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Effect of neonatal gene therapy on lumbar spine disease in mucopolysaccharidosis VII dogs

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Abstract

Mucopolysaccharidosis VII (MPS VII) is due to deficient β -glucuronidase (GUSB) activity, which leads to accumulation of chondroitin, heparan, and dermatan sulfate glycosaminoglycans in various tissues including those of the spine. Associated spine disease can be due to abnormalities in the vertebrae, the intervertebral discs, or other spine tissues. The goal of this study was to determine if neonatal gene therapy could prevent lumbar spine disease in MPS VII dogs. MPS VII dogs were injected intravenously with a retroviral vector (RV) expressing canine GUSB at 2 to 3 days after birth, which resulted in transduction of hepatocytes that secreted GUSB into blood. Expression was stable for up to 11 years, and mean survival was increased from 0.4 years in untreated dogs to 6.1 years in treated dogs. Despite a profound positive clinical effect, 6-month-old RV-treated MPS VII dogs still had hypoplastic ventral epiphyses with reduced calcification in the lumbar spine, which resulted in a reduced stiffness and increased range of motion that was not improved relative to untreated MPS VII dogs. At six to 11 years of age, ventral vertebrae remained hypoplastic in RV-treated MPS VII dogs, and there was desiccation of the nucleus pulposus in some discs. Histochemical staining demonstrated that discs did not have detectable GUSB activity despite high serum GUSB activity, which is likely due to poor diffusion into this relatively avascular structure. Thus, neonatal gene therapy cannot prevent lumbar spine disease in MPS VII dogs, which predicts that enzyme replacement therapy (ERT) will similarly be relatively ineffective even if started at birth.

Keywords

Lumbar spine; mucopolysaccharidosis VII; gene therapy; bone; intervertebral disc; gibbus deformity

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1. Introduction

The mucopolysaccharidoses (MPS) are a subset of lysosomal storage disorders characterized by deficiencies in enzymes that contribute to the degradation of glycosaminoglycans (GAGs) [1]. Disease manifestations can occur in the skeleton, liver, cardiorespiratory system, eyes, ears, central nervous system, and other sites, although the severity in different tissues varies with the specific type of MPS. The pathogenesis may involve binding of GAGs to the TLR4 and/or components of the complement cascade, resulting in induction of inflammatory cytokines and destructive enzymes [2, 3].

Major manifestations of most types of MPS are skeletal abnormalities, collectively referred to as dysostosis multiplex, which are prevalent and adversely impact the patient's quality of life [4-6]. Odontoid hypoplasia can occur in the cervical spine and is associated with neck instability. The superior-anterior aspect of lumbar and thoracic vertebrae can be hypoplastic, resulting in collapse of a vertebral body anteriorly to create a wedge-shaped vertebrae that may be associated with deformity such as kyphosis (or gibbus), and/or scoliosis. Intervertebral discs can degenerate leading to nerve root or spinal cord compression. Finally, thickening of ligaments, dura, and bony structures can also contribute to spinal cord compression [7-12]. Spine disease that is severe enough to require surgery occurs in ~10% of patients with the severe form of MPS I (Hurler syndrome) at a median age of 4 years, ~15% of patients with intermediate-severity MPS I at a median age of 13 years, and ~15% of attenuated-severity MPS I at a median age of 21 years [13]. The apparent lower incidence in patients with more severe disease likely reflects their reduced survival.

MPS VII (Sly Syndrome) is due to deficient β -glucuronidase (GUSB) activity, which leads to accumulation of chondroitin, heparan, and dermatan sulfate GAGs [9, 10]. Although data on the frequency of spinal disease in patients with MPS VII are not available due to the low incidence of MPS VII (~1:1,000,000), gibbus deformities have been reported [8, 9]. The naturally-occurring MPS VII dog has a missense mutation (R166H) in the GUSB gene and exhibits many of the musculoskeletal manifestations of the disorder observed in humans [14-17]. In recent studies, we demonstrated that the lumbar vertebrae of 6-month-old MPS VII dogs had radiolucent cartilaginous lesions with reduced calcium and increased GAG content in the ventral and dorsal regions, suggestive of a failure to convert cartilage to bone during development [18]. This was likely responsible for the reduced stiffness and increased range of motion found in lumbar spine segments from MPS VII dogs [18]. Additionally, the disc annulus fibrosus (AF) contained elevated levels of GAGs compared to those in normal animals [18].

Treatments that are currently used clinically for MPS include hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT), while gene therapy is being investigated in animal models. The efficacy of HSCT is likely due in large part to migration of enzyme-sufficient normal blood-derived cells into tissues, which secrete enzyme with mannose 6-phosphate (M6P) locally that can be taken up by nearby cells via the M6P receptor, although secretion of enzyme into blood could also play a role. HSCT has not prevented spine disease [19], which may be because of the relatively avascular nature of many spine components and the paucity of blood-derived cells. ERT is only available for certain types of MPS and involves intravenous injection (IV) of M6P-modified enzyme into blood, which can diffuse to other tissues and be taken up via the M6P receptor. The effect of ERT on spine disease is somewhat difficult to gauge, as the first drug was only approved in 2003, and many of the patients had manifestations of skeletal disease at the time of therapy initiation. Some have reported that spine stability is adversely affected in MPS by ERT, which may relate to clearance of GAGs from soft tissues, allowing structural instability from

ligament and bone abnormalities to be manifest [20]. One gene therapy approach involved intravenous (IV) injection of a gamma retroviral vector (RV) expressing canine GUSB to newborn MPS VII animals, which led to transduction of liver cells and secretion of M6P-modified GUSB into blood, from where enzyme could diffuse to other organs and be taken up via the M6P receptor [21]. Gene therapy has shown promise in correcting some musculoskeletal manifestations of MPS, as RV-treated MPS VII mice and/or dogs have improved growth plate morphology, partially normalized lengths of bones including the vertebral bodies, improved facial morphology, and reduced erosions of femoral heads [22, 23]. However, the effect of gene therapy on the biomechanical function of the spine has not been addressed. The objective of this study was to determine if neonatal RV-mediated gene therapy of MPS VII dogs could reduce lumbar spine disease.

2. Methods

2.1. Animals and sample preparation

The animals used in this study were raised at the School of Veterinary Medicine at the University of Pennsylvania, under NIH and USDA guidelines for the care and use of animals in research. At the start of the project, dogs that were heterozygous for MPS VII (GUSB^{+/-}) were bred to generate affected GUSB^{-/-}, heterozygous GUSB^{+/-}, or homozygous GUSB^{+/+} dogs that were identified by PCR of blood cell DNA at birth and confirmed with GUSB enzyme assay of serum. The gamma Moloney murine leukemia-based LNL6 vector designated hAAT-cGUSB-WPRE contained an intact LTR with the canine GUSB cDNA downstream of the human α_1 -antitrypsin promoter [21, 24]. It was packaged in the amphotropic GP+AM12 cells and concentrated by ultrafiltration followed by gel filtration to remove low molecular weight contaminants as reported previously [24]. The vector was titrated by performing GUSB staining on GUSB-deficient murine fibroblast-derived 3521 cells that were transduced after serial dilution of the vector. Some MPS VII dogs were injected IV at 2 to 3 days of age with 3 to 10 $\times$ 10⁹ transducing units (TU)/kg of hAAT-cGUSB-WPRE and are designated as RV-treated dogs. One MPS VII dog (M1287) received hepatocyte growth factor (HGF) prior to injection of a higher dose of RV (2 $\times$ 10¹⁰ TU/kg) as detailed previously [21], and is designated as the HGF/RV-treated dog. Later, RV-treated GUSB^{-/-} males were bred with heterozygous females to generate affected MPS VII and heterozygous unaffected dogs. Normal dogs at 6 months of age or earlier included both homozygous GUSB^{+/+} and heterozygous GUSB^{+/-} animals, that were all phenotypically normal. Most unaffected controls were littermates of MPS VII dogs or had at least one parent in common. Older normal dogs were heterozygous GUSB^{+/-} females that had been used for breeding. Radiographs were obtained while under anesthesia with intramuscular injection of 0.02 mg/kg of atropine (Phoenix Pharmaceutical, St. Joseph MO) and 0.1 mg/kg of hydromorphone (Elkins-Sinn, Cherry Hill NJ), and IV injection of 2 mg/kg of propofol (Abbott, Chicago IL). Euthanasia was performed for animals with substantial clinical manifestations, or for collection of tissues, using 80 mg/kg of sodium pentobarbital (Veterinary Laboratories, Lenexa, KS) in accordance with American Veterinary Medical Association guidelines. Kaplan-Meier log-rank survival analysis was performed for untreated and RV-treated MPS VII dogs that were born between June of 2000 and June of 2010.

2.2. GUSB assay and urine GAG assays

Serum GUSB activity was measured using 4-methylumbelliferyl (4-MU)- β -D-glucuronide substrate and determination of the fluorescent product 4-MU using a fluorometer, as described previously [21]. Extracts from the annulus fibrosus (AF) were prepared by homogenization of samples as described [25], and GUSB activity was normalized to the amount of protein in the sample. Enzyme activity of 1 Unit (U) indicates conversion of 1

nmole of substrate to product in one hour at 37°C. Eight μm -thick frozen sections were stained for GUSB activity using naphthol AS-BI- β -D-glucuronide as described [21].

2.3. Histology and biomechanics

Lumbar spines (T12-sacrum) were dissected out immediately following euthanasia and frozen. L2-L3 spine segments were thawed, fixed for 1 week in buffered 10% formalin, then decalcified for 1-2 weeks in formic acid/EDTA (Formical-2000; Decal Chemical Corporation, Tallman, NY). A 5 mm thick, mid-sagittal slab was processed into paraffin and 10- μm -thick sections were double-stained with alcian blue and picrosirius red to demonstrate GAG and collagen, respectively [18]. Histology was undertaken for 6-month old normal, untreated MPS VII and RV-treated MPS VII animals, and for older normal and RV-treated MPS VII animals.

L1-L2 spine segments (vertebra-disc-vertebra) were thawed and lateral radiographs taken using a fluoroscope (Hologic, Bedford, MA, USA). Biomechanical testing was carried out according to techniques published previously by our laboratory [18]. Briefly, following overnight equilibration in PBS, samples were tested in 20 tension-compression loading cycles between 45 and -90 Newtons (N) (Instron, Norwood, MA, USA) at a strain rate of 0.3 mm/second. Compressive and neutral zone stiffness (N/mm) and range of motion (mm) were calculated as described previously [18]. Biomechanical testing was performed for 3 RV-treated dogs at 6 months of age, and were compared with those for age-matched normal and untreated MPS VII dogs that were previously evaluated using the same personnel and equipment [18].

2.4. GAG, water, and calcium content

Samples were obtained from whole nucleus pulposus (NP) and outer ventral AF by sharp dissection of the L5-L6 disc and by bone shears to obtain cortical and trabecular bone of the ventral epiphysis of the L6 vertebral body. Epiphysis samples were divided into two equal portions and pulverized. Specimens were weighed to determine wet weight, dried overnight at 60°C, and re-weighed to determine dry weight and calculate the water content. NP and AF samples and half of the epiphysis were then digested overnight at 56°C using proteinase K. Sulfated GAG was measured using the dimethylmethylene blue assay [26]. The second half of the epiphysis was incubated overnight in 0.5 M HCl, and total calcium content was measured using the α -cresolphthalein complexone technique [27]. Sulfated GAG and calcium were normalized to the sample dry weight.

2.5. Statistical analysis

Values for normal, untreated MPS VII, and RV-treated MPS VII dogs were compared using ANOVA with Holm-Sidak post-hoc analysis (Sigma Plot 12; Systat Software Inc, Chicago, IL, USA). Kaplan-Meier log-rank survival analysis was also undertaken using this software.

3. Results

3.1. MPS VII dog survival with RV treatment

MPS VII dogs were injected IV at 2 to 3 days after birth with the RV designated hAAT-cGUSB-WPRE. This was previously demonstrated to result in transduction of liver cells, which could secrete M6P-modified GUSB into serum, after which the enzyme was taken up by cells in other tissues in a fashion analogous to ERT [21]. Twenty dogs have been treated with neonatal RV since the initiation of this gene therapy project in 2000. M1287 is designated as the HGF/RV-treated MPS VII dog, which was treated as detailed in the methods, and was sacrificed at 11 years of age for poor mobility.

The other 19 treated dogs did not receive any stimulus for hepatocyte replication, and are designated RV-treated MPS VII dogs. One RV-treated dog (M2159) had a marked fall in expression at approximately 2 months after gene transfer that we hypothesize was due to a cytotoxic T lymphocyte response to the normal canine GUSB protein (similar to that seen in MPS I cats [28]), and was sacrificed at 4 months without any further clinical evaluation. Four dogs died suddenly of an unclear etiology after gene transfer (two at 1 week, and two at 3 months). For the other 14 RV-treated MPS VII dogs, 3 are still alive at 2 to 3 years of age, and 7 were sacrificed at 6 months to 5 years after gene transfer for the collection of tissues, but did not have any serious clinical signs. Of the 4 other RV-treated dogs, two were sacrificed at 6 or 10 years for poor mobility, one was sacrificed at 8 years for a benign hemangioma that turned out to express the RV (KPP and MEH, unpublished data), and one was sacrificed at 8 years for a forelimb cellulitis that did not heal with antibiotics. The Kaplan-Meier log-rank survival curve of untreated and treated (both RV- and HGF/RV-treated) MPS VII dogs is shown in Fig. 1A. The mean survival for the untreated MPS VII dogs was 0.4 years, while the mean survival for the treated dogs was dramatically increased to 6.1 years ($p < 0.001$). For normal (GUSB^{+/-} and GUSB^{+/+}) dogs (not shown), approximately 10% died in the first month, but there were few spontaneous deaths thereafter. Normal dogs were generally sacrificed while in good health.

3.2. Serum GUSB activity

Serum GUSB activity (Fig. 1B) is a simple measure of the level and stability of expression, although achieving normal levels in blood does not necessarily mean that enzyme can diffuse to all tissues. The average serum GUSB activity for the entire RV-treated group was 884 ± 1093 U/ml (3.6-fold normal). The RV/HGF-treated MPS VII dog had 15,935 U/ml of GUSB activity in serum over his lifetime, which was 65-fold normal, and more than 3-fold that for any other treated animal.

3.3. Urinary GAGs

Urine GAGs are elevated in patients with MPS, and their reduction is a commonly-used biomarker of improvement with therapy. For example, urine GAGs were reduced by 63% with ERT in patients with MPS I [29] and by 49% with ERT in patients with MPS II [30], although urine GAGs remained above normal in most patients in both of these previous studies. In this study, untreated MPS VII dogs that were 6 months or older had urine GAG levels that were 119 ± 23 μ g of GAG/mg of creatinine (N=20), which was 17-fold ($p < 0.001$) the value of 7.1 ± 5.5 μ g of GAG/mg of creatinine in normal dogs (N=55), as shown in Fig. 1C. Treated MPS VII dogs (both HGF/RV- and RV-treated) had 32 ± 23 μ g of GAG/mg of creatinine in urine at 6 months or older (N=23, as the same dog was sometimes assayed more than once), which represented a 74% reduction from the value in untreated MPS VII dogs ($p < 0.001$ vs. MPS VII), but values remained 4-fold normal ($p < 0.001$ vs. normal).

3.4. Radiographic analysis of RV-treated dogs at 3 months to 1 year of age

Radiographs were used to evaluate the time course of the appearance of abnormalities in the lumbar spine. Representative radiographs from 3 months to 1 year after birth for normal, untreated MPS VII, and RV-treated MPS VII dogs are shown in Figure 2. The end-plate of the epiphysis of normal dogs was smooth at all ages, and calcification extended all the way to the periphery. In contrast, calcification was absent in the ventral and to a lesser extent the dorsal epiphysis at 3 and 6 months in the untreated MPS VII dogs, and was probably also reduced at 1 year, although the irregularities of the end-plate surface made this difficult to evaluate. RV-treated MPS VII dogs still had reduced calcification in the ventral epiphysis at all of these ages, although the middle region of the epiphysis had a smoother surface than the untreated MPS VII dogs. The radiographs shown were representative of those from a total of 20 different normal (17 female and 3 male), 22 different MPS VII (9 female and 13

male), and 14 different RV-treated (4 female and 10 male) dogs at 6 months or older. Radiographs from the HGF/RV-treated MPS VII dog M1287 were similar to those of RV-treated dogs. There were no apparent differences between males and females for any of the groups.

3.5. Histological and radiographic analysis of RV-treated dogs at 6 months of age

Lumbar spines (L2-L3) from normal, untreated MPS VII, and RV-treated MPS VII dogs were evaluated histologically and radiographically after dissection at 6 months of age (Fig. 3). The specific RV-treated MPS VII dogs that were evaluated in detail here had an average serum GUSB activity of 792 ± 507 U/ml (2.8-fold normal), which was similar to the average of 3.6-fold normal for the entire RV-treated group. These dogs had 142 ± 64 U/mg of GUSB activity in the liver (46% of normal), which was similar to the activity observed previously in RV-treated dogs of 182 ± 38 U/mg [21]. These dogs had increases in body weights, improvements in gait, and increases in vertebral body heights (data not shown) that were similar to those reported previously for dogs with similar levels of serum GUSB activity [21, 23].

Alcian blue (GAGs are blue) and picrosirius red (collagen is red) staining revealed large cartilaginous zones in the ventral and to a lesser extent the dorsal epiphyses of RV-treated MPS VII dogs (Fig. 3C), which were only slightly improved compared to spines of untreated MPS VII dogs (Fig. 3B). Corresponding radiographs (Fig. 3E-F) indicated that these lesions were not calcified. The central region of the epiphyses contained trabecular bone in both the untreated and RV-treated MPS VII dogs.

3.6. Mechanical properties of lumbar spines at 6 months of age

Biomechanical testing was performed to assess functional differences for L1-L2 bone-disc-bone spine segments between normal, untreated MPS VII, and RV-treated MPS VII dogs at 6 months of age (Fig. 4). Compressive stiffness is the elastic resistance to compressive loading, neutral zone stiffness is elastic resistance within the range over which the segment moves with minimal resistance, and range of motion is the magnitude of motion that occurs between the peak compressive and tensile loads applied. Spine segments from untreated MPS VII dogs had compressive stiffness that was 66% of normal ($p=0.05$ vs. normal) and a neutral zone stiffness that was 19% of normal ($p=0.04$ vs. normal). RV-treated MPS VII dogs had a compressive stiffness that remained low at 75% of normal [not significant (NS) vs. normal ($p=0.15$) or MPS VII ($p=0.45$)] and a neutral zone stiffness that was 41% of normal [NS vs. normal ($p=0.12$) or MPS VII ($p=0.45$)]. The range of motion for the MPS VII dogs was elevated at 2.2-fold normal ($p=0.008$ vs. normal), and remained somewhat elevated in RV-treated animals at 1.6-fold normal [NS vs. normal ($p=0.12$) or MPS VII ($p=0.11$)]. These data suggest that RV treatment results in at best marginal improvement in biomechanical function of MPS VII spine segments, although failures to find significance for some comparisons may have been related to the small sample size.

3.7. GAG, water, and calcium content of lumbar spines at 6 months of age

GAG and water content were determined for the NP, the outer AF, and the vertebral epiphysis, while calcium content was determined for the vertebral epiphysis (Fig. 5). In the NP, the GAG and water contents were not significantly different between the 3 groups. In the outer AF, there were no significant differences in GAG levels per wet weight between the 3 groups using ANOVA, although the value in untreated MPS VII dogs was 2.3-fold that of normal dogs, which was significant with a Student's t-test ($p=0.04$), as reported previously [18]. Outer AF GAG for the RV-treated MPS VII dogs was 2.2-fold normal [NS vs normal, $p=0.21$ and NS vs untreated MPS VII ($p=0.91$) when compared using Student's t-tests]. The water content of the ventral outer AF of MPS VII animals was increased at 1.3-

fold normal ($p < 0.001$ vs. normal, ANOVA with Holm-Sidak post-hoc analysis), while in RV-treated animals it was 1.1-fold normal ($p = 0.07$ vs. normal) and 84% of untreated MPS VII ($p = 0.004$ vs. MPS VII). Since a primary function of GAGs is to bind water, the modest reduction in GAGs in the outer AF of RV-treated dogs may be responsible for their reduced water content.

In the ventral vertebral epiphyses, the GAG content of the MPS VII animals was markedly increased to 8-fold normal ($p = 0.02$ vs. normal), which remained elevated in RV-treated animals at 9.4-fold normal [$p = 0.02$ vs. normal but NS vs. untreated MPS VII ($p = 0.51$)]. The water content of MPS VII animals was elevated to 2.2-fold normal ($p < 0.001$ vs. normal) and remained elevated in RV-treated animals at 2.0-fold normal [$p < 0.001$ vs. normal, NS vs. untreated MPS VII ($p = 0.06$)]. The calcium content of MPS VII animals was reduced to 46% of normal ($p = 0.002$ vs. normal) and remained low in RV-treated animals at 44% of normal [$p = 0.002$ vs. normal, NS vs. untreated MPS VII ($p = 0.85$)]. These data confirm the histological data and demonstrate that the ventral epiphyses from RV-treated dogs still have marked increases in GAGs, which are likely responsible for the increase in water content. The reduction in calcium content in the epiphyses of RV-treated MPS VII dogs correlates well with the absence of radiographic calcification in this region, and the failure to normalize biomechanical properties.

3.8 Radiographic analysis of older RV- treated MPS VII dogs

Spines from some normal and treated MPS VII dogs were evaluated at 6 to 11 years. Radiographs shown in Fig. 6 demonstrate that a normal dog continues to have smooth vertebral body margins and intact intervertebral spaces at 8 years. In contrast, both the RV-treated dogs and the HGF/RV-treated dog had marked abnormalities, which included irregularities of the vertebral endplate and reduced dimensions of the intervertebral space. These observations suggest that disc degeneration occurred. Histochemistry (Fig. 7) shows that a normal dog has well-formed vertebral bodies with a smooth surface and an intact disc. Both the RV-treated dogs and the HGF/RV-treated dog still had some cartilaginous lesions in the ventral epiphyseal region, collapse of the L2-L3 intervertebral disc space, and a highly disorganized structure of the AF. Older untreated MPS VII dogs could not be evaluated, as they did not survive beyond 2 years of age.

3.9. GUSB activity in spines

GUSB activity was determined in the AF of lumbar spines using extracts that were homogenized from samples. Normal dogs had 125 ± 81 U/mg of GUSB activity (data not shown), which was ~50% of the level observed in normal liver. GUSB activity was not detectable in AF from MPS VII dogs, and there were not sufficient amounts of samples from the RV-treated dogs to test GUSB activity from lumbar AF. However, samples from the cervical spine were available, and frozen sections were stained for GUSB activity, as shown in Supplementary Figure 1. This demonstrated that cells in the normal AF and NP had a strong red signal indicative of GUSB activity, while untreated MPS VII and RV-treated MPS VII dogs had no detectable red signal. These data suggest that GUSB is not able to diffuse into the AF or the NP of treated MPS VII dogs despite high serum GUSB activity. GUSB staining was not performed on the epiphysis, as frozen sections are difficult to obtain from calcified tissues.

4. Discussion

4.1. Long-term expression after neonatal RV-mediated gene therapy

Gene therapy has the potential to result in long-term correction of disease manifestations in MPS. We have previously reported that neonatal IV injection of an RV can result in high

serum GUSB activity and correct many manifestations in MPS VII dogs, but long-term expression has not been previously reported, and the effect on spine disease has not been evaluated in detail. We report here that the mean survival was increased in the treated MPS VII dogs (both HGF/RV- and RV-treated) to 6.1 years, up from 0.4 years in untreated MPS VII dogs. Although the survival was shortened by performing euthanasia for animals with substantial clinical manifestations, as required ethically, similar criteria for euthanasia was applied to both treated and untreated MPS VII dogs. The quality of life of treated MPS VII dogs was substantially improved, as all could walk throughout their life, albeit with some difficulty after approximately 6 years, while untreated MPS VII dogs could not stand or walk beyond 6 months.

The kidney is a site that does not express substantial levels of the RV after gene therapy [31], and GUSB activity in this organ can be used to estimate the delivery to a vascular tissue and compare it with the levels achieved after ERT. The level of GUSB activity achieved in the kidney of RV-treated dogs of 11 ± 10 U/mg [31] at 6 months after transfer is similar to the level achieved in kidney at one week after repeated administration of 4 mg/kg of GUSB enzyme to MPS VII mice of 8 ± 1 U/mg [32]. This leads us to conclude that the amount of enzyme produced in RV-treated MPS VII dogs approximates or is even higher than that achieved with ERT, although there are numerous caveats to this estimation, while the production in the HGF/RV-treated dog almost certainly exceeds that delivered with ERT. The only adverse event noted to date was the development of a benign hemangioma in the spleen of one dog that expressed RV sequences (KPP and MEH, unpublished data). Overall, the risk/benefit of this gene therapy appears to be very beneficial, although the hemangioma is a major concern.

Reductions in urine GAGs are a commonly-used biomarker for correction of disease [29, 30], although urine GAGs have generally not been normalized, and it has been unclear as to whether or not this is predictive of correction of clinical manifestations. In this study, urine GAGs were reduced to a similar extent in the RV-treated dogs and the HGF/RV-treated dog. The average urine GAGs in the treated dogs was reduced to 74% of that seen in untreated MPS VII dogs, although they remained elevated at 4-fold normal. It is not clear whether or not urine GAGs are derived from blood GAGs that are filtered through the kidney, or from GAGs that accumulate in the kidney. From a prior study, we know that GAGs remained in both the cortex and the medulla of the kidney of RV-treated MPS VII dogs based on histochemical analysis [31], so it is possible that this failure to normalize urine GAGs was due to the failure to prevent GAG accumulation in the kidney.

4.2. Neonatal gene therapy does not prevent hypoplastic vertebrae

Spine disease is a major source of morbidity in patients with MPS, and has been very difficult to treat. Previously, we demonstrated that the ventral epiphyses of untreated MPS VII dogs had reduced calcium and that motion segments had reduced stiffness to forces and increased range of motion compared with those in normal dogs [18]. The stiffness measured from indentation tests was most abnormal in the non-calcified regions of the epiphysis, while the AF had normal tensile stiffness, leading to the conclusion that the abnormal calcification of the epiphysis was the major factor that increased the joint laxity in the lumbar spine in MPS VII dogs at 6 months of age.

The current study demonstrates that RV treatment failed to normalize calcification of the ventral epiphysis of the vertebral bodies at 6 months, as demonstrated histochemically, radiographically, and biochemically. In addition, GAGs remained elevated in the ventral epiphysis, while neutral zone and compressive stiffness of lumbar motion segments were not significantly increased and the range of motion was not significantly reduced relative to untreated MPS VII dogs. The region that will become the epiphysis consists of cartilage

early during development, and calcification initiates in the secondary ossification center of the middle of this region, then spreads to the periphery. We speculate that conversion of epiphyseal cartilage to bone is somehow inhibited by GAG accumulation, and that the avascular nature of cartilage makes it difficult for the 300 kD GUSB tetramer to diffuse into this region, which is about 3 mm thick at 3 months. Biomechanical studies were not performed in older RV-treated animals, as limited numbers of samples were available, and untreated controls could not be obtained as they cannot live beyond 2 years. However, radiographs demonstrated that non-calcified regions with GAG accumulation persisted in the region of the ventral epiphysis as late as 11 years after birth in treated dogs, although the central regions were calcified.

The failure to prevent vertebral body hypoplasia contrasts with our previous demonstration that lumbar bone lengths were partially corrected in RV-treated MPS VII dogs, where L5 vertebral body lengths were 67% of normal in untreated MPS VII dogs, and were increased to 93% of normal with gene therapy [23]. In this study, the RV-treated dogs whose spines were evaluated with biomechanics at 6 months had similar improvements in their L5 vertebral bone lengths (data not shown). Although the growth plate that is responsible for long bone growth is also cartilage and hence avascular, the thickness of the growth plate is only ~0.5 mm in dogs at 3 months, which may allow some GUSB enzyme to diffuse into the growth plate and potentiate growth.

Kyphoscoliotic deformities, such as a gibbus, are a major source of morbidity in patients with MPS, and are associated with spinal cord compression and back pain. It is likely that the hypoplasia that is seen radiographically in the anterior epiphysis in humans with MPS VII [8, 9] is a major factor in the development of gibbus deformities, and that this is due to a failure to convert cartilage into bone, as is the case in the MPS VII dogs. The absence of gibbus deformities in MPS VII dogs may relate to quadruped locomotion, which would reduce the mechanical stress placed upon the non-calcified regions of the ventral epiphysis compared with the analogous anterior epiphysis in biped humans. The failure to normalize calcification of the ventral epiphysis in RV-treated MPS VII dogs leads to the prediction that gene therapy, or by analogy ERT, may not prevent gibbus deformities in humans.

4.3. Neonatal gene therapy does not prevent intervertebral disc degeneration

Another important aspect of spine disease in MPS is disc degeneration. Discs may herniate and cause spinal cord compression, or contribute to subluxation due to vertebral misalignment [33]. In this study, the discs that were evaluated at 6 months were relatively intact with histochemical evaluation in untreated and RV-treated MPS VII dogs. This is consistent with our previous study demonstrating that the biomechanical function of the AF was normal in MPS VII dogs at 6 months [18]. However, the discs were severely abnormal at 6 and 8 years of age in RV-treated MPS VII dogs, and at 11 years in the HGF/RV-treated MPS VII dog, where NPs were absent, AFs were markedly abnormal histochemically, and the intervertebral space was frequently collapsed. As shown in the accompanying paper [Smith et al, Pathogenesis of Lumbar Spine Disease in Mucopolysaccharidosis VII], disc degeneration may be caused by upregulation of proteases such as cathepsin B, which can cleave aggrecan and collagen, which are important components of the NP and AF, respectively. These data suggest that neonatal gene therapy, and by analogy ERT starting at birth, will not prevent disc disease in patients. The disc is relatively avascular, which may explain why GUSB activity was not detected within either the AF or the NP.

4.4. Summary and implications

In this study we have demonstrated that neonatal gene therapy results in stable expression for up to 11 years and prolongs life in MPS VII dogs. However, it fails to prevent lumbar

spine disease. Since our method of gene therapy likely works primarily by secreting enzyme by liver into blood and subsequent uptake of enzyme by other tissues, this leads to the prediction that ERT will not prevent spine disease even if started in the neonatal period. This failure is likely due to the relatively avascular nature of the disc and the cartilage precursor of the epiphysis, making it difficult for enzyme to diffuse to these sites from the blood. Vertebral body disease might be treated by drugs that potentiate cartilage to bone transition in the epiphysis, if such a drug can be identified. Intervertebral disc disease might be treated by drugs that inhibit destructive proteases, or that inhibit the signal transduction cascade or inflammatory cytokines that are likely responsible for the upregulation of these destructive proteases. The MPS VII dog is an excellent model in which to study the effect of novel therapies on the spine, as it reproduces many of the abnormalities that are seen in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Expression of β -glucuronidase in blood is stable for up to 11 years in MPS VII dogs after neonatal intravenous injection of a retroviral vector and survival is increased compared with that in untreated dogs.
2. Lumbar spines of treated MPS VII dogs still have reduced calcification and increased laxity at 6 months due to the inability of GUSB to diffuse into spine tissues.
3. Gene therapy treated MPS VII dogs have intervertebral disc and vertebral body bone degeneration at 8 to 11 years.

Fig. 1. Survival curves, serum GUSB activity, and urine GAGs

A. Survival of treated and untreated MPS VII dogs. MPS VII dogs were either untreated, or were injected with the RV designated hAAT-cGUSB-WPRE at 2 to 3 days after birth. One dog (M1287) received HGF prior to injection of RV to attempt to induce hepatocyte replication and potentiate transduction, while 19 dogs received RV without HGF. The survival of the indicated number of dogs using Kaplan-Meier log-rank survival analysis is shown. For the untreated MPS VII dogs, 34% died spontaneously at a mean age of 1 month, 5% developed liver shunts with high blood ammonia and were euthanized at a mean age of 3 months, 43% were euthanized for other clinical indications such as poor mobility, sepsis, or pneumonia at a mean age of 4 months, and 18% were sacrificed at a mean age of 6 months for the collection of tissues and were censored from the survival curve. For the treated MPS VII dogs, 4 dogs (20%) died spontaneously at a mean age of 2 months, 4 dogs (25%) were euthanized for musculoskeletal abnormalities at a mean age of 8.6 years (including the HGF/RV-treated dog M1287 at 11 years), 1 dog (5%) was euthanized for a hemangioma at 8 years (dog M1328), 1 dog (5%) lost expression of GUSB at an early age and was killed without a clinical indication and was censored, 7 dogs (35%) were killed for the collection of tissues without clinical signs and were censored, and 3 dogs (15%) are still alive and were censored. **B. Serum GUSB activity.** Serum GUSB activity for the HGF/RV-treated MPS VII dog M1287 and for the RV-treated MPS VII dogs at the indicated ages is shown. Normal dogs had 284 ± 55 U/ml (SD) as indicated by the yellow shaded box, while untreated MPS VII dogs had <1 U/ml, as indicated by the low initial value. **C. Urine GAG.** Urine GAGs were normalized to creatinine levels for the indicated number of animals from each group at 6 months of older. The values were compared with ANOVA with Holm-Sidak post-hoc analysis, and all groups were statistically different from each other with a p value <0.001 .

Fig. 2. Radiographs at 3 months to 1 year

Lateral radiographs of the lumbar spine were obtained from normal, untreated MPS VII, or RV-treated MPS VII dogs at the indicated age. The growth plate appears as a non-calcified cartilaginous region between the epiphysis and the diaphysis of the vertebral body and is identified at 3 months for the normal dog with a black arrow. The caudo-ventral and the cranio-ventral epiphyses are identified with white arrows and white arrowheads, respectively. For the normal dog, the growth plate had almost disappeared at the time of puberty of ~6 months, and was absent at 1 year. For the untreated MPS VII dog, the epiphyses were irregular, and failed to fully extend ventrally even as late as 1 year after birth. For the RV-treated MPS VII dog, the endplates were better-defined than for the untreated MPS VII dog, but a lack of calcification in the ventral region of the caudal aspect of L2 was still present, creating a sharp point referred to as beaking.

Fig. 3. Histochemistry and radiographs of lumbar spines at 6 months

Normal, untreated MPS VII, or RV-treated MPS VII dogs were evaluated at 6 months of age. **A-C. Histology.** Mid-sagittal sections of the L2-L3 spine level were stained with alcian blue and picrosirius red to identify GAGs and collagen, respectively. **D-F. Radiographs.** Lateral radiographs of the L1-L2 spine from the same animal. Labels: af = annulus fibrosus, np = nucleus pulposus, e = epiphysis, vb = vertebral body, gp = growth plate. On all images, ventral is to the left and dorsal is to the right. Radiolucent cartilaginous lesions (*) in the epiphyses are identified in some animals. Three animals were evaluated for the RV-treated MPS VII dogs and 4 dogs were evaluated for each of the other groups and gave similar results to those shown here.

Fig. 4. Biomechanical Properties

Spine segment biomechanical testing for an L2-L3 motion segment (vertebrae-disc-vertebrae) was performed for the indicated number of normal (2 male and 2 female), untreated MPS VII (2 male and 2 female) and RV-treated MPS VII dogs (3 male) at 6 months of age. **A. Compressive stiffness. B. Neutral zone stiffness. C. Range of motion.** Values were compared using ANOVA with Holm-Sidak post-hoc analysis; * indicates that values in the groups connected with a bracket had a p value of 0.01 to 0.05, while ** indicates a p value <0.01.

Fig 5. Biochemical Composition

Samples were obtained at 6 months of age from normal, untreated MPS VII, and RV-treated MPS VII dogs, and composition analysis was performed. GAG and water content were determined for the NP and outer AF of the L5-L6 disc, while GAG, water, and calcium content were determined for the ventral epiphysis (E) of L6, with normalization to the dry weight. **A. GAG content. B. Water content. C. Calcium content.** The same number of animals was evaluated as in Fig. 4, and statistical analysis used the same techniques.

Fig. 6. Radiographs of older dogs

Normal or RV-treated MPS VII dogs were evaluated at the indicated age. No untreated MPS VII dogs were evaluated as they cannot survive beyond 2 years. The number of the vertebral body is identified. White arrows indicate sites of unequivocal loss of intervertebral space.

Fig. 7. Histochemistry of older dogs

Alcian blue-picrosirius red staining (for GAG and collagen respectively) of L1-L2 spines (A-D) and corresponding radiographs (E-H) were obtained at the indicated age from normal and from RV-treated and HGF-RV-treated dogs with the indicated average serum GUSB activity. Labels: af = annulus fibrosus, np = nucleus pulposus, vb = vertebral body. On all images, ventral is to the left and dorsal is to the right. Radiolucent cartilaginous lesions (*) in the epiphyses are identified in some animals.