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Pathogenesis of Mitral Valve Disease in Mucopolysaccharidosis VII Dogs

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Abstract

Mucopolysaccharidosis VII (MPS VII) is due to deficient activity of α -glucuronidase (GUSB) and results in the accumulation of glycosaminoglycans (GAGs) in lysosomes and multisystemic disease with cardiovascular manifestations. The goal here was to determine the pathogenesis of mitral valve (MV) disease in MPS VII dogs. Untreated MPS VII dogs had a marked reduction in the histochemical signal for structurally-intact collagen in the MV at 6 months of age, when mitral regurgitation had developed. Electron microscopy demonstrated that collagen fibrils were of normal diameter, but failed to align into large parallel arrays. mRNA analysis demonstrated a modest reduction in the expression of genes that encode collagen or collagen-associated proteins such as the proteoglycan decorin which helps collagen fibrils assemble, and a marked increase for genes that encode proteases such as cathepsins. Indeed, enzyme activity for cathepsin B (CtsB) was 19-fold normal. MPS VII dogs that received neonatal intravenous injection of a gamma retroviral vector had an improved signal for structurally-intact collagen, and reduced CtsB activity relative to that seen in untreated MPS VII dogs. We conclude that MR in untreated MPS VII dogs was likely due to abnormalities in MV collagen structure. This could be due to upregulation of enzymes that degrade collagen or collagen-associated proteins, to the accumulation of GAGs that compete with proteoglycans such as decorin for binding to collagen, or to other causes. Further delineation of the etiology of abnormal collagen structure may lead to treatments that improve biomechanical properties of the MV and other tissues.

Keywords

Lysosomal storage disease; mucopolysaccharidosis; mitral regurgitation; collagen; dog

1. Introduction

The mucopolysaccharidoses (MPS)¹ are lysosomal storage diseases with an overall incidence of 1:27,000 that are due to deficient activity of an enzyme that contributes to the degradation of glycosaminoglycans (GAG) [1-2]. Cardiovascular disease is a prominent component of most types of MPS including MPS VII [3-6], although other organs such as lung, bones, joints, and brain are also affected. Mitral valve (MV) disease occurs at a young age, and can result in mitral regurgitation (MR) and the need for valve replacement [7], a major surgical procedure. MPS VII is due to deficient activity in α -glucuronidase (GUSB) and results in the accumulation of the GAGs heparan, dermatan, and chondroitin sulfates. The canine model of MPS VII has a missense mutation (R166H) in the *GUSB* gene [8] and closely resembles the disease seen in humans [Online Mendelian Inheritance in Man (OMIM#253220)]. Current treatments for some types of MPS such as hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT) have not prevented cardiac disease [3, 9-10]. Gene therapy is being tested in animals with MPS [11]. Neonatal intravenous (IV) administration of a gamma retroviral vector (RV) has reduced cardiovascular disease in the canine models of MPS VII as shown previously [12-15] and in the accompanying manuscript, but also does not prevent all aspects of cardiovascular disease. A better understanding of the pathogenesis of heart disease in MPS could lead to development of ancillary therapies.

MR in MPS could be due to a thickening of structures from GAG accumulation and failure of MV leaflets to oppose, and/or to changes in extracellular matrix (ECM) proteins such as collagen. Collagen is the major structural protein of the MV and represents 55% of the non-fat dry weight [16]. Collagen I is the most prevalent type at 74% of the total, although collagen III and V represent 24% and 2% of the total, respectively [17]. For collagen I, 3 polypeptides intertwine to form one trimer that is 1.5 nm in diameter and 300 nm long, which is then secreted, cleaved at the N- and C-terminus to form tropocollagen, assembled, and cross-linked into a collagen fibril, which can be 30 to 500 nm in diameter and, thus, contain 20 to 333 tropocollagen molecules assembled in a parallel fashion [18]. Collagen fibrils then associate via small leucine-rich proteoglycans (SLRPs) such as decorin or lumican [19], or other proteoglycans such as aggrecan, to form collagen fascicles, which can be 50 to 300 μ m in diameter and contain 1000 or more fibrils aligned in a parallel fashion along one dimension.

Collagen abnormalities could be due to a failure to synthesize or assemble collagen, or to upregulation of enzymes that degrade collagen or collagen-associated proteins. Patients with MPS I had interstitial cells in the MV with large amounts of GAGs [20] and collagen within lysosomes [21], while the MV annulus had fragmented collagen fibrils and calcification in humans with MPS I at a mean age of 10 years [21]. The goal of this project was to identify the pathogenesis of MV disease in MPS and to determine if neonatal gene therapy could prevent any changes. We demonstrate here that MR was likely due to abnormalities of collagen structure in the MV and the chordae tendinae (CT), and that enzymes that degrade collagen or collagen-associated proteins were upregulated.

¹Mucopolysaccharidosis (MPS); glycosaminoglycan (GAG); mitral valve (MV); mitral regurgitation (MR); α -glucuronidase (GUSB); Online Mendelian Inheritance in Man (OMIM); hematopoietic stem cell transplantation (HSCT); enzyme replacement therapy (ERT); intravenous (IV); retroviral vector (RV); extracellular matrix (ECM); small leucine-rich proteoglycan (SLRP); chordae tendinae (CT); National Institutes of Health (NIH); hepatocyte growth factor (HGF); red light units (RLU); electron microscopy (EM); α -L-iduronidase (IDUA); cathepsin (Cts); ethylenediaminetetraacetic acid (EDTA); dithiothreitol (DTT); benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-AMC); matrix metalloproteinase (MMP); reverse-transcription (RT); Toll-like receptor 4 (Tlr4); interleukin (Il); oncostatin M (Osm); chemokine (C-C motif) ligand (Ccl); and tumor necrosis factor- α (TNF).

2. Materials and Methods

2.1 Materials

Materials were purchased from Sigma-Aldrich Chemical (St. Louis, MO) unless otherwise stated.

2.2 Animals care

National Institutes of Health (NIH) and United States Department of Agriculture guidelines for the care and use of animals in research were followed in the animal colony of the School of Veterinary Medicine, University of Pennsylvania. For this outbred colony, RV-treated MPS VII (*GUSB*^{-/-}) males were bred with *GUSB*^{+/-} females to generate litters where 50% of the dogs were affected and the other half were heterozygous normal. The body size was generally ~20 kg for phenotypically normal dogs. Some MPS VII dogs were treated with neonatal IV injection of the gamma RV designated hAAT-cGUSB-WPRE at 2 to 3 days after birth, as reported previously [12] and as described in the accompanying manuscript, one of which received hepatocyte growth factor (HGF) IV to attempt to potentiate hepatocyte replication, and thus transduction, prior to the injection of RV. For post-mortem collection of samples, dogs received IV injections of 2 mg/kg of Propofol (Abbott, Chicago IL) and 80 mg/kg of sodium pentobarbital (Veterinary Laboratories, Lenexa, KS) in accordance with American Veterinary Medical Association guidelines, and hearts were dissected prior to freezing or after fixation.

2.3 Histopathology

Hearts were fixed in buffered 10% formalin or in phosphate buffered saline with 4% paraformaldehyde and 2% glutaraldehyde, and embedded in plastic (Epon; Miller-Stephenson Chemical Co, Danbury CT) after incubation with osmium tetroxide or paraffin. For paraffin-embedded samples, 6 µm-thick sections were stained with Masson's trichrome or picrosirius red. For the latter, sections were analyzed with polarized light, which results in a yellow to red color for structurally-intact collagen due to the ability of highly organized collagen to rotate light, but no color for most proteins or collagen that is not structurally intact [22-26]. For quantification of the collagen signal, photographs were taken at an exposure time where the signal was linearly related to the time of exposure, regions of the slide with myocardium, annulus, or no tissue were subtracted from the image using the Magic Wand tool, and the red light units (RLU) per area of image were quantified on a scale from 0 to 251 with computer software using PHOTO-PAINT X5 (Corel Inc., Mountain View, CA). For illustrations, all photographs of picrosirius-stained sections used for a particular figure were taken at the same exposure time, which is indicated in the legend. For Epon-embedded samples, 1 µm-thick sections were stained with toluidine blue and basic fuchsin and evaluated with light microscopy, and 100 nm-thick sections were evaluated with electron microscopy (EM).

For Masson's trichrome-stained samples, the severity of GAG accumulation was scored from 0 to +3, where 0 represented no GAG storage, +1 modest accumulation of GAGs in some but not other areas, +2 storage in most cells but <50% of the total area had storage, and +3 marked storage accumulation with >50% of the total area containing GAGs. Collagen was scored from +3 to 0, where +3 represented a strong blue signal throughout the valve, +2 a modest reduction in signal, +1 a marked reduction in signal, and 0 no blue signal.

2.4 Biochemistry

The MV and the CT were dissected and frozen immediately on dry ice. For *GUSB*, -L-iduronidase (IDUA), GAG, and cathepsin (Cts) assays, one third of the anterior leaflet of the

MV was homogenized with a hand-held homogenizer (Kimble-Kontes; Vineland, NJ) in 100 mM sodium acetate pH 5.5 containing 2.5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, and 2.5 mM dithiothreitol (DTT), centrifuged at 10,000 g for 5 minutes at 4°C, and the supernatant was transferred to a new tube and aliquoted. The protein concentration was determined with the Bradford assay (BioRad Laboratories, Hercules CA). Enzyme assays were performed with chromogenic substrates at pH 4.5 for GUSB (4-methylumbelliferyl- β -L-glucuronide), pH 3.5 for IDUA [4-methylumbelliferyl- β -L-iduronide (Toronto Research Chemicals, North York, Canada)], pH 7.5 for total Cts [benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-AMC) from Anaspec (San Jose, CA)], CtsB [Z-Arg-Arg-AMC; Bachem (Torrance, CA)], and Cts K [2-aminobenzoic acid-HPGGPQ-N-(2,4-dinitrophenyl)-ethylenediamine (Abz-HPGGPQ-EDDnp) from Anaspec], and pH 4.0 for CtsD [7-methoxycoumarin-4-acetyl (Mca)-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys-2,4 nitrophenyl (Dnp)-D-Arg-NH₂], as described previously [27]. One unit of enzyme activity converted 1 nmole of substrate to product per hour at 37°C. The CtsB inhibitor Ac-Leu-Val-Lysinal (product #219385) from Calbiochem (San Diego, CA) was incubated with some sample for 10 minutes prior to starting the assay. GAG content was determined using the commercial kit Blyscan (Biocolor, Carrickfergus, UK) using 30 μ g of protein or less from each sample [28]. A matrix metalloproteinase (MMP)12 assay kit from Anaspec (Sensolyte™ 490 MMP12) was used to test posterior MV leaflet samples that were homogenized in the assay buffer provided as described previously [27]. The manufacturer states that this substrate could also be cleaved by MMP1, 2, 3, 8, and 13.

For assessment of collagen levels, MV were diced to 1 mm cubes with a razor blade and incubated in 0.5 M acetic acid for 24 hours and the supernatant 1 (Spt 1) was collected. The remaining sample was incubated in 0.5 M acetic acid with pepsin at 0.1 mg/mL for 24 hours and collected as supernatant 2 (Spt 2) to identify newly synthesized collagen. Collagen levels in the supernatant were assessed using a soluble collagen assay designated Sircol™ from Biocolor Ltd. that was based on colorimetric analysis after incubation with picosirius red using collagen as the standard. To measure insoluble collagen, the remaining sample was homogenized in 6N HCl, incubated overnight at 100°C, neutralized with NaOH, lyophilized, and resuspended in water. Hydroxyproline content was measured at 595 nm after adding chloramine-T and Elrich's reagent [29]. Calculation of total insoluble collagen assumed that hydroxyproline corresponds to 14% of the collagen weight.

2.5 RNA Analysis

One third of the anterior leaflet of the MV was homogenized in Trizol with a Braun Mikro-Dismembrator as described previously [15]. Samples were treated with DNase I to degrade DNA, and RNA was isolated on a Qiagen column. Reverse transcription (RT) was performed on 1 μ g of DNase I-treated RNA with an oligo (dT) 20 primer using a Superscript III kit from Invitrogen Corp. (Carlsbad, CA) in a 20 μ l volume, followed by real-time PCR on 1% of each cDNA sample per well using SYBR green reagents from Applied Biosystems (Foster City, CA). The primers and the procedure for testing the percent of a test RNA to that of β -actin are listed in Supplementary Table 1.

2.6 Statistics

The Student's t test and the Mann-Whitney test compared continuous and non-continuous values, respectively, between 2 groups, while ANOVA with Tukey post-hoc analysis or Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA on ranks) compared continuous and non-continuous values, respectively, between 3 groups using Sigma Plot 12 software (Systat Software, Inc., Point Richmond, CA).

3. Results

3.1 Histochemistry of paraffin-embedded samples

The MR that is observed in untreated MPS VII dogs could be due to abnormalities in the structure of collagen or other ECM proteins in the MV, irregularities of the valve edges that affect its ability to close, or to other causes. To investigate the first possibility, different histological techniques were used to assess the structure of the MV. The anterior leaflet of the MV from a normal dog obtained at 2 years of age was embedded in paraffin, and one section was stained with Masson's trichrome (Fig. 1A and 1C), which demonstrated that the collagen (blue) was tightly packed and fibroblasts were small and without large vacuoles. An adjacent section was stained with picrosirius red and evaluated with polarized light (Fig. 1B and 1D), which identifies structurally-intact collagen as yellow to red due to its highly ordered structure and ability to rotate polarized light, but does not recognize structurally-disrupted collagen or most other proteins [22-26]. This stain demonstrated a strong collagen signal for the normal dog. An anterior MV leaflet from an untreated MPS VII dog obtained at 2 years of age was analyzed in a similar fashion. Masson's trichrome analysis (Fig. 1E and 1G) demonstrated that the valve was thickened, fibroblasts were engorged with GAGs, and the blue collagen signal was reduced, while picrosirius red/polarized light evaluation (Fig. 1F and 1H) demonstrated that the structurally-intact collagen signal was markedly decreased.

Eight MPS VII dogs were treated with neonatal IV injection of a gamma RV alone (RV-treated) while one MPS VII dog was given hepatocyte growth factor (HGF/RV-treated) prior to administration of RV (HGF/RV-treated) as discussed in the accompanying article. Evaluation of an MV from an RV-treated dog obtained at 2 years of age (Figs. 1I to 1L) demonstrated that GAG accumulation was reduced, and the collagen signal was more intact than for the untreated MPS VII dog. A normal dog had a strong collagen signal (Fig. 1M-1P) at 10 years of age, while the collagen signal was modestly reduced in the HGF/RV-treated dog M1287 at 11 years of age (Figs. 1Q to 1T).

Blood vessels were identified in the MV of 92% of untreated MPS VII dogs that were evaluated at 6 months or older (N=13), as shown for one Masson's trichrome-stained sample at 2 years of age at low magnification in Fig. 1E and at higher magnification in Fig. 1Y, which were absent from the normal dog shown in Fig. 1A and 1U. Neovascularization was never seen in any of the normal dogs, and was either absent or minimal in the treated dogs.

3.2 Histochemistry and EM of plastic-embedded samples

The histochemistry shown above for paraffin-embedded samples suggested that collagen structure was abnormal in untreated MPS VII dogs. To further evaluate this hypothesis, some samples were also examined by EM. Basic fuchsin and toluidine blue staining of a plastic-embedded section of the posterior MV leaflet of the same normal dog whose anterior MV leaflet was evaluated above demonstrated that the collagen (pink) was densely packed (Fig. 1V; pink), while EM confirmed that the collagen fibrils were closely packed with few gaps (Fig. 1W-1X). In contrast the same 2 year-old untreated MPS VII dog whose anterior leaflet was evaluated above had fibroblasts that were massively engorged with vacuoles, and that the collagen signal outside the cells was reduced and was separated by large gaps (Fig. 1Z). EM confirmed that fibroblasts contained enlarged lysosomes, as shown in Fig. 1AA. Although individual collagen fibrils appeared to have a near-normal diameter at ~50 nm (Fig. 1BB), clusters of collagen that contained ~40 fibrils or less in one dimension (and thus were ~2 µm or less in width) were separated by large gaps (Fig. 1AA). These data suggest that higher order collagen structure may be abnormal in untreated MPS VII dogs and that

analysis of paraffin-embedded samples with Masson's trichrome stain and picrosirius red stain/polarized light is a simple and reliable approach to assess collagen structure.

3.3 Quantification of GAG and collagen in MV

MV samples that were processed with paraffin and stained with Masson's trichrome, as shown in Fig. 1, were scored subjectively for the amount of GAGs where 0 represented no GAGs, and +3 that at least 50% of the area was lysosomal storage, as shown in Fig. 2A. Samples were analyzed from 19 normal, 17 untreated MPS VII, and 9 treated MPS VII dogs that were collected over the past decade and were of various ages, as summarized in Supplementary Table 2. For normal dogs, the GAG score was 0 at all ages. For untreated MPS VII dogs, the GAG score was low at 0.2 months at 0.5 ± 0 , increased to 2.0 ± 0 at 3 months and 2.4 ± 0.2 at 6 months, and remained elevated at similar or higher levels thereafter. Values for MPS VII dogs at 6 months were significantly higher than for normal dogs at 6 months ($p=0.01$ to 0.05) and at 1 and 2 years were significantly higher than for normal dogs at 2 years ($p<0.01$ for both comparisons). For RV-treated MPS VII dogs, the GAG score of 1.0 ± 0.5 at 6 months ($N=3$) was lower than for untreated MPS VII dogs at 6 months, although this was not significantly different from values in either normal or untreated MPS VII dogs. At 8 years of age, treated dogs had a GAG score of 1.0 ± 0.2 , which was higher than the value in normal dogs at 7 years ($p=0.03$), but was significantly lower than the value in untreated MPS VII dogs at 2 years ($p=0.03$).

The Masson's trichrome-stained samples were also evaluated for collagen (Fig. 2B), where +3 represents a strong blue collagen signal, and 0 represents no blue color. Normal dogs had an increase in signal from 2.6 ± 0.8 at birth to 3.0 ± 0 at 6 months or later. MPS VII dogs had a lower signal at birth with a score of 0.6 ± 0.5 than did normal dogs (not significant), which increased at 3 months to 0.8 ± 0.0 ($p=0.04$ vs. normal) and at 6 months to 1.2 ± 0.6 ($p<0.05$ vs. normal), but then fell to 1.0 ± 0 at 1 year and 0.5 ± 0 at 2 years ($p<0.01$ vs. normal at 2 years for both comparisons). RV-treated dogs had a trichrome collagen score at 6 months of 2.2 ± 1.0 , which was slightly lower than in normal dogs and was higher than in untreated MPS VII dogs, although neither of these was significant. The collagen signal in RV-treated dogs at 8 years of 2.1 ± 0.2 was lower than the value at 7 years in normal dogs of 3.0 ($p=0.03$), but was higher than the value in untreated MPS VII dogs at 2 years ($p=0.03$).

Picrosirius red-stained samples were evaluated with polarized light microscopy as shown in Fig. 1, and the red signal was used to quantify the structurally-intact collagen, as shown in Fig. 2C. The average signal in normal dogs was 31 ± 3 RFU at 0.2 months, which increased to 322 ± 116 RLU at 6 months, and was maintained at 206 ± 36 RLU at 7 years of age. For MPS VII MVs, the signal of 10 ± 0.3 RLU at birth was only 34% of the value for normal dogs, although this was not significant. Similarly, the RLU at 3 months of 25 ± 24 units in untreated MPS VII dogs was 22% of normal ($p=0.06$), while the signal at 6 months of 30 ± 21 RLU was only 9% of normal ($p<0.001$). At 1 and 2 years, the signal of 28 ± 12 and 11 ± 3 RLU in untreated MPS VII dogs was even lower, and represented only 12% ($p<0.001$) and 4% ($p=0.002$), respectively, of values in normal dogs at 2 years. For the RV-treated dogs, the signal at 6 months of 156 ± 93 RLU was 48% of normal ($p=0.48$ vs. normal), which was 5-fold the value in untreated MPS VII dogs ($p=0.05$). The signal in the treated dogs at 8 years was 125 ± 44 RLU, which was 61% of the value in normal dogs at 7 years ($p=0.05$), and was 11-fold the value at 2 years of age in untreated MPS VII dogs ($p=0.004$). Collectively, these data demonstrate that the collagen structure was markedly abnormal in untreated MPS VII dogs, and was improved in treated MPS VII dogs.

3.4 Biochemistry

MV samples collected at 6 months of age, when MR was present in MPS VII dogs, were homogenized and biochemical assays were performed. Extracts from MPS VII dogs had markedly reduced GUSB activity at 0.3 ± 0.1 U/mg (1% normal; $p < 0.05$ vs. normal), as shown in Fig. 3A. GUSB activity was increased to 6.7 ± 0.2 U/mg (25% normal) in the RV-treated dogs, although this was not significantly different from the values in untreated MPS VII dogs.

Elevation of other lysosomal enzymes such as IDUA and of GAGs are markers of lysosomal storage disease, while normalization of these abnormalities is a good measure of correction of disease. Indeed, IDUA activity was markedly increased to 23.5 ± 19.1 U/mg (19.9-fold normal) in MPS VII extracts ($p = 0.008$ vs. normal), as shown in Fig. 3B, and was reduced to 1.8-fold normal in the RV-treated dogs ($p = 0.02$ vs. MPS VII, not significant vs. normal). MPS VII dogs also had marked elevation in GAGs at 97.7 ± 44.9 μ g GAG/mg protein (37.1-fold normal; $p < 0.01$), as shown in Fig. 3C, and this was reduced in RV-treated dogs to 6.2 ± 4.7 μ g GAG/mg protein (2.4-fold normal; not significant vs. normal) which was lower than in untreated MPS VII dogs ($p < 0.01$). The GAG levels were consistent with the histochemical analysis shown in Fig. 1, which demonstrated that GAGs were markedly reduced in treated MPS VII dogs compared with untreated MPS VII dogs, but were still visible.

3.5 Analysis for proteases

One possible explanation for the reduced signal for structurally-intact collagen in MPS VII dogs is that there is an upregulation of enzymes that degrade collagen or associated proteins. Therefore, extracts were tested for activity of members of the cathepsin and MMP families using fluorogenic substrates. Cysteine cathepsins are lysosomal enzymes that can be secreted and degrade various ECM proteins. Although most are maximally active at an acidic pH, some maintain activity at neutral pH, and activity at neutral pH would likely be most relevant to the degradation of ECM proteins in the extracellular space. Fig. 3D demonstrates that MV extracts from MPS VII dogs had high activity against a general cysteine cathepsin substrate (Z-Phe-Arg-AMC) at pH 7.5 with $3,413 \pm 1,555$ U/mg (17-fold normal, $p < 0.01$). This was reduced to 288 ± 50 U/mg in RV-treated dogs (1.4-fold normal, $p < 0.01$ vs. MPS VII, not significant vs. normal).

Additional assays were performed to further assess the specific cathepsin responsible for the activity. Fig. 3E shows that 100 nM of a CtsB-specific inhibitor could reduce the activity found against a general Cts substrate in MPS VII dogs by 98.2% ($p < 0.01$ when compared with the values without the inhibitor), while Fig. 3F shows that extracts from MPS VII dogs had 19-fold normal activity against Z-Arg-Arg-AMC, a substrate that is reported to be specific for CtsB, which was reduced to 2-fold normal in treated MPS VII dogs. In contrast, extracts from MPS VII dogs had very little activity against a CtsK-specific substrate (data not shown). Altogether, these data suggest that CtsB is the major enzyme responsible for the cysteine cathepsin activity in MPS VII MV.

CtsD is an aspartyl protease that can activate other cathepsins such as CtsB [30], although it has no activity against substrates that are cleaved by cysteine cathepsins. CtsD activity at pH 4.0 (Fig. 3G) was 12-fold normal in untreated MPS VII dogs at 3650 ± 2205 U/mg ($p = 0.003$), suggesting a mechanism for activation of CtsB. CtsD activity was reduced to 900 ± 553 U/mg ($p = 0.03$ vs. untreated MPS VII dogs) in RV-treated dogs, which remained 3-fold normal, although this was not significant. MMP activity was not elevated in untreated MPS VII dogs (data not shown).

3.6 Quantification of collagen content in the MV

A reduction in the collagen signal with picrosirius red evaluation could be due to a failure to synthesize collagen or to a failure of collagen to form a highly ordered structure that can rotate polarized light. Therefore, MV were tested for easily dissociated and insoluble collagen as detailed in the methods section and as shown in Fig. 3H. This demonstrated that collagen levels were low in the supernatants from MPS VII dogs, and were reduced to 40% of normal ($p=0.04$) in the pellet. RV-treated MV had soluble collagen levels that were 105% of normal (not significant vs. normal, $p=0.05$ vs. MPS VII). These data suggest that the reduction in collagen in the MV of untreated MPS VII dogs is relatively modest, and is unlikely to account for the marked reduction in the signal for structurally-intact collagen.

3.7 RNA analysis in the MV in MPS VII Dogs

The data shown above indicate that collagen structure is abnormal in the MV of MPS VII dogs. This could be due to a failure to synthesize or assemble collagen, or to upregulation of proteases that degrade collagen or associated proteins. Real-time reverse-transcriptase PCR was performed on RNA isolated from MV collected at 6 months of age to determine the expression of genes that could affect collagen structure (Fig. 4A). RNA was not evaluated from treated dogs, as samples were either not collected at the time of post-mortem, or the mRNA was partly degraded. Collagen I is the major type of collagen in the MV. In the MPS VII dogs, Col1-1 and Col1-2 were reduced to 36% and 41% of normal, respectively, although this was not significant. Col2a1 and Col3a1 were reduced to 11% of normal ($p=0.02$) and 6% normal ($p=0.01$), respectively, although these collagens were expressed at relatively low levels. Decorin (Dcn), lumican (Lum), and biglycan (Bgn) were the most abundant of the SLRP members studied at 322%, 412%, and 155% of the levels of β -actin, respectively. mRNA for Dcn and Lum were reduced to 36% ($p=0.01$) and 49% ($p=0.03$) of normal, respectively, in untreated MPS VII dogs, while Bgn levels were not affected. Aggrecan (Acan) mRNA levels were relatively low at 2% of β -actin in normal dogs, and were not significantly altered in MPS VII dogs. We conclude that there were modest reductions in mRNA levels for minor collagens and for some SLRPs in the MV of MPS VII dogs.

mRNA for proteolytic enzymes was also determined, as one hypothesis is that an enzyme(s) could degrade collagen or collagen-associated proteins. Cysteine cathepsins analyzed included CtsB, CtsK, CtsL, CtsL2, CtsS, CtsW, CtsZ, and the poorly characterized enzyme legumain (Lgmn), while the aspartyl protease analyzed included CtsD. CtsB was the most abundant mRNA of the cysteine cathepsins, being present at 76% of the level of β -actin in normal dogs, and increased to 3-fold normal in untreated MPS VII dogs ($p=0.002$), as shown in Fig. 4A. There were also significant increases in mRNA for CtsK, CtsL, CtsS, CtsW, and Lgmn. CtsD, which can activate cathepsins by proteolytic cleavage, was also increased to 5-fold normal. mRNA for an inhibitor of cysteine cathepsins, cystatin 3 (Cst3), was not detectable. MMPs are another family of enzymes that can degrade ECM components. mRNA for MMP9 and MMP12 were increased to 4-fold ($p=0.03$) and 23-fold ($p<0.001$) normal, respectively, although the absolute levels were relatively low at 0.05% and 5% of the levels of β -actin, respectively. Tissue inhibitor of metalloproteinase (Timp)1 and Timp2 are proteins that can inhibit MMPs; their mRNA was relatively high and was not significantly affected by MPS VII. mRNA for osteopontin (Spp1), a protein that has been reported to activate MMPs in a non-proteolytic fashion, was not affected. ADAMTS4 levels were not affected by MPS VII, while ADAMTS5 levels were not detectable.

mRNA for genes whose protein products are involved in signal transduction were also evaluated, as shown in Fig. 4B. Toll-like receptor 4 (Tlr4), which has been proposed to bind to GAGs and activate signaling, was increased to 2-fold normal ($p=0.002$), and was present

at 1% of the level of β -actin in untreated MPS VII MV. Levels for mRNA for a number of interleukin 6 (IL6)-like cytokines and their receptors were evaluated. Oncostatin M (Osm) was elevated to 5-fold normal ($p=0.003$) and its receptors (dimers of IL6st with Lifr or IL6st with Osmr) were abundant, so it is possible that Osm is important for signaling. Tumor necrosis factor- α (Tnf) has been elevated in other tissues in MPS animals [31-33]. Tnf was elevated to 4-fold normal ($p=0.01$), although its levels remained low at 0.01% of β -actin. A Tnf receptor family member (Tnfrsf14) was elevated to 6-fold normal ($p<0.001$), while IL1 α and Ccl4 were elevated to 3-fold ($p<0.001$) and 6-fold ($p<0.001$) normal, respectively. Complement gene mRNAs have been elevated in various tissues in MPS animals [27, 34]. mRNA for an early component of complement, C1qa, was elevated to 2-fold normal ($p=0.007$), while mRNA for a receptor that can bind to C5a (C5ar) and transduce a signal was elevated to 7-fold normal ($p=0.001$).

3.8 Chordae tendinae (CT)

CT are another important component of the MV apparatus. The gross evaluation shown in the accompanying manuscript demonstrated that the CT were thickened in an MPS VII dog and to a varying extent in the treated MPS VII dogs. The average diameter of the CT in untreated MPS VII dogs at 6 months of age was 0.50 ± 0.28 mm, which was 1.5-fold the value of 0.33 ± 0.21 mm in normal dogs ($p=0.01$ to 0.05). RV-treated dogs had CTs with a diameter of 0.46 ± 0.16 mm, which was not significantly different from values in the other groups. Histochemical analysis (Supplementary Fig. 1 and Fig. 2), demonstrated that GAGs were increased in CTs of untreated MPS VII dogs at 6 months, with an average storage score of 1.7 ± 0.4 , which was significantly higher ($p=0.01$ to 0.05) than the scores in normal and RV-treated dogs. The red signal for structurally intact collagen was reduced to 59 ± 44 RLU at 6 months in untreated MPS VII dogs (14% normal, $p<0.001$), while the signal in RV-treated MPS VII dogs of 258 ± 239 was 60% of normal at 6 months, which was significantly higher than in untreated MPS VII dogs ($p=0.004$), but remained lower than in normal dogs ($p=0.01$).

Biochemical analysis of CT (Supplementary Fig. 3) demonstrated that the untreated MPS VII dogs had only 0.4% of normal GUSB activity, while RV-treated dogs had 10% of normal GUSB activity, although this was not significantly higher than for untreated MPS VII dogs. Untreated MPS VII dogs had an increase in IDUA activity to 9-fold normal ($p<0.01$), an increase in GAGs to 51-fold normal ($p<0.01$), and an increase in general cathepsin activity to 15-fold normal ($p=0.01$ to 0.05) for which 100% of the activity was inhibited with 100 nM of a CtsB inhibitor ($p<0.01$). In addition, extracts from MPS VII CT had CtsB activity that was elevated to 10-fold normal ($p<0.01$), CtsK activity that was 3-fold normal (not significant), and CtsD activity that was 8-fold normal ($p<0.01$). CT from RV-treated dogs had significant improvements in IDUA activity, GAG levels, and in general cathepsin, CtsB, and CtsD activity.

3.9 Mitral valve histochemistry in a patient with MPS VII

An MV was obtained at post-mortem from a young man with MPS VII who died suddenly at age 19 years. He was the first reported patient with MPS VII and had ~2% of normal GUSB activity in white blood cells [5] and ~1% of normal GUSB activity in organs obtained at post-mortem [6]. He was never treated for MPS VII, and MR of unclear severity was observed with echocardiogram one year prior to death and may have been present earlier. At post-mortem, no evidence of myocardial infarction was found, and the apparent cause of death was aspiration. Figs. 5A-5B and 5D-5E demonstrate that many regions of the MV had a reduced signal for structurally-intact collagen, while Fig. 5B demonstrates that GAGs accumulated in fibroblasts and there was neovascularization in the MV. The CT maintained a stronger collagen signal than did the MV, as shown in Figs. 5A, 5C-5D, and

5F, although some lysosomal storage was visible in the fibroblasts. The MV annulus had numerous regions with amorphous pink precipitates that are labeled Ca^{++} in Fig. 5A and 5D as they were identified to be calcium with alizarin red stain (data not shown).

4. Discussion

The goal of this study was to evaluate the pathogenesis of MR in MPS VII dogs. MR is a frequent finding in patients with various types of MPS including MPS VII, as discussed in the introduction.

4.1. The collagen structure in the MV is abnormal in MPS VII dogs

Untreated MPS VII dogs consistently have MR at 6 months of age or older, and some develop MV prolapse, suggesting that the strength of the valve might be reduced. In this study, untreated MPS VII dogs had a significant reduction in their signal for structurally-intact collagen. Therefore, we believe that abnormal collagen structure in the MV is a major cause of MR. EM showed that the diameter of individual collagen fibrils in untreated MPS VII dogs was relatively normal, but relatively few fibrils were assembled into parallel arrays of 2 μm or less, and the arrays that were present were separated by gaps. In contrast, normal dogs had arrays of fibrils that were up to 500 μm thick, and contained tightly packed collagen fibrils throughout. This suggests that the higher order collagen structure is disrupted in the MPS VII MV, which is a likely explanation for the abnormal signal with picrosirius red. This is consistent with the finding that the collagen structure was abnormal in the cornea of animals and patients with MPS [35-36].

4.2 Possible causes of abnormal collagen structure in MPS VII

There are several possible explanations for abnormal collagen structure in the MV of MPS VII dogs: 1) reduced synthesis of collagens, proteoglycans, or other proteins that are responsible for assembly of fibrils; 2) competitive inhibition of collagen fibril:proteoglycan association by the presence of excess GAGs; or 3) degradation of collagen or associated proteins. Biochemistry performed at 6 months, when MR and collagen abnormalities were apparent, demonstrated that the amount of hydroxyproline per wet weight (an indirect measure of the amount of collagen) in the MV of MPS VII dogs was only modestly reduced to 40% of normal. In addition, real-time RT-PCR demonstrated that mRNA for Col1a1 and Col1a2 were 36% and 41% of normal, respectively, while mRNA for Col3a1 was reduced to 16% of normal. Thus, a modest reduction in the synthesis of collagen could contribute to reduced strength of the valve, although the signal for structurally intact collagen was reduced far more to only 9% of normal at 6 months, suggesting that alterations in synthesis cannot account for all the abnormalities.

This study also demonstrated that mRNA levels of some proteoglycans known to be involved in assembly of collagen fibrils into a higher order structure were modestly reduced. For example, decorin and lumican mRNA were significantly reduced in MPS VII MV at 36% and 49% of normal, respectively. Reduced expression of decorin is associated with MR in dogs [37] and with a Marfan-like syndrome with MR in human patients [38], while decorin deficiency causes fragile skin [39] and biglycan deficiency causes aortic dissection [40] in mice.

A second possibility is that the accumulation of GAGs inhibits the ability of proteoglycans to help assemble collagen into a higher order structure, as GAGs are important for the function of SLRPs [41], although the leucine-rich domain of SLRPs can also bind to collagen. While GAGs accumulate in lysosomes, it is also likely that some GAGs are released by exocytosis and when a cell degenerates. Future studies will attempt to evaluate

the structure of collagen and proteoglycans using electron microscopy with anti-proteoglycan-specific antibodies.

A third possible explanation for abnormal collagen structure is that proteases such as cathepsins or MMPs are upregulated and degrade collagen or collagen-associated proteins [42-44]. This study shows that CtsB enzyme activity was increased to 17-fold normal and CtsB mRNA was abundant and was 3-fold normal in the MPS VII dogs. The discrepancy between the degree of elevation of cathepsin enzyme activity and the mRNA levels of CtsB could be due to increased activation of CtsB by CtsD, which was increased. Alternatively, GAG accumulation itself could activate CtsB, as occurs *in vitro* [45]. Although MMP12 was the most upregulated gene in the MPS VII MV as its mRNA was 23-fold normal, MMP enzyme activity was not elevated.

A human patient with MPS VII also had a reduced signal for collagen in the MV, while dogs with MV prolapse due to other causes had abnormal MV collagen structure [46]. MPS VII dogs also had a reduced signal for collagen in the CT, although the magnitude of the abnormality was lower than for the MV. It is also possible that irregularities of the leaflet margin could contribute to abnormal coaptation of the leaflets.

4.3. Effect of MPS VII on signal transduction pathways

The leading hypothesis regarding the pathogenesis of disease in bones and joints in MPS is that GAGs bind to a receptor such as Tlr4 [31-33], which results in upregulation of signaling, cytokines, and genes whose proteins can degrade ECM components. This study demonstrates that mRNA for Tlr4 is increased to 2-fold normal at 6 months in MPS VII valves, while mRNA for cytokines (Osm, Tnf, IL1a, and Ccl4), complement components (C1qa), and receptors for cytokines (Tnfrsf11) and complement components (C5ar) were also upregulated. Further studies will attempt to define the specific pathways and genes that are affected in MV in MPS VII, which might lead to the testing of drugs that can affect these pathways for their effect on cardiac disease.

4.4. Neonatal gene therapy improved collagen structure

The improvement in echocardiographic parameters in the treated MPS VII dogs was associated with an improvement in the picrosirius red/polarized light collagen signal in the MV, which was maintained at 61% of normal at 8 years. This was likely due to the ability of some enzyme to diffuse into the valve, as the GUSB enzyme activity was 21% of normal, and some GUSB enzyme activity was visible in the valve with a histochemical stain in the accompanying manuscript. In addition, although mRNA from the treated dogs was of poor quality and was not assessed here, the CtsB activity in treated dogs was only 10% of the value in untreated MPS VII dogs. Despite these improvements, there was some lysosomal storage material visible with light microscopy and GAGs were elevated to 2-fold normal in extracts, leading us to predict that collagen structure may deteriorate over a longer period of time.

4.5 Implications for treatment of MV disease

These studies suggest that MR in MPS VII dogs is associated with abnormal collagen structure, which is likely due primarily to upregulation of proteases that degrade collagen or associated proteins, although reduced synthesis of collagen components or inhibition of higher order collagen structure by excess GAGs is possible. Further studies will attempt to better define the cause of abnormal collagen in these dogs, which might identify a drug that would reduce valve disease. Neonatal IV injection of a gamma RV that programs the liver to secrete GUSB can markedly improve the function of the MV and improves the signal for structurally-intact collagen. We believe that similar results will be seen in patients that

receive ERT, as the levels of expression in the gene therapy-treated dogs are likely similar to the amounts of enzyme that can be delivered with ERT [13]. Gene therapy could be a simple and effective way to reduce cardiovascular manifestations of MPS if safety concerns can be addressed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

The collagen structure is abnormal in the mitral valve of mucopolysaccharidosis VII dogs.

Abnormal collagen structure might be due to upregulation of proteases that degrade collagen or collagen-associated proteins

Neonatal gene therapy with a gamma retroviral vector improves the structure of collagen in the mitral valve

Fig. 1. Histopathological evaluation of mitral valve (MV)

MV leaflets were collected at the indicated age in years (Y) from normal, untreated MPS VII, RV-treated, or HGF/RV-treated dogs as indicated. **Panels A-T, U, and Y. Anterior MV leaflets processed with paraffin.** Fixed anterior MV leaflets were embedded in paraffin and some 6 μ m-thick sections were stained with Masson's trichrome (Trichrome) and analyzed with light microscopy, which identifies collagen as blue, lysosomal GAGs as white vacuoles in the cytoplasm (yellow arrow), and nuclei as dark red. Adjacent sections were stained with picrosirius red and analyzed with polarized light, which identifies highly-ordered collagen as yellow to red, and photographed with a 640 msec exposure. For low power images, the heart is at the left, the tip of the valve is on the right, and the annulus is visible in some panels. For the 2 year-old MPS VII dog, a blood vessel (BV; black arrow) seen at low power in panel E is shown at higher power in panel Y; blood vessels were absent in a normal dog at 2 years (panels A and U). **V-X and Z-BB. Posterior MV leaflets processed in plastic.** Posterior MV leaflets from the same 2 year-old normal and untreated MPS VII dogs shown in earlier panels were processed in plastic. In panels V and Z, 1 μ m-thick sections were stained with toluidine blue and basic fuchsin and evaluated with light microscopy. Collagen appears pink, nuclei appear blue (green arrows), and GAG accumulation in lysosomes appears as white vacuoles in the cytoplasm (black arrow). In panels W-X and AA-BB, electron microscopy (EM) was performed. An engorged lysosome in a fibroblast is identified with a short black arrow in panel AA and individual collagen fibrils are identified with red arrows in panels X and BB. For all panels, size markers are indicated.

Fig. 2. Quantification of GAGs and collagen in the MV. A. GAG score from Masson's trichrome MV were processed with paraffin and sections were stained with Masson's trichrome as in Fig. 1 and scored for the amount of lysosomal GAGs using the system detailed in the methods section were 0 represents no GAGs (normal) and +3 indicates that >50% of the area was GAGs. The number of dogs evaluated at each time point for each group is shown, and details about the groups are found in Supplementary Table 2. **B. Collagen score from Masson's trichrome.** The amount of collagen from Masson's trichrome-stained sections of the MV was scored from +3 (dense collagen, which is normal) to 0 (no blue visible, which is abnormal). For data in panels A and B, Kruskal Wallace one-way analysis of variation on ranks was used to compare values at 6 months, while Mann-Whitney Rank Sum Test was performed when only 2 groups were evaluated at the other ages. * represents a p value of 0.01 to 0.05 and ** represents a p value <0.01 when values in other groups were compared with those in normal dogs. **C. Collagen signal from picosirius red stain.** Sections of MV were stained with picosirius red and evaluated with polarized light, as shown in Fig 1. Photographs were obtained at an exposure where the red light signal was linear with respect to the time of exposure and the red light units (RLU) were normalized to a 160 msecond exposure and plotted as the average $\pm$ SD. One-way Analysis of Variance with Tukey post-hoc analysis was used for statistical comparisons when there were 3 groups, and Student's t-test was performed when there were 2 groups of the same age, and values in other groups were compared with those in normal dogs.

Fig. 3. Biochemistry of MV

Extracts were prepared from MV of 6 month-old normal (N=7), untreated MPS VII (N=4), and RV-treated MPS VII (N=4) dogs. **A-D. GUSB activity, IDUA activity, GAG levels, and total cathepsin activity.** Enzyme activity and GAG levels were determined and normalized to the mg of protein in the sample. For the total cathepsin assay, the substrate Z-Phe-Arg-AMC (Phe-Arg) was used at pH 7.5. * and ** indicate that the values in the other groups were statistically different at $p=0.01$ to 0.05 or $p<0.01$, respectively, from values in samples from untreated MPS VII dogs. **E. Effect of Cathepsin B (CtsB) inhibitor on cathepsin activity in MPS VII dogs.** Samples from untreated MPS VII dogs were incubated with the non-specific Z-Phe-Arg-AMC substrate with or without a CtsB inhibitor at the indicated final concentration, and the activity relative to that in samples without the inhibitor was determined. ** indicates that the values were statistically different from values obtained for the same samples without the inhibitor. **F-G. CtsB and CtsD activity.** Enzyme activity was determined using the CtsB-specific substrate Z-Arg-Arg-AMC (Arg-Arg) at pH 7.5, and CtsD activity was determined at pH 4.0. **H. Collagen content of MV.** A piece of MV obtained from 7 normal, 7 untreated MPS VII, and 4 RV-treated dogs at 6 months of age was incubated overnight with 0.5 M acetic acid, and the supernatant (Spt 1) was collected. The MV was then incubated with 0.5 M acetic acid with 0.1 mg/ml of pepsin, and the supernatant (Spt 2) was collected. Spt1 and Spt 2 were tested for soluble collagen using a picosirius red assay. The pellet was then incubated with 6N HCl at 100°C for 14 hours, and the hydroxyproline content determined using an amino acid assay. Values were normalized to the initial wet weight of the MV, and the mean $\pm$ SD determined.

Fig. 4. RNA Analysis

RNA was isolated from 7 normal and 5 untreated MPS VII dogs at 6 months of age and real time RT-PCR with different primer sets was used to determine the percent of each test mRNA in each sample relative to that of β -actin as detailed in the methods. **A. mRNA for extracellular matrix (ECM) genes and proteases.** This shows amounts of mRNA for extracellular matrix proteins [collagen (Col), decorin (Dcn), lumican (Lum), biglycan (Bgn), aggrecan (Acan), and elastin (Eln)], proteases of the cathepsin family [cathepsin (Cts) and legumain (Lgmn)], an inhibitor of cysteine cathepsins [cystatin C (Cst3)], proteases of the matrix metalloproteinase (MMP) family, tissue inhibitor of metalloproteases (TIMP) members, osteopontin (Spp1; this may activate MMPs), and proteins of the ADAMTS family that have aggrecanase activity. **B. mRNA for signal transduction genes.** mRNA levels for genes involved in signal transduction such as Toll-like receptors (Tlr), interleukin 6 (IL6)-like cytokines [IL6, IL11, oncostatin M (Osm), cardiotrophin-like cytokine factor 1 (Clcf1), ciliary neurotrophic factor (Cntrf), cardiotrophin 1 (Ctfr1), leukemia inhibitory factor (Lif)], receptors for IL6-like cytokines [glycoprotein 130 (Il6st), interleukin 11 receptor alpha (Il11ra), oncostatin M receptor (Osmr), ciliary neurotrophic factor receptor (Cntrf), and leukemia inhibitory factor receptor alpha (Lifra)], members of the tumor necrosis family signaling [tumor necrosis factor alpha (Tnf), tumor necrosis factor receptor subfamily 14 (Tnfrsf14)], interferon gamma (Infg), transforming growth factor beta 1 (Tgfb1), chemokine (C-C motif) ligand (Ccl), complement component C1qa, and the receptor for C5a component of complement (C5ar). Student's t-test was used to compare values between the 2 groups where * indicates a p value of 0.01 to 0.05, and ** indicates a p value <0.01.

Fig. 5. Histochemical evaluation of an MV from a human patient with MPS VII

An MV was obtained at post-mortem from a patient with MPS VII who died suddenly at age 19, fixed with formalin, embedded in paraffin, and adjacent sections were obtained. In the low magnification images in panels A and D, the tip of the valve is on the left (distal) and the proximal region is on the right. Regions of the MV and the CT that are shown at higher power in subsequent panels are identified with orange and green arrows, respectively. Regions labeled Ca^{++} were found to be calcium with alizarin red stain. **A-C. Masson's trichrome stain.** The section was stained with Masson's trichrome. In panel B, GAG storage in the fibroblasts of the MV is identified with a black arrow, the collagen signal (blue) is markedly reduced, and a blood vessel is present, indicating neovascularization. In panel C, the CT had some fibroblasts with modest amounts of GAG storage, as indicated with the white arrow. **D-F. Picrosirius red stain.** An adjacent section to that shown in panels A-C was stained with picrosirius red and analyzed with polarized light, and photographs were exposed for 640 mseconds.