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Pathogenesis of mitral valve disease in mucopolysaccharidosis VII dogs

Paul W. Bigg [a], Guilherme Baldo [b], Meg M. Sleeper [c], Patricia A. O'Donnell [d], Hanqing Bai [a], Venkata R.P. Rokkam [a], Yuli Liu [a], Susan Wu [a], Roberto Giugliani [b], Margret L. Casal [c], Mark E. Haskins [c,d], Katherine P. Ponder [a,e,*]

[a] Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO, USA
[b] Programa de Pos-Graduacao em Genetica e Biologia Molecular, Universidade Federal do Rio Grande do Sul, RS, Brazil
[c] Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA
[d] Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA
[e] Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO, USA

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ABSTRACT

Mucopolysaccharidosis VII (MPS VII) is due to the deficient activity of β-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.

1. Introduction

The mucopolysaccharidoses (MPS) are lysosomal storage diseases with an overall incidence of 1:27,000 that are due to the deficient activity of an enzyme that contributes to the degradation of glycosaminoglycans (GAGs) [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 the deficient activity in β-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 the development of ancillary therapies.

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

* Corresponding author at: Department of Internal Medicine, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA. Fax: +1 314 362 8813.
E-mail address: kponder@dom.wustl.edu (K.P. Ponder).

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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 collagens 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 in length, 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, contains 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 proteoglcyans such as aggrecan, to form collagen fascicles, which can be 50 to 300 μm 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 tendineae (CT), and that enzymes that degrade collagen or collagen-associated proteins were upregulated.

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 in an 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, α-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 min 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 chromogeneic substrates at pH 4.5 for GUSB (4-methylumbelliferyl-β-L-glucuronide), pH 3.5 for IDUA [4-methylumbelliferyl-α-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 nmol 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 min prior to starting the assay. GAG content was determined using the commercial kit Blyscan (Biocolor, Carrickfergus, UK) using 30 μg of protein or less from each sample [28]. A matrix metalloproteinase (MMP)12 assay kit from Anaspec (SensolyteTM 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 h 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 h 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 the colorimetric analysis after incubation with picrosirius red using collagen as the standard. To measure insoluble collagen, the remaining sample was homogenized in 6 N HCl, incubated overnight at 100 °C, neutralized with NaOH, lyophilized, and resuspended