storage, but the higher IV and the IT treatments may be potential treatments for
the CNS components of these disorders based on the response to therapy of these secondary accumulations.

**Introduction**

The Mucopolysaccharidoses (MPSs) are a group of 7 distinct genetic lysosomal storage disorders in which affected individuals lack a specific lysosomal enzyme leading to intercellular storage of glycosaminoglycans (GAGs). Symptoms and phenotypes of the diseases vary widely with the specific GAG stored and amount of residual enzyme activity and may include somatic or neurological manifestations or a combination thereof. Mucopolysaccharidosis type IIIB (Sanfilippo B syndrome, MPS IIIB) and severe cases of MPS type I (also called Hurler’s syndrome, MPS IH) present clinically with profound mental retardation in children, possibly as a consequence of the heparan sulfate that is stored due to the lack of α-L-iduronidase (IDU) and α-N-acetyl-glucosaminidase (Naglu), respectively, in these diseases. In addition, secondary accumulation of gangliosides in the central nervous system (CNS) is also a component of the histopathology of the CNS of the neuropathic MPSs. It is not entirely clear why gangliosides are stored in these MPSs, as the missing hydrolayses do not function in the degradation of these glycosphingolipids (11). Regardless of the mechanism of storage, their storage, at least as primary stored substrates leads to neurodegeneration as seen in the gangliosidosis diseases. It has been hypothesized that accumulating GAGs may inhibit activity of lysosomal enzymes that break down gangliosides. Notably, heparan sulfate, a GAG stored in both MPS I and IIIB, selectively binds *in vitro* to hydrolases reducing their enzymatic activity towards their original target substrate (129-131).
Gangliosides normally reside on the plasma membrane of cells and are expressed at high levels in the nervous tissues. These glycosphingolipids are important in cell signaling (132), though excess accumulation in the cell has severe consequences for neuronal health. The storage leads to altered neuronal function due to storage in the neuronal perikarya, dendrites, and axons (leading to growths termed ectopic dendrites), but does not cause cell death in early disease (133). Simultaneously, rises in GM2 levels lead to the production of new dendrites (13, 67) while normal dendrites regress. Additionally, neuroaxonal dystrophy occurs as organelles are blocked from normal axoplasmic transport forming swelling of the cell. These swellings and abnormal growths of the neurons are the major cellular consequences of lysosomal dysfunction and are thought to play an important role in neurodegeneration (133). Increasing GM3 levels in cell lines have been shown to induce neuronal cell death (134) and high levels of GD3 act as a mitochondrial toxin and induce apoptosis (135).

Interestingly, initial burden caused by excess ganglioside is not detrimental to the individual as some degree of ganglioside accumulation can occur before a decrease in neuronal function occurs (136). GM2 levels are high in times of dendritic proliferation (6, 67) and mylenation (132) in the normal developing brain, however, these increased levels are not associated with lysosomal accumulation. Decreases in the gangliosides are seen as the brain matures.

GM2 and GM3 gangliosides have been found in elevated levels in MPS I (137-140) and MPS IIIB (140, 141) patients while both are virtually undetectable in normal patients (133). Ganglioside storage has also been evaluated in MPS I canines (142, 143) and accumulation was found to be elevated as well, but less than found in human patients (143).
This is the first large report of quantification of ganglioside storage in MPS IIIB canines (a single case (144) and small-scale analysis (145) have been previously reported). Although MPS I canines do not display clear signs of CNS disease, clear neurological problems have been noted in the MPS IIIB canine model including wide stance, ataxia, head tilt, and decreased menace and nystagmus (146-148).

Our study aimed to quantify the amounts of GM2, GM3, and GD3 gangliosides in unaffected and affected MPS I and IIIB animals, the natural history of these gangliosides in the young canine brain, and MPS I affected canines treated with enzyme replacement therapy (ERT) via intravenous (IV) and intrathecal (IT) administration. Levels of all three gangliosides were elevated in both MPS disease groups when compared to normal animals. Treatment of MPS I animals from birth with rhIDU decreased the secondary storage burden of gangliosides. Conventional dosage did not lead to statistical reduction in ganglioside storage, but increased intravenous dosages and the IT treatments may be potential treatments for the CNS components of these disorders based on the response to therapy of these secondary accumulations.

Methods

The MPS I and IIIB canine colonies were maintained and cared for at Iowa State University (ISU) under approved IACUC protocols, and in accordance with the requirements of the NIH and USDA. The MPS I ISU colony was founded by two MPS I allele bearing Plott hounds with admixture of beagles and cross-bred dogs (113) whilst the MPS IIIB colony was founded by a Schipperke mated with cross-bred animals (146).
MPS I dogs were diagnosed within 24-48 hours of birth by plasma iduronidase assay (96). The genotype of all dogs used was confirmed by subsequent PCR diagnosis (96, 102). Affected dogs began enzyme replacement therapy with rhIDU at 3 to 24 days of age (Fig 2.1a-b). Fourteen dogs were enrolled in the study: 4 dogs received 0.58 mg/kg weekly IV rhIDU plus quarterly 0.058 mg/kg (maximum 1 mg) IT injections (24), 6 dogs received 0.58 mg/kg rhIDU weekly IV injections, and 4 dogs received 1.57 mg/kg rhIDU weekly IV injections. Also included in the study were untreated MPS I affected and carrier dogs. Three untreated MPS I animals ranged from 11-19 (15.9 ± 4.41) months and nine carrier animals ranged from 11-19 (24.0 ± 17.9) months and were used for comparison with these MPS I animals.

MPS IIIB canines were diagnosed within 72 hours of birth by genomic PCR (149). Animals were aged 1-65 (26.4 ± 21.5) months, both before and after clinical signs of the disease were present. They received no treatment. Twelve normal and carrier age-matched controls for these animals were 3-69 (20.1 ± 17.9) months.

Young carrier and normal animals were also assessed to determine the natural history of ganglioside storage. Two animals were evaluated within the first week of life, three at 3 weeks, three at 2 months, four at 3 months, two at 4 months, and five at 5.5 months. The 4 and 5 month animals (152 ± 18 days, n = 7) were used for comparison to the 2.0 mg/kg/week rhIDU IV treated MPS I animals (n=4, all aged 130 days). MPS I affected untreated animals (n=3) for this group were aged 171 ± 32 days.
Administration of enzyme replacement therapy

Lot numbers 295292, 4986252, 5930176, and P10502 of rhIDU in formulation buffer (100 mM sodium phosphate, 150 mM sodium chloride, 0.001% polysorbate 80, pH 5.5 – 5.6) were donated by BioMarin Pharmaceutical (Novato, CA) and stored at 4°C. The rhIDU was prepared within an hour of infusion by diluting the enzyme in 0.9% saline (96). Each dog received 10 ml per kilogram weight. For the conventional dosage (0.58 mg/kg), dogs received 2.5% dose during the first hour, and 97.5% of the enzyme over the second and third hours. For 1.57 mg/kg dosage, 2.5% dose was administered during the first hour, 12.5% in the second hr, and 85% in the third hour. No immunosuppressive drugs were administered throughout the study. All dogs received 2.2 mg/kg diphenhydramine (antihistamine) pre-treatment.

Ganglioside quantification

Ganglioside extraction, purification, and quantification was conducted as previously published (150, 151). Briefly, 200-400 mg of gray matter was dissected from the caudal third of the cerebrum, obtained from a sagittal half of a frozen brain. Total lipid extraction (151) was followed by fractionation based on polarity (150). Quantification of gangliosides was then conducted by high performance thin layer chromatography by staining for neuraminic acids and scanning densitometry. Resultant bands were calculated as mole %.

Tissue preparation. Canine brains were collected at time of necropsy. Brains were halved sagittally, covered with a frozen slurry of tris EDTA to protect from cold induced dessication, and frozen at -80°C. One day prior to dissection, brains were moved to -20°C.
The protective ice covering was carefully chipped from a section of the cerebrum, and gray matter (200-400 mg) was dissected from the white at 4°C.

**Total lipid extraction.** Lipids were extracted based on the method of Svennerholm and Fredman (152), modified by Svennerholm et al. (153). Tissue was minced to uniformity and transferred to a glass Potter-Elvenhjem homogenizer with 0.6 ml water. Gray matter was then homogenized with 2 ml methanol and 1 ml chloroform and vortexed. After 30 minutes, the extract was centrifuged at 1000g for 10 minutes and supernatant was removed. The pellet was resuspended in 0.6 ml water and 3 ml chloroform/methanol 1:2 (v/v). After another 30 minutes, extract was centrifuged at 1000g for 10 minutes; supernatants were combined and brought up to 8 ml with chloroform/methanol 1:2 (v/v) and stored at 4°C.

**Fractionation.** Fractionation based on polarity was conducted as described by Kyrklund (150). Column elution was conducted under a constant vacuum of 3-5 mmHg which yielded a flow rate of approximately of 1-2 ml/minute. Nylon stopcocks were used to ensure the columns never ran dry. Bond Elut C18 columns were preconditioned with 6 ml chloroform/methanol 1:2 (v/v) and 2 ml methanol. Six of the 8 ml total lipid extract was added to 8 ml methanol/water 1:1 (v/v), mixed, and passed through the column and collected in a 50 ml glass tube. The first tube was washed with 8 ml methanol/0.9% saline 1:1 (v/v), added to the eluate, and passed through the column again. After second elution was collected, the previous step was repeated once more. After the third pass through the column, the eluate was saved and stored at 4°C and the column was rinsed with 2 ml water. Polar lipids were then eluted with 8 ml methanol/water 12:1 (v/v). Non-polar lipids were eluted second using 8 ml chloroform/methanol 1:2 (v/v). Both eluates were dried under heat and nitrogen to 5 ml and stored at 4°C.
**Visualization.** GM2 standard was obtained from Alexis Biochemicals (Farmingdale, NY). A volume of the polar fraction equivalent to three mg of tissue was applied to a Merck HPTLC silica gel 60 matrix plate with a Camag Linomat IV (Muttenz, Switzerland). After 30 minutes of drying, the plate was developed in a chloroform/methanol/0.2% calcium chloride (55:45:10, v/v/v) solution for 1 hour. After 30 minutes of drying, the plate was stained for neuraminic acid residues using resourcinol agent (154). The plates were sprayed and placed in a humidifying chamber in a 140 ºC oven. The humidifying chamber consisted of a glass pan filled with sand, covered with glass, and containing resourcinol reagent/water (1:1, v/v) to allow the solution to reach the plate. Plates were thus charred for about 20 minutes. Resultant bands were then quantified via densitometry using an Alpha Innotech imager at 580 nm (Figure 3.1) and AlphaEaseFC software. Areas under the curve for the various species of gangliosides were converted to mole percent, based on the neuraminic acid residue number of the specific gangliosides species. Hence ganglioside values were reported as mole %.

**Statistics**

Means and standard deviations were calculated in standard fashion. Values were assessed by ANOVA and Tukey-Kramer post-hoc test for multiple comparisons with p<0.05 considered significant. Analysis was performed using SAS statistical software. Animals were pooled for comparison based on their age as previously discussed; normal and carrier dogs are grouped together as unaffected animals.
Figure 3.1 Ganglioside HP-TLC plate

HP-TLC plate shows separation of gangliosides. GM2 standard (lane 1), unaffeted canine (lane 2), MPS I affected (lane 3), and MPS IIIB affected (lane 4). Increased levels of GM2 and GM3 seen when comparing diseased canines (lanes 3 and 4) to normal (lane 2).
Figure 3.2 GM2, GM3, and GD3 ganglioside storage in canines

Unaffected (a), MPS I affected (d), and MPS IIIB (g) GM2 ganglioside storage in canines. Unaffected animals showed a clear logarithmic decay with time (a) while MPS I animals showed a steady increase of GM2 with age (d). Unaffected (b), MPS I affected (e), and MPS IIIB (h) GM3 ganglioside storage in canines. Note the increased storage in MPS I (d-f) and MPS IIIB (g-i) when compared to the unaffected animals (a-c). Dotted lines on graphs indicate 95% confidence intervals for unaffected canines over 50 days of age. Logarithmic (a-c) and linear (d-i) general trends are shown.
Results

**GM2 levels decrease with age while GM3 remains relatively steady in normal canines**

Normal and carrier canine ganglioside levels were assessed from 1 to 255 days of age. There was a clear trend of logarithmic reduction of GM2 ganglioside ($R^2 = 0.725$) with age (Figure 3.2a). This is expected as elevated GM2 during development is instrumental in dendritic proliferation (67) and mylenation (132) during times of brain development. GD3 levels showed a reduction with age as well (Figure 3.2c). GM3 showed virtually unchanged levels in days 60-186, though high values were seen in the first day of life (9.0 mol %) and a decrease was seen around 3 weeks of age (Figure 3.2b). MPS I affected untreated animals (n=8) showed a clear upward trend in their GM2 ganglioside storage with age (Figure 3.2d, $R^2 = 0.764$), a trend opposite that of the normal canines (Figure 3.2a). This increase in GM2 levels is attributed to lysosomal storage (133). GM3 levels in MPS I and GM2, GM3, and GD3 levels in MPS IIIB affected canines showed no clear correlation of storage with age, though they are clearly elevated in relation to the unaffected animals (Figure 3.2e-i).

**MPS IIIB canines have elevated GM2, GM3, and GD3 levels in the brain**

MPS IIIB canines displayed elevated GM2, GM3, and GD3 ganglioside storage when compared to age-matched unaffected controls (Figures 3.2a-c, g-i and 3.3a). The MPS IIIB levels averaged across all ages were: 3.17 ± 1.27 mole % GM2, 7.96 ± 2.49 mole % GM3, and 1.33 ± 1.39 mole % GD3 (n=18). Levels of all three gangliosides were statistically (p≤0.02) higher than age matched normal and carrier animals: GM2 0.86 ± 0.26, GM3 3.84 ±
GM2, GM3, and GD3 levels are statistically higher in MPS IIIB animals when compared to unaffected canines (a). Intrathecal and higher-than-conventional intravenous treatment in MPS I leads to reduction in GM3 levels (b-c). GM2 and GD3 levels are not statistically significantly different between untreated MPS I and unaffected animals (b-c).
0.55, and $0.35 \pm 0.17$ GD3 mole % ($n=12$) (Figure 3.3a). GD3 levels were also statistically elevated when compared to unaffected animals: $1.34 \pm 1.39$ vs. $0.35 \pm 0.17$ mole % ($p = 0.02$). Increased ganglioside levels are seen as early as 29 days of age, much younger than the 3-month-old pups previously analyzed (145).

**MPS I animals show reduction of gangliosides with ERT**

MPS I affected canines ($n=3$) showed statistically ($p=0.0003$) elevated levels in GM3 ($12.26 \pm 6.09$) levels when compared to unaffected age-matched controls ($3.8 \pm 0.56$, $n=9$), (Figures 3.2c-d, 3.3b). GM2 levels, though not statistically significant ($p = 0.59$) showed a trend towards increased storage in MPS I canines ($2.35 \pm 1.0$) when compared to normal animals ($0.86 \pm 0.3$) (Figure 3.3b). Similarly, GD3 levels were elevated in affected animals ($1.19 \pm 0.41$ vs. $0.32 \pm 0.13$, $p = 0.1$).

MPS I affected animals receiving weekly intravenous (IV) enzyme replacement therapy (ERT) from birth with recombinant human iduronidase (rhIDU) for 56-81 weeks had decreased secondary storage in the brain. Animals treated intravenously with the conventional dosage of 0.58 mg/kg displayed lower ganglioside storage; $1.95 \pm 1.24$ mole % GM2 and $8.95 \pm 1.53$ mole % GM3. A higher dosage (1.57 mg/kg) yielded lower average storage of GM2 and GM3; $1.41 \pm 0.62$ and $6.84 \pm 2.32$ mole % respectively ($n=3$). MPS I affected animals receiving quarterly 0.058 mg/kg intrathecal (IT) injections with rhIDU in addition to 0.58 mg/kg weekly IV infusions further reduced GM3 storage to $6.78 \pm 0.22$ mole % though GM2 levels were much the same as the other treated groups at $1.93 \pm 0.88$ mole %.

Statistically significant decreases from affected levels were seen in GM3 ($p = 0.04$) in the IT
treatment group, and the 1.57 mg/kg group also showed a trend of reduction \( (p = 0.064) \) (Figure 3.3b). GD3 levels were virtually unchanged from untreated MPS I values.

Animals treated from birth for 18 weeks with 2.0 mg/kg rhIDU weekly IV showed similar results. Unaffected canines aged 152 ± 18 days had GM2 levels of 0.82 ± 0.13, GM3 levels of 4.12 ± 0.38, and GD3 levels of 0.32 ± 0.09 mole % \( (n=7) \) while untreated affected MPS I animals aged 171 ± 32 days had 1.54 ± 0.50 mole % GM2, 9.26 ± 3.40 mole % GM3, and 1.02 ± 0.37 mole % GD3 \( (n=3) \). The GM3 levels between these two groups were statistically significant \( (p=0.002) \), but the GM2 and GD3 levels were not \( (p=0.20 \text{ and } p=0.10, \text{ respectively}) \). Treated MPS I affected animals \( (n=4) \) had slightly lower GM3 levels \( (6.05 ± 1.39) \) when compared to untreated affected animals but the reduction was not significant \( (p=0.065) \). GM2 and GD3 levels for this group \( (2.52 ± 0.97 \text{ and } 1.52 ± 0.84 \text{ mole %, respectively}) \) were higher than both untreated and unaffected animals (Figure 3.3c).

**Clinical signs in MPS I canines may correlate with skewed ganglioside storage**

Two MPS I affected animals, one in the 1.57 mg/kg treatment group and one untreated, developed cerebellar ataxia believed to be linked to a post-distemper virus encephalitis which was seen in an MPS I unaffected animal in the colony and was suspected in its littermate. This ataxia is not believed to be associated with canine MPS I.

Upon analysis of ganglioside levels in their brains, it was revealed that these two animals were highest in their groups for the gangliosides of interest. For the treated individual, GM3 levels were 13.4%, much higher than the other animals in the same treatment group \( (5.4, 5.6, \text{ and } 9.5\%) \). GM2 levels for this animal were intermediary in the group. Conversely, the untreated MPS I animal GM3 levels were the median of the group.
while the GM2 levels were more than twice the other values (7.5% vs. 2.5, 3.1, 3.3, and 3.5%). It is unclear why these animals exhibited high ganglioside levels, though similar results have been seen in previous encephalitis reports (155). Regardless, these animals were removed from analysis because of their clear neurological signs not seen in any of the other MPS I animals.

Discussion

Natural history of the course of ganglioside storage may be beneficial to determining when treatment of MPS diseases would be most advantageous. To this end, we have begun to analyze animals at different time points to determine when ganglioside storage becomes a burden for the developing brain. In the MPS I dog, GM2, GM3, and GD3 levels are elevated as early as 130 days of age. Elevated levels of the same three gangliosides are also seen in the MPS IIIB canine by 30 days of age. Younger and more animals affected with both of these diseases need to be analyzed to determine when increased storage begins. By determining the time point when excess ganglioside starts to accumulate in the brain, we can more effectively determine when treatment would be most beneficial in treating CNS disease associated with these MPSs, though the interval from treatment initiation to establishment of enzyme activity in the brain also needs to be evaluated.

Neurological dysfunction is clinically present in MPS IIIB (Sanfilippo) and severe MPS I (Hurler) human patients. Although neurological manifestations such as ataxia and dysmetria occur in MPS IIIB dogs and manifest between 18 and 36 months of age (147, 148, 156), these signs are not seen in clinical disease of MPS I canines. This can perhaps be attributed to phenotype of the MPS I canine model as it is believed to be a model of MPS
IH/S (100) and not IH, though it is difficult to make an absolute correspondence and correlation between the two species. That being said, two of our MPS I affected dogs (one untreated and one in the 1.57 IV dosage group) exhibited clear signs of cerebellar ataxia.

A potential confounding finding is the possibility of post-distemper virus encephalitis in one untreated affected and one treated affected, each with the highest GM levels in their respective groups. These abnormal ganglioside levels are consistent with previous findings of encephalitis (155).

While GM3 levels were significantly reduced in the intrathecal treatment group, we saw a trend towards significance in both of the higher dosage treatment groups (1.57 and 2.0 mg/kg groups). Conversely, the conventional 0.58 mg/kg weekly IV dosage was not different from untreated animals (p = 0.36). GM2 or GD3 levels, on the other hand, did not reach significance when comparing untreated affected and unaffected animals (p = 0.59 and p = 0.11, respectively) though levels of these gangliosides were elevated in nearly all animals.

Successful treatment of GM3 and not GM2 or GD3 may be a result of the location of the gangliosides in the brain, as it has been shown that gangliosides localize to different cellular locations in MPS (140). GM3 is found near blood vessels of the brain, and not located as deep as the GM2 and GD3 (77, 140). It therefore is reasonable that the response we see to IV or IT ERT is a result of enzyme reaching areas near vasculature or the surface of the brain where the enzyme would come into contact with diseased cells.

Our MPS I affected animal ganglioside levels are comparable to previously published studies. Early quantification of a single MPS I affected animal yielded 4.3 mole % GM3, 3.8% GM2, and 9.2% GD3 levels in the gray matter while the normal counterpart displayed 1.9, 2.6, and 3.1 mole % of GM3, GM2, and GD3, respectively (143). While our numbers
vary a bit from these results, they are similar to a more recent study encompassing 12 animals using comparable quantification methods as used in our study. This publication \cite{142} showed GM2 levels of 4.6 ± 0.5 and GM3 15.7 ± 1.3 for affected MPS I canines while normals were GM2 1.9 ± 0.1 and GM3 5.5 ± 0.4. Animals in the same study treated with gene therapy reduced their ganglioside storage in MPS I affected animals to 1.9 ± 0.1 mole % GM2 and 7.0 ± 0.5 mole % GM3 \cite{142}. Ganglioside levels in the IV and IV/IT treated animals in the current study are comparable to these gene therapy findings, however our animals are older (7.09 ± 2.63 vs. 13.2 ± 5.47 months) so we may in fact be preventing detrimental neuronal storage for a longer period of time. Additionally, it is noted that their animals were unable to stand, eat, and were losing weight at the end of their study \cite{142} causing variability in their endpoints; our animals’ endpoints were predetermined based on their treatment group and all 16 were able to eat and stand at time of necropsy.

Secondary GM2, GM3, and GD3 CNS storage in MPS I and IIIB patients is also seen in canine patients. Treatment of MPS I animals from birth with rhIDU decreases this secondary storage burden. Conventional dosage does not lead to statistical reduction in ganglioside storage, but the 1.57 and 2.0 mg/kg IV and the IT treatments may be potential treatments for the CNS components of these disorders based on the response to therapy of these secondary accumulations. These findings are important in advocating for the higher dosage and intrathecal administration of enzyme for human patients at a young age to help prevent irreversible damage in the MPS brain.
CHAPTER 4. GENERAL CONCLUSIONS

General discussion

The canine model of MPS I has been a very effective model for human disease and was first used when developing ERT in human patients (91). Successes in earlier ERT studies in canines have led to positive therapeutic results in human patients in clinical trials and have led to the currently approved treatments. Because of past accomplishments with the correlate of canine MPS I to human disease, we would expect that our findings in this study would also translate well to patients.

Our findings in dogs treated from birth suggest that the combination of early and higher-dose treatment may increase the efficacy of enzyme replacement therapy for MPS I. Dogs with MPS I that began treatment with intravenous ERT between 3 to 23 days of age did not develop an immune response to ERT. The high dose treated dogs were, with the exception of corneal disease, indistinguishable from normal, age-matched control animals in outward appearance, and radiographic and cardiologic findings. Biochemical analysis showed normal GAG levels in the heart valve of high dose treated dogs, confirmed by histopathology, which was not achieved previously with ERT. All other systemic internal organs and tissues evaluated for GAG storage showed normal GAG levels and normal histopathology in every neonatally treated dog analyzed –an outcome not seen in prior IV rhIDU studies in which treatment was initiated in older animals (92, 96, 116). The only non-CNS pathology evaluated that did not show complete or significant resolution or improvement was corneal disease.
CNS disease was also reduced as evidenced by decreased GM3 ganglioside storage present in the brain. Though full analysis of GAG storage and IDU activity in the CNS is still pending, these preliminary results indicate that early ERT intervention was beneficial in these animals. Intrathecal administration of enzyme was superior to intravenous administration in reducing GM3 storage; however increased IV dosages were clearly more effective than the conventional dosage. This speaks to the increased ability of the enzyme to cross the BBB in higher dosages and through the CSF, though penetration deep within the brain is still not achieved.

**Recommendations for future research**

**Increased ERT dosage**

Our study illustrates the potential benefit of increased enzyme delivery in immunotolerant animals, suggesting higher than currently approved enzyme levels may be beneficial in disease treatment. This has been seen in previous MPS I canine studies(96) but not in human patients, perhaps because of antibody production (93). Our animals were treated from birth and did not develop antibodies to the enzyme, most likely due to the naivety of the immune system at this young age. This possible change to the protocol may help improve the efficacy of treatment if antibodies are not produced, which would require early administration or immunomodulation.

Our next study will look to determine if the increased ERT dosage is required as a life-long therapy. The current weekly administration of IDU is expensive, and increased dosage would cost more to patients. The most damaging and irreversible lysosomal storage occurs in developmental stages of life (I), so perhaps the increased dosage is only required
during peak growth phases. We will begin neonatal administration of rhIDU in the MPS I dogs at the higher dose and continue during development followed by the conventional dose in the adult phase. This will help us to determine if the higher dosage is effective in treating disease during development or if the dosage is required as a long-term therapy. Additionally, we need to extend our study for a longer period of time to determine if the higher dosage is safe in a long-term situation.

Another approach would be to combine the higher dosage IV ERT with the IT treatment. In our study, the higher IV dosage was the most beneficial treatment for somatic tissues while the IT treatment was most efficacious in reducing secondary GM3 storage in the brain. By combining these two treatments beginning in the canine MPS I neonatal period, perhaps we can more effectively ameliorate disease in both somatic and CNS tissues.

**Characterization and treatment of corneal disease in MPS I**

The only somatic tissue not showing significant improvement in our study was the cornea. Although this tissue has historically been a difficult compartment to treat, we have very little understanding of the development and time course of corneal clouding in MPS I. Future work would characterize corneal disease progression in the MPS I canine and also provide a clear benchmark for assessing severity of corneal disease. We have seen corneal clouding apparent in animals as young as 130 days; it is unknown when the first symptoms of ocular disease are present in these animals.

Because the corneal disease is not corrected by the higher IV dosages, we may need to treat the cornea using a different therapy. One approach is a gene therapy/stem cell treatment in which transduced stem cells expressing IDU would be injected into the cornea.
This would allow a tissue-specific response to therapy and hopefully generate a more favorable response in the cornea.

Natural history of ganglioside storage

Though normal and carrier brains were also evaluated to quantify the storage of GM2, GM3, and GD3 during development in this study, this venue needs to be further explored. GM3 mole % levels appear as a clear biomarker for both MPS I and IIIB at all timepoints tested, while GM2 is an appropriate biomarker of disease in MPS IIIB only. Our results indicate that increased storage can be seen as young as 29 days in MPS IIIB and 130 days in MPS I animals, though younger affected animals were not evaluated. By assessing when the onslaught of ganglioside storage begins in MPS I and IIIB individuals, perhaps we can better understand the importance of early therapies and also fuel the newborn screening efforts. Our numbers can serve as a starting point, but more research is needed before definitive conclusions can be drawn.

Implications for human clinical research and treatment

This study provides the LSD community with promising somatic results not previously seen with ERT. Although it may be difficult to execute a clinical trial without first implementing neonatal screening, it is reasonable to enroll patients with a family history of MPS I as they would most likely be screened and diagnosed shortly after birth. Families in this situation, if given the opportunity, may be likely to enter their young children in such a study given the promising results shown in our research.

Aside from MPS I treatment, our study implies that any ERT administered at birth may be able to circumvent an immune response and provide more effective treatment of
disease. Eight other LSDs utilize ERT and may benefit similarly to neonatal administration of treatment, though our results cannot be expected to be duplicated in every LSD as the diseases, enzymes, and enzyme delivery varies. Additionally, more enzyme therapies may emerge in light of improved efficacy and given its relatively safe history. For example, no ERT is currently available for MPS III patients as it is a CNS disease and ERT has not been shown to be beneficial in the brain in humans. Our results of reduced GM3 storage in the brain and our pending GAG storage and IDU activity analysis coupled with recent promising IT findings in MPS IIIA canines(86) may lead to the development of IT ERT for these diseases. Although we would not expect ERT to be a complete treatment for these individuals, it would be a huge stepping stone as these patients currently have very limited treatment options with inadequate results.

**Neonatal screening**

Early administration may be the solution to more completely treating MPS I and many other LSDs; it may help the deficient individual to accept the foreign enzyme as self and reduce the immune response to the antigen. Early administration benefits the individual by reducing the initial burden sustained by tissues before treatment. This, however, would require early detection of the disease and newborns without a family history of LSDs are currently not screened though diagnoses can be made in utero (7). There is a push within the LSD community to change newborn screening to include LSDs (1, 7) and simple blood spot tests may be available in the near future to help diagnose young patients (109, 128, 157, 158). In addition to the ease of the testing, LSDs are twice as common as PKU, a disease routinely
screened for after birth using blood collected from a heel prick, suggesting that screening for all LSDs is economically feasible (42).
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