Molecular Genetics and Metabolism 107 (2012) 153–160

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Pathogenesis of lumbar spine disease in mucopolysaccharidosis VII

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ARTICLE INFO

Article history:
Received 19 March 2012
Accepted 19 March 2012
Available online 30 March 2012

Keywords:
Lumbar spine
Mucopolysaccharidosis VII
Bone
Intervertebral disk
Cathepsin
Inflammation

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 kypho-scoliotic deformity and spinal cord compression due to disease of intervertebral disks, vertebral bodies, and associated tissues. We have previously demonstrated in MPS VII dogs that intervertebral disks 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 disk 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 these 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. The spine obtained at autopsy from a young man with MPS VII exhibited similar increased cartilage in the vertebral bodies adjacent to the end plates, disorganization of the intervertebral disks, 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 cathepsins. Inhibition of destructive proteases, such as cathepsins, might reduce spine disease in patients with MPS VII or related disorders.

1. Introduction

The mucopolyaccharidoses (MPS) are a subset of lysosomal storage disorders characterized by deficiencies in enzymes that contribute to degradation of glycosaminoglycans (GAGs), and which exhibit multisystemic 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 disk 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 disk 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

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doi:10.1016/j.ymgme.2012.03.014

cathepsins (Cts) is 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 NFκB 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 wedging of the vertebral body and contribute to a gibbus deformity. The spine also exhibits broad destructive changes in the vertebral bodies and intervertebral disks 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, therefore, was to perform this analysis on a spine that was obtained from a 19-year old man 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 the 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 disk. 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 from the NP, AF and ventral vertebral epiphysis from 4 normal and 4 MPS VII dogs 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 10,000 *g* 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 nmol 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 from 5 normal and 5 MPS VII dogs were were homogenized for 30 s 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 $= (100) \times 2^{\Delta CT}$. 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 $p < 0.05$. All results are expressed as mean ± standard deviation.

2.5. Radiological and histological analysis of human MPS VII spinal tissue

Lumbar spine tissue comprising the intervertebral disk 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 had been obtained prior to death at age 18.