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Pathogenesis of lumbar spine disease in mucopolysaccharidosis VII

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Abstract

Mucopolysaccharidosis type VII (MPS VII) is characterized by deficient β -glucuronidase (GUSB) activity, which leads to accumulation of chondroitin, heparan and dermatan sulfate glycosaminoglycans (GAGs), and multisystemic disease. MPS VII patients can develop kyphoscoliotic deformity and spinal cord compression due to disease of intervertebral discs, vertebral bodies, and associated tissues. We have previously demonstrated in MPS VII dogs that intervertebral discs degenerate, vertebral bodies have irregular surfaces, and vertebral body epiphyses have reduced calcification, but the pathophysiological mechanisms underlying these changes are unclear. We hypothesized that some of these manifestations could be due to upregulation of destructive proteases, possibly via the binding of GAGs to Toll-like receptor 4 (TLR4), as has been proposed for other tissues in MPS models. In this study, the annulus fibrosus of the intervertebral disc of 6 month-old MPS VII dogs had cathepsin B and K activities that were 117- and 2-fold normal, respectively, which were associated with elevations in mRNA levels for cathepsins as well as TLR4. The epiphyses of MPS VII dogs had a marked elevation in mRNA for the cartilage-associated gene collagen II, consistent with a developmental delay in the conversion of the cartilage to bone in this region. A spine from a human patient with MPS VII exhibited similar increased cartilage in the vertebral bodies adjacent to the end plates, disorganization of the intervertebral discs, and irregular vertebral end plate morphology. These data suggest that the pathogenesis of destructive changes in the spine in MPS VII may involve upregulation of

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cathepsins. Inhibition of destructive proteases, such as cathepsins, might reduce spine disease in patients with MPS VII or related disorders.

Keywords

Lumbar spine; mucopolysaccharidosis VII; bone; intervertebral disc; cathepsins; inflammation; dog

1. Introduction

The mucopolysaccharidoses (MPS) are a subset of lysosomal storage disorders characterized by deficiencies in enzymes that contribute to degradation of glycosaminoglycans (GAGs), and which exhibit multi-systemic disease manifestations [1]. Spine abnormalities are prevalent, can require surgery, and significantly impact patients' quality of life [2, 3]. MPS VII (Sly Syndrome) is characterized by deficient β -glucuronidase (GUSB) activity, which leads to accumulation of chondroitin, heparan and dermatan sulfate GAGs [4, 5]. The impact of MPS VII on the spine can include odontoid hypoplasia, vertebral body collapse, thoracolumbar kyphosis and scoliosis, intervertebral disc degeneration, and spinal cord compression [4–9]. The naturally occurring MPS VII dog has a missense mutation (R166H) in the GUSB gene [10] and exhibits many of the musculoskeletal manifestations of the disorder observed in humans [11–13]. In recent studies, we demonstrated that the lumbar spines of 6-month-old MPS VII dogs had radiolucent, cartilaginous lesions in the ventral and dorsal regions of the vertebral epiphyses, suggestive of a failure to convert cartilage to bone during development [14], and which was consistent with a reduction in calcium in those regions. In addition, the vertebral endplates and intervertebral disc annulus fibrosus (AF) of MPS VII dogs contained elevated levels of GAG compared to those from normal animals [14]. Functionally, lumbar spine segments from MPS VII dogs exhibit reduced stiffness (increased laxity) and increased range of motion, which are likely due to a combination of the abnormalities in structure and composition [14].

The molecular bases of these abnormalities in MPS VII lumbar spines have not been described, limiting the development of new therapeutic strategies. In other tissues, such as joints and the aorta, upregulation of destructive enzymes such as matrix metalloproteinases (MMPs) and cathepsins (Cts) are believed to play an important role in the pathogenesis of MPS [15, 16]. These enzymes degrade key components of the extracellular matrix including elastin, collagens and proteoglycans [17]. Elevation of destructive enzymes in MPS may be due to induction of inflammatory pathways, such as those induced by Toll-like receptor 4 (TLR4) [18–20]. While liposaccharide (LPS) is the classical ligand of TLR4, heparan sulfate, one of the GAGs that accumulates in MPS VII, acts as an endogenous ligand of TLR4 and activates an inflammatory response via the NF κ B signaling pathway [21, 22]. TLR4/MPSVII double knockout mice have improved cranial and long bone morphology and growth plate organization compared to MPS VII mice [23]. TLR4 expression is significantly elevated in the aorta of MPS VII dogs [15] and in cells of the articular cartilage and synovium of MPS VI rats [20]. The first goal of this study was to determine if upregulation of proteolytic enzymes, inflammatory cytokines, and/or TLR4 contributes to extracellular matrix breakdown and altered biomechanical function in the lumbar spines of MPS VII dogs.

Humans with MPS VII are known to have hypoplastic anterior vertebrae [4], which can result in the collapse of the vertebral body and contribute to a gibbus deformity. The spine also exhibits broad destructive changes in the vertebral bodies and intervertebral discs that can result in pain and contribute to joint instability. However, histopathological evaluation

of a spine from a patient with MPS VII has never been reported. The second objective of this study was to perform this analysis on a spine that was obtained from a 19-year old patient with MPS VII.

2. Methods

2.1. Animals

The dogs 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. Females that were heterozygous for MPS VII (GUSB^{+/-}) were bred with retroviral vector-treated GUSB^{-/-} males to generate heterozygous GUSB^{+/-} or homozygous GUSB^{-/-} dogs, which were identified by PCR of blood cell DNA at birth and confirmed with GUSB enzyme assay of serum. Most normal controls were littermates of MPS VII dogs or had at least one parent in common. Radiographs of the lumbar spines were obtained while under anesthesia with an 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 an 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.

Lumbar spines (T12-sacrum) were dissected out immediately following euthanasia. For enzyme activity and mRNA analyses, the entire nucleus pulposus (NP) and the ventral AF were removed via sharp dissection from the T12-L1 disc. Samples from the ventral epiphysis of the L1 vertebral body that contained both cortical and trabecular bone were removed using bone shears.

2.2. Enzyme Activity Assays

For cathepsin activity assays, samples were homogenized with a hand-held homogenizer in 100 mM sodium acetate pH 5.5 containing 2.5 mM ethylenediaminetetraacetic acid (EDTA), 0.01% Triton X-100, and 2.5 mM dithiothreitol (DTT), and centrifuged at 10,000g for 5 min at 4°C as described previously [16]. For the total cathepsin assay, approximately 0.3 µg of the supernatant was incubated with 100 µM benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-AMC) from Anaspec (San Jose, CA) at pH 7.5 in 100 mM sodium acetate with 2.5 mM EDTA, 0.01% Triton X-100, and 2.5 mM DTT in a microtiter plate at 37 °C. The amount of product was determined by excitation at 355 nm and emission at 460 nm using kinetic readings and comparison with 7-amino-4-methylcoumarin (AMC) standards from Anaspec. One unit (U) of enzyme produced 1 nmole of the product per hour at 37 °C. The protein concentration was determined with the Bradford assay (BioRad Laboratories; Hercules CA). The cathepsin B assay was performed using the same extracts and the substrate Z-Arg-Arg-AMC (Bachem; Torrance, CA) at pH 7.5. CtsK activity was measured at pH 7.5 with 10 µM of the substrate 2-aminobenzoic acid-HPGGPQ-N-(2,4-dinitrophenyl)-ethylenediamine (Abz-HPGGPQ-EDDnp) (Anaspec), which is cleaved by CtsK but not other cathepsins, and 2-aminobenzoic acid was the standard. The CtsD assay was performed at pH 4.0 with 10 µM of the substrate 7-methoxycoumarin-4-acetyl (Mca)-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys-2,4-nitrophenyl (Dnp)-D-Arg-NH₂, which can also be cleaved by CtsE, with Mca-Pro-Leu-OH (Enzo Life Sciences) as the standard. CtsK and CtsD assays were read at 320 nm for excitation and 420 nm for emission. Inhibition assays were performed as described previously [16] using cathepsin inhibitors obtained from Calbiochem (San Diego, CA) and included the CtsK inhibitor I [1,3-Bis (N-carbobenzoyloxy-L-leucyl) amino acetone; #219377] and the CtsB inhibitor Ac-Leu-Val-Lysinal (#219385). Samples were incubated

with the inhibitor for 10 min prior to starting the assay. An MMP-12 enzyme activity assay was performed using a kit from Anaspec as detailed previously [16].

2.3. Messenger RNA Levels

Frozen tissue samples from the NP, AF and ventral vertebral epiphysis were homogenized for 30 seconds in a Mikro-Dismembrator (Braun Biotech International, Melsungen, Germany), then 1 ml of Trizol was added, and RNA was isolated using a Qiagen column as described previously [16]. Reverse transcription was performed on 1 µg of DNase I-treated RNA with an oligo (dT) 20 primer using a Superscript III kit (Invitrogen Corp; Carlsbad, CA) in a 20 µl volume, followed by real-time PCR on 0.4 µl of each cDNA sample per well using SYBR green reagents (Applied Biosystems; Foster City, CA). Primers are listed in Supplementary Table 1. The percent of a test RNA to that of β-actin was calculated by subtracting the cycle to reach the threshold (C_T) for a gene from the C_T for a separate real-time assay using β-actin primers to determine the ΔC_T , and the formula: Percent β-actin = $(100) \times 2^{\Delta C_T}$. PCR reaction efficiency was determined for each primer by running serial dilutions of two control samples, and the slope demonstrated to be similar to that of β-actin. In addition, data were only accepted if the dissociation curve observed at the end of 40 PCR cycles was sharp and consistent for all the samples.

2.4. Statistical Analysis

Values for samples from normal and MPS VII dogs were first compared using unpaired Student's t-tests and Sigma Plot 12 software (Systat Software, Inc; Chicago, USA). If normality or equal variance tests failed, values were compared using the Mann-Whitney U test. Significance was defined as $p < 0.05$.

2.5. Radiological and Histological analysis of Human Spinal Tissue

Lumbar spine tissue comprising the intervertebral disc and vertebral bone was obtained at post-mortem at age 19 and fixed with formalin, decalcified, and embedded in paraffin. Four µm-thick sections were either stained with Masson's trichrome, double stained with alcian blue and picrosirius red [14], or stained with picrosirius red alone and viewed under polarized light [15]. Thoracolumbar radiographs were obtained prior to death at age 18.

3. Results

3.1. Enzyme Activity

We demonstrated previously that the endplate surface is markedly irregular at 6 months [14], and demonstrate in the accompanying article [Smith et al, "Effect of neonatal gene therapy on lumbar spine disease in mucopolysaccharidosis VII dogs"] that several intervertebral discs had degenerated at 8 to 11 years of age in MPS VII dogs that were treated with neonatal gene therapy with a retroviral vector. To test the hypothesis that these abnormalities were due to upregulation of destructive enzymes, cathepsin activities in extracts of AF, NP and epiphyseal bone in normal and untreated MPS VII samples were evaluated using the fluorogenic substrate Z-Phe-Arg-AMC (Figure 1A), which releases the fluorescent product AMC upon cleavage by a cathepsin. This particular substrate is cleaved by most cysteine cathepsins, and, thus, this represents a general cathepsin assay, although some cathepsins have reduced activity at neutral pH. General cathepsin activity in MPS VII dogs was significantly elevated in the AF at 11,663 $\pm$ 3922 U/mg (110-fold normal; $p = 0.001$). General cathepsin activity was readily detectable in the NP at 88 $\pm$ 65 U/mg in MPS VII dogs, which was 440-fold normal ($p < 0.001$), although the absolute level was not as high as in the AF. General cathepsin activity was also high in the epiphysis of MPS VII dogs at 9,166 $\pm$ 269 U/mg, or 9-fold normal ($p < 0.001$).

The promiscuity of the general cathepsin assay made it difficult to determine which of the 11 cysteine cathepsins contributed to activity. Therefore, assays were performed with inhibitors known to be relatively specific for distinct cathepsins, as shown in Figures 1B and 1C. For samples from the AF and the epiphysis, the majority of the enzyme activity was inhibited with 10 nM of a CtsB inhibitor, suggesting that most of the activity was due to CtsB. The CtsK inhibitor had slight inhibition of the CtsK activity at 100 to 1000 nM, but this is a concentration where the inhibitor has some activity against CtsB [16]. The inhibitor assay was not performed for the NP samples, as the amount of material was more limited and the enzyme activity was lower. The specificity of the cathepsin present in the extracts was also assessed using the substrate Z-Arg-Arg-AMC, which is relatively specific for CtsB, as shown in Figure 1D. The enzyme activity was very similar to that observed for the general cathepsin assay, further confirming the hypothesis that the majority of the activity was due to CtsB.

CtsK is a very important enzyme for this study, as it is a potent collagenase. Although the majority of the cathepsin activity was clearly due to CtsB, it remained possible that some CtsK activity was present and was masked by the CtsB activity. Therefore, we performed a CtsK assay using a substrate that is reported to be specific for CtsK, which we verified experimentally [16]. Figure 1E demonstrates that CtsK activity in the AF was 48 ± 2 U/mg, which was elevated at 2-fold normal ($p < 0.001$ vs. normal), but was only 0.5% of the activity of CtsB. CtsK activity was not elevated in the epiphysis. Finally, cathepsin D (CtsD) is an aspartyl protease that can cleave and activate CtsB. CtsD activity (Figure 1F) was elevated to 1683 ± 783 U/mg (26-fold normal, $p < 0.001$) in the AF of MPS VII dogs. MMP-12 activity was also tested using a fluorogenic substrate, and was not elevated in either the AF or the epiphysis (data not shown).

3.2. Messenger RNA Levels

3.2.1. Annulus Fibrosus—Messenger RNA from the AF of the lumbar spine was evaluated to determine if changes in enzyme activity were due to changes in mRNA levels of those enzymes and to determine if expression of other genes was affected (Figure 2). Neither collagen I α 1 nor collagen II α 1 were altered at the mRNA level in the AF of MPS VII dogs compared with normal dogs. Small leucine-rich proteoglycans (SLRP) are proteoglycans that associate with collagens and contribute to their assembly. In MPS VII dogs, the SLRP decorin was reduced to 7% of the level in normal dogs ($p = 0.01$) although it remained abundant at 1.9-fold that of β -actin in the MPS VII dogs. Messenger RNA levels of lumican and biglycan were not significantly affected in MPS VII dogs. Levels of the large aggregating proteoglycan, aggrecan, were reduced to 24% of normal ($p = 0.04$). Levels of elastin were low and were not affected by MPS VII.

Levels of mRNA for several cathepsins were evaluated in the AF. CtsB is the cysteine cathepsin whose enzyme activity was noted to be elevated above. CtsB mRNA was abundant in normal dogs at 70% of β -actin, and was 7-fold normal in MPS VII dogs ($p = 0.002$). Other cysteine cathepsin mRNAs that were elevated in MPS VII dogs included CtsK (37-fold normal; $p = 0.001$ vs. normal), CtsS (5-fold normal, $p = 0.002$), and CtsW (2-fold normal, $p = 0.002$). In addition, mRNA for an aspartyl cathepsin, CtsD, was elevated to 5-fold normal in untreated MPS VII dogs ($p = 0.002$), consistent with the enzyme data shown above.

Levels of mRNA for several MMPs were examined in the AF. In MPS VII dogs, MMP12 mRNA was elevated to 25-fold normal ($p = 0.03$) and was reasonably abundant at 33% of the level of β -actin. However, tissue inhibitor of metalloproteinase 2 (TIMP-2) was even higher at 18-fold that of β -actin in MPS VII dogs, which may explain why the MMP-12 activity was not elevated. Osteopontin (OPN) is a protein that has been reported to activate MMPs in

a non-proteolytic fashion, although it can also play a role in signal transduction. Levels of mRNA for OPN were not affected in MPS VII dogs. Levels of mRNA for ADAMTS4, a protease that cleaves aggrecan, were also not different between normal and MPS VII dogs.

Various cytokines have been proposed to play a role in the pathogenesis of MPS in the aorta and synovium [15, 19]. Interleukin-6 (IL-6) mRNA was 8-fold normal in MPS VII dogs, although this did not reach significance, which may relate to the small sample size. Levels of mRNA for TGF- β , TNF- α , and IL-1 α were not elevated. The only signal transduction pathway gene that was tested by mRNA analysis that was significantly elevated in MPS VII dogs was TLR4, which was present at 2% of the level of β -actin in MPS VII dogs, and was 5-fold normal ($p=0.05$). Thus, TLR4 is present and should be capable of responding to GAG accumulation with expression of cytokines. Levels of mRNA for a number of other genes such as ADAMTS5, IL-6-like cytokines, receptors for IL-6 and related cytokines, and TLR5 did not exhibit differences in expression levels between normal and MPS VII samples in the AF, as shown in supplementary Table 1.

3.2.2. Nucleus Pulposus—The NP was also tested for mRNA levels, as shown in Supplemental Figure 1. There were no apparent differences between MPS VII and normal dogs for any of the genes that were tested, although relatively low yields of mRNA made it difficult to test many genes. Cathepsin B, with enzyme activity that was elevated in NP in MPS VII compared with normal dogs, was not elevated at the mRNA level, raising the possibility that enzyme may diffuse from the AF to the NP.

3.2.3. Vertebral Epiphysis—For the epiphysis (Figure 3), values in samples from each group were more variable than for the AF or the NP, which likely reflected technical difficulties in obtaining and homogenizing samples from a calcified structure. Collagen II α 1, which is abundant in cartilage but not in bone, was elevated to 52-fold normal in MPS VII dogs ($p=0.03$). This is consistent with previously published histological data showing that the epiphysis contained cartilaginous lesions in place of bone, and with biochemical data demonstrating a reduction in calcium in these regions [14]. Messenger RNA levels for CtsK and CtsS were significantly elevated in MPS VII dogs, while mRNA levels for CtsB and CtsD were somewhat elevated in MPS VII samples, although differences were not significant. Messenger RNA levels for MMP12 were also significantly ($p=0.001$) elevated in the epiphyses of MPS VII dogs.

3.3. Analysis of Radiographic Abnormalities in MPS VII Dogs During Postnatal Growth

We previously demonstrated that the ventral and to a lesser extent the dorsal epiphyses had cartilaginous lesions with reduced calcium content compared with normal dogs at 6 months of age, which correlated with the reduced calcification on radiographs [14]. Radiographs were used to evaluate the time course of abnormalities in calcification of the epiphysis in the lumbar spine (Figure 4). At 1 week after birth, lateral radiographs of the lumbar spine appeared similar for normal and untreated MPS VII dogs. However, reduced calcification of the epiphysis was apparent in the untreated MPS VII dogs at 1 and 2 months compared with normal controls. These data illustrate that abnormal calcification of the epiphysis is apparent as early as 1 month after birth in MPS VII dogs, and that shortening of the lumbar vertebral body lengths is apparent by 2 months.

3.4. Radiographic and Histological Assessment of Lumbar Spine Disease in Human MPS VII

We undertook radiographic and histological analyses to determine if the lumbar spine in a human patient with MPS VII had morphological abnormalities that resembled those reported previously for MPS VII dogs. The patient examined was the first reported patient with MPS

VII, who was diagnosed at 7 weeks of age when he presented with feet that turned inward and was noted to have other abnormalities including kyphosis that were consistent with MPS, and white blood cell GUSB enzyme activity that was 2% of normal [4]. As shown in the previous publication, at 1 year of age the patient had a gibbus abnormality at T12, and underdevelopment of the anterior-superior portions of T10, T11, and L1 to L4. Lateral radiographs of the spine of the same patient when he was 18 years old (Figure 5A) demonstrated a sharp angle at the position of T12 consistent with a gibbus deformity, although T12 itself could not be identified. The anterior-superior aspect of L1 was still hypoplastic, although other vertebrae appeared to have more complete anterior calcification than was present at 1 year of age. There were also increased lumbar intervertebral spaces and irregular endplates, similar to those reported in MPS VII dogs [24]. The posterior-anterior radiograph shown in Figure 5B demonstrates marked scoliosis in the thoracic spine and degenerative changes.

The patient died suddenly at age 19, possibly due to aspiration. A post mortem examination was performed. Grossly, the spine had a very irregular bone-cartilage interface, as previously reported [5]. The paraffin block of the lumbar spine that was prepared at the time of autopsy was retrieved from the pathology department for this study, and histochemical stains were performed, as shown in Figure 6. Low magnification images of the entire section in Figures 6A, D, and G are stained with alcian blue and picosirius red, picosirius red only (polarized light) and Masson's trichrome, respectively. With the alcian blue-picosirius red stain (Figure 6A), cartilage appears blue due to its GAG content, as indicated with asterisks, while bone and disc appear red due to collagen content. The cartilage was 1 to 2 mm thick for most of the interface between the vertebral body and the intervertebral disc, which is markedly abnormal. The region at the bone:cartilage interface indicated with the red arrow in Figure 6A is shown at higher power in Figure 6B, and illustrates cells with a characteristic morphology of chondrocytes. This region had a relatively low yellow/red signal when a picosirius red-stained slide was evaluated with polarized light (Figure 6E), which indicates that the collagen was not highly aligned. This region did, however, stain blue with Masson's trichrome (Figure 6H), demonstrating that it contained collagen. In contrast, the red-staining region of the intervertebral disc indicated with the green arrow in Figure 6D was highly birefringent in Figure 6F, as would be expected for AF. These data indicated that there are large cartilaginous regions at the bone:intervertebral disc interface in a human patient with MPS VII, which resembles what occurs in MPS VII dogs, and would likely result in structural abnormalities similar to those found the dog model.

4. Discussion

4.1. Role of Proteolytic Enzymes and Extracellular Matrix in the Pathogenesis of Lumbar Spine Disease in MPS VII

The principal extracellular matrix (ECM) molecules responsible for maintaining tissue integrity in the AF include collagen types I and II, the large aggregating proteoglycan, aggrecan, and elastin [25, 26]. In the NP, aggrecan is critical for maintaining tissue hydration which enables the NP to evenly transfer and distribute loads between the vertebrae, while in the bone, collagen I is the major ECM protein. These tissues also contain SLRP such as biglycan, decorin and lumican [27, 28], which perform important structural and biological functions by interacting with collagens, elastin and various cytokines.

In this study, we hypothesized that abnormal structure and function in the lumbar spine of MPS VII dogs was due in part to upregulation of proteolytic enzymes in the vertebral bone and substructures of the intervertebral disc. The lysosomal cysteine cathepsins B, K, L, S and W can degrade one or more of these matrix molecules [17, 29–32] and are associated with a number of degenerative musculoskeletal conditions, including osteoarthritis [31] and

rheumatoid arthritis [33]. In addition, previous studies have suggested that they play an active role in the destructive changes in the aorta of mice and dogs with MPS [15, 16, 34]. In the AF, NP, and epiphysis, enzyme activity for CtsB was very high at 110-fold, 440-fold, and 9-fold normal, respectively, while CtsB mRNA was elevated in the AF at 7-fold normal. CtsB is a potent aggrecanase [30] and we propose that CtsB is an excellent candidate for an enzyme that contributes to dessication of the NP. CtsB also has some activity against collagen I and II, although it is a less potent collagenase than CtsK [35]. Thus, CtsB could contribute to degradation of collagen I in bone and AF, collagen II in AF and endplate cartilage, and aggrecan in the endplate and AF. Of note was that the increase in enzyme activity was far greater than the increase in mRNA levels for CtsB. CtsD is an enzyme that cleaves and activates CtsB, and both mRNA and enzyme activity for CtsD were elevated in the MPS VII AF.

CtsK is able to cleave aggrecan, collagen II, and collagen I, key constituents of the NP, AF, and bone [29–31]. CtsK is upregulated in AF during the course of age-associated disc degeneration, and likely contributes to progressive loss of aggrecan [36, 37]. Collagenolytic activity of cathepsin K is enhanced by chondroitin and keratan sulfates, whereas heparan and dermatan sulfates inhibit it [35, 38]. Since MPS VII results in accumulation of the CtsK-activating chondroitin sulfate and the CtsK-inhibiting heparan and dermatan sulfates, the effect of these GAGS on CtsK activity requires further study. In the AF, CtsK activity was shown to be 2-fold normal in MPS VII dogs, while the mRNA was 37-fold normal. Thus, CtsK could contribute to degenerative changes, although the absolute level of CtsK activity appeared rather low and was <1% of the activity of CtsB.

Elastinolytic activity of MMP-12 and cathepsin S have previously been suggested to be associated with aortic disease in MPS I mice, and in MPS I and MPS VII dogs [15, 34], although a more recent study demonstrated that deficiency of these enzymes did not protect against aortic dilatation, and further characterization of the cathepsin activity suggested that CtsB was actually responsible for what was initially thought to represent CtsS [16]. In this study, there were also some increases in mRNA for other cathepsins, but the absence of a specific assay and limited information regarding their specificity made it difficult to predict if they might contribute to degenerative changes. Although mRNA for MMP12 was elevated in MPS VII dogs, enzyme activity was not elevated. This could reflect the high levels of the MMP-inhibitor TIMP2.

Abnormal synthesis or assembly could contribute to ECM protein abnormalities. The inner AF normally contains significant quantities of aggrecan [26]. Aggrecan mRNA was 24% of normal in AF of MPS VII animals, pointing to potentially abnormal proteoglycan biosynthesis. Similarly, mRNA levels of the SLRP, decorin, were only 7% of normal in MPS VII animals. Although not significant, biglycan was 17% of normal. These proteoglycans perform important roles in collagen fibrillogenesis and fibril organization [39], and their dysregulation could disrupt AF structural development and mechanical function.

4.2. Role of Inflammation in the Pathogenesis of Lumbar Spine Disease in MPS VII

The mechanism by which proteases are upregulated in spinal tissues in MPS VII is very important, as the failure to prevent spine disease with hematopoietic stem cell transplantation, ERT, or gene therapy leads us to predict that a new approach will be required to treat this particular manifestation. Upregulation of destructive proteases in synovium and aorta of MPS animals has been proposed to be due to induction of an inflammatory cascade via TLR4. In addition to its classical ligand, lipopolysaccharide, TLR4 also recognizes extracellular matrix breakdown products, such as hyaluronan fragments, heparan sulfate, fibrinogen, fibronectin extra domain A, lung surfactant protein

A, and high-mobility group box 1 (HMGB1) proteins [40–43]. In this study we found that mRNA for TLR4 was significantly upregulated in the AF of MPS VII affected animals, while our previous study demonstrated that GAGs were 2-fold normal in the AF [14]. Upregulation of TLR4 has been demonstrated in articular chondrocytes and synoviocytes from MPS VI cats [20], and TLR4/MPSVII double knockout mice had improved cranial and long bone morphology, and growth plate organization compared to MPS VII mice [23]. It is possible that the TLR4 receptor on AF cells recognizes accumulated GAG fragments, initiating and sustaining an inflammatory response that drives increased activity of catabolic enzymes. It is also likely that TLR4 is upregulated as part of a feedback loop in which damage-associated molecular pathogens are generated as a consequence of increased matrix catabolism within the pre-existing local inflammatory environment. TLR4 may, therefore, present an attractive upstream therapeutic target for combating disc degeneration in MPS VII [44].

In this study, mRNA for cytokines such as IL-6, TNF- α , and TGF- β that have been elevated in other tissues in MPS were not significantly elevated in the AF in MPS VII dogs. It is possible that the relatively small number of samples analyzed here contributed to our failure to identify significant differences, as some genes such as IL-6 were 8-fold normal in MPS VII dogs. Alternatively, this may indicate that a different set of cytokines are expressed in the AF compared with the synovium or aorta. Further studies will attempt to test additional candidates for cytokines that may be playing an important role in the upregulation of destructive enzymes. For this study, the limited amount of RNA obtained per sample precluded testing additional genes.

4.3. Pathogenesis of Poorly-Calcified Vertebral Epiphyses in MPS VII Spine

Previously, we demonstrated that the ventral and dorsal epiphyses were poorly calcified at 6 months, but the developmental progression of this abnormality was not clear. We now have demonstrated that the epiphysis of normal dogs exhibits calcification at 1 month and that calcification is already reduced at this age in the MPS VII dogs. mRNA analysis at 6 months demonstrated that collagen 2 (Col2 α 1) expression was 52-fold normal in the epiphyses of MPS VII dogs (collagen II is abundant in cartilage and low in bone), which is consistent with previous radiological and histological reports at 6 months. This abnormality may be a major cause of the reduced stiffness of spine motion segments at 6 months, and may also contribute to the vertebral body collapse that can occur in the thoracolumbar spine in MPS VII patients [9]. Ongoing work will seek to further address why the conversion from cartilage to bone is abnormal using samples from 1 month-old normal and MPS VII dogs, as differences between calcification in these groups appears most profound at that age. Abnormal cartilage to bone conversion may reflect delayed endochondral ossification during development, perhaps due to dysregulation of growth factor signaling by accumulating GAGs, altered vascular supply, or other factors.

4.4. Radiographic and Histological Presentation of Lumbar Spine Disease in Human MPS VII

This is the first report of radiographic and histochemical evaluation of the spine in an adult human patient with MPS VII. Radiographs demonstrated severe degenerative abnormalities of the vertebral bodies and kyphoscoliosis that was substantially more severe than was observed at 1 year of age from the same patient [4]. Histopathology demonstrated that there were residual cartilage remnants adjacent to the vertebral end plates, which likely relate to a failure to convert cartilage to bone during development, as occurred in the MPS VII dog model, although the possibility that this represents a thickening of the articular cartilage of the endplate cannot be ruled out. The marked irregularities of the vertebral body surface were also similar to what occurred in the MPS VII dog model, and may be due to

upregulation of destructive proteases. These findings suggest that abnormalities in the spine are similar in humans and dogs with MPS VII, and strongly support the continued use of the canine model for studying spine disease pathogenesis and outcomes of therapeutic interventions in MPS VII.

4.5. Summary and Implications

Upregulation of destructive enzymes in MPS VII likely contributes to intervertebral disc and vertebral bone degeneration, which may be induced by the TLR4 pathway. Possible treatments for this manifestation of disease could include the inhibition of proteases such as CtsB and CtsK that are responsible for the destructive changes, or inhibition of the TLR4 pathway or downstream cytokines. The failure to fully convert cartilage to bone in the epiphysis is likely due to a different mechanism and results in regions in the vertebral bodies that are not calcified and are less resistant to forces, contributing to spine instability. Developing treatments for this aspect of disease will require a better understanding of the underlying biological processes that are abnormal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Increased mRNA expression and enzyme activity of destructive proteases, such as the cathepsins B and K in the annulus fibrosus and vertebral endplates, may contribute to progression of spine disease in mucopolysaccharidosis VII dogs.
2. Abnormal bone formation in the secondary centers of ossification of the vertebral bodies is apparent from 1 month of age in MPS VII dogs.
3. Cartilaginous lesions adjacent to the vertebral endplates are present in a human MPS VII patient, and may contribute to the kypho-scoliotic deformity observed radiographically.

Figure 1. Cathepsin enzyme activity

Samples from the indicated number of normal and untreated MPS VII dogs that were collected at 6 months of age were homogenized and enzyme assays performed using a fluorogenic substrate. Activity was normalized to the amount of protein in the sample. **A. General cathepsin assay. B. General cathepsin assay of Annulus Fibrosus (AF) samples using cathepsin inhibitors.** Samples from the AF of 4 MPS VII dogs were incubated with Z-Phe-Arg-AMC without or with the indicated concentrations of a CtsB or CtsK inhibitor, and the activity relative to that in the absence of an inhibitor determined and average percent remaining activity $\pm$ SD was determined. **C. General cathepsin assay of epiphysis samples with inhibitors of specific cathepsins. D. CtsB assay. E. CtsK assay. F. CtsD assay.** Student's t test was used to compare values between normal and MPS VII samples in panels A, D, E, and F; Student's t-test was used to compare values in samples that were incubated with an inhibitor with those in the same samples without an inhibitor in panels B and C. * $p < 0.05$, ** $p < 0.005$.

Figure 2. Annulus fibrosus (AF) mRNA

Messenger RNA was isolated from the AF at the T12/L1 level for samples obtained from 6 month-old dogs. Reverse transcription was followed by real-time PCR, and levels of specific genes relative to that of β -actin in individual animals was determined as detailed in the methods section, and the mean $\pm$ SD was determined. Genes for extracellular matrix molecules, catabolic enzymes, cytokines and receptors were assessed. Samples from 5 normal and 5 MPS VII dogs were analyzed. Values that were statistically different between the groups are indicated, where * $p < 0.05$, ** $p < 0.005$.

Figure 3. Vertebral epiphysis mRNA

Messenger RNA from the anterior T12 vertebral epiphysis was analyzed for samples obtained from 6-month-old dogs. Reverse transcription was followed by real-time reverse transcriptase PCR as detailed in the legend for Figure 3. Samples from 5 normal and 5 MPS VII dogs were analyzed.

Figure 4. Analysis of calcification of the lumbar spine in normal and MPS VII dogs during postnatal growth

Lateral radiographs of the lumbar spine from L1 to more caudal regions were obtained at the indicated age after birth from normal or untreated MPS VII dogs. The ventral aspect is at the bottom and dorsal aspect at the top. At one week after birth, the length of the vertebrae was similar for the two groups, and epiphyses were not present in either group. For normal dogs, the caudal epiphysis of L1 (long arrow) and the cranial epiphysis of L2 (short arrow) were readily seen at 1 month after birth, and by 2 months calcification of the ventral endplates extended as far as edge of the vertebral body. For MPS VII dogs, the size of the epiphyses was markedly reduced at 1 month, and the epiphyses remained hypoplastic at 2 months, which was most marked in the ventral region. At 2 months, the vertebral body lengths were clearly shorter in the MPS VII compared to the normal dogs. The radiographs shown are representative of those seen for 3 dogs for each age per group.

Figure 5. Radiographs of the thoracolumbar spine of an 18 year-old human MPS VII patient
 A. Lateral radiograph. The positions of the lumbar vertebrae (L1 to L5) are indicated. There is a gibbus deformity of the spine at the position of T12 as indicated by the arrow, although a vertebral body cannot be identified at that position. The lumbar intervertebral space is increased relative to that found in a normal spine, and the surfaces of the endplates are irregular. The superior anterior aspect of L1 is hypoplastic. B. Posterior-anterior radiograph. The positions of L3 to L5 vertebrae are indicated. Severe scoliosis of the thoracic spine is indicated with an arrow.

Figure 6. Histological evaluation of the lumbar spine of a 19 year-old human MPS VII patient Sections containing a vertebral body and adjacent intervertebral discs were stained with alcian blue and picrosirius red (panels A–C; GAGs are blue and collagen is red), picrosirius red viewed under polarized light (panels D–F; triple-helical collagen is red to yellow), and Masson's trichrome stain (panels G–I; collagen is blue). The regions identified with the red arrow for the low power images contain an area of bone:cartilage interface, and are shown at higher power in panels B, E, and H. The region identified with the green arrow for the low power images contains an area with disc, most likely AF, and are shown at higher power in panels C, F, and I.