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# Pathogenesis of aortic dilatation in mucopolysaccharidosis VII mice may involve complement activation

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ABSTRACT

Mucopolysaccharidosis VII (MPS VII) is due to mutations within the gene encoding the lysosomal enzyme β-glucuronidase, and results in the accumulation of glycosaminoglycans. MPS VII causes aortic dilatation and elastin fragmentation, which is associated with upregulation of the elastases cathepsin S (CtsS) and matrix metalloproteinase 12 (MMP12). To test the role of these enzymes, MPS VII mice were crossed with mice deficient in CtsS or MMP12, and the effect upon aortic dilatation was determined. CtsS deficiency did not protect against aortic dilatation in MPS VII mice, but also failed to prevent an upregulation of cathepsin enzyme activity. Further analysis with substrates and inhibitors specific for particular cathepsins suggests that this enzyme activity was due to CtsB, which could contribute to elastin fragmentation. Similarly, MMP12 deficiency and deficiency of both MMP12 and CtsS could not prevent aortic dilatation in MPS VII mice. Microarray and reverse-transcriptase real-time PCR were performed to look for upregulation of other elastases. This demonstrated that mRNA for complement component D was elevated in MPS VII mice, while immunostaining demonstrated high levels of complement component C3 on surfaces within the aortic media. Finally, we demonstrate that neonatal intravenous injection of a retroviral vector encoding β-glucuronidase reduced aortic dilatation. We conclude that neither CtsS nor MMP12 are necessary for elastin fragmentation in MPS VII mouse aorta, and propose that CtsB and/or complement component D may be involved. Complement may be activated by the GAGs that accumulate, and may play a role in signal transduction pathways that upregulate elastases.



## 1. Introduction

Mucopolysaccharidoses (MPS) are a group of 11 diseases caused by mutations in genes that encode lysosomal enzymes that degrade glycosaminoglycans (GAGs) [1]. MPS is associated with accumulation of GAGs throughout the body and multisystemic disease. The focus of this project was to better understand the pathogenesis of aortic disease in MPS using the murine model of MPS VII, which is an autosomal recessive disease due to β-glucuronidase (GUSB) deficiency (Online Mendelian Inheritance in Man #253220). An elegant model for the pathogenesis of MPS involves the binding of GAGs to Toll-like Receptor 4 (TLR4), which upregulates cytokines such as tumor necrosis factor α (TNFα), Ccl4 (MIP-1β), and interleukin 6 (IL-6), which in turn upregulate destructive proteases [2–4]. Adult humans with attenuated MPS I have aortas that are 122% of normal diameter [5] and reduced elasticity [6], while one patient with MPS VII required an aortic graft [7]. Mice with MPS I [8–10] and MPS VII [11], cats with MPS I and MPS VI [12], and dogs with MPS I [13–15] and MPS VII [14,16,17] also have aortic dilatation.

Elastin represents 30% of the dry weight of the aorta [18]. Tropoelastin monomers are secreted and then crosslinked into elastic fibers in a process that involves elastin binding protein, extracellular matrix microfibrils, and crosslinking enzymes [19]. Elastin was fragmented in the ascending aorta of humans, mice, and dogs with MPS I, and in humans and dogs with MPS VII [10,13,20–22]. Hinek et al. demonstrated that exogenous administration of dermatan sulfate, a GAG that accumulates in many types of MPS, reduced elastin binding protein levels and inhibited elastin assembly *in vitro*, and proposed that reduced assembly caused elastin defects in MPS I [23]. Alternatively,

*Abbreviations:* MPS, mucopolysaccharidosis; Cts, cathepsin; MMP, matrix metalloproteinase; GUSB, β-glucuronidase; GAGs, glycosaminoglycans; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor alpha; IL-6, interleukin 6; M6P, mannose 6-phosphate; ERT, enzyme replacement therapy; RV, retroviral vector; PBS, phosphate buffered saline; VVG, Verhoeff Van Gieson; AMC, 7-amino-4-methylcoumarin; IDUA, α-L-iduronidase; TBS, tris-buffered saline; BP, blood pressure; STAT3, signal transducer and activator of transcription 3.

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we reported in MPS I mice and dogs and in MPS VII dogs [10,14] that elastin fragmentation was temporally associated with increases in RNA and enzyme activity for two elastases, cathepsin S (CtsS) and matrix metalloproteinase 12 (MMP12), which contribute to aortic aneurisms in mouse models [24,25], and proposed that degradation was the major factor leading to elastin fragmentation. Although collagen is another important extracellular matrix protein of the aorta, collagen fibrils appeared to be relatively intact with histochemical stains in MPS I and MPS VII dogs [14].

Hematopoietic stem cell transplantation can reduce clinical manifestations of MPS, as hematopoietic cells migrate into tissues and secrete mannose 6-phosphate (M6P)-modified enzyme that can be taken up via the M6P receptor by nearby cells [26]. This has reduced, but not prevented, accumulation of GAGs, elastin fragmentation, and/or dilatation of the aorta in MPS VII mice [27] and dogs [16], MPS VI rats [28], and MPS I dogs [15] and cats [29]. Enzyme replacement therapy (ERT) involves intravenous (IV) injection of M6P-modified enzyme that can diffuse to organs and be taken up via the M6P receptor [30,31], although ERT is not available for MPS VII. ERT had little effect on lysosomal storage accumulation in aortic smooth muscle cells in MPS VI cats [32]. Gene therapy is also being tested in animal models [33]. One approach involves neonatal IV injection of a retroviral vector (RV) expressing the appropriate enzyme, which results in transduction of liver cells and secretion of enzyme into blood [33–35]. This reduced aortic dilatation, but MPS I mice required very high expression for a full therapeutic effect [8–10], and MPS VII dogs developed aortic dilatation after 5 years [14].

The data that CtsS and MMP12 are upregulated in MPS aortas led us to hypothesize that deletion of CtsS and/or MMP12 might reduce elastin fragmentation. To test this hypothesis, $CtsS^{-/-}$ and $MMP12^{-/-}$ mice were crossed with MPS VII ($GUSB^{-/-}$) mice and the effect upon the aorta diameters was determined. In addition, microarray analysis was performed to determine if other genes that could contribute to aortic dilatation were upregulated. The results demonstrate that CtsS and MMP12 are not essential for aortic dilatation, but a related cathepsin, CtsB, may contribute. These studies also demonstrate that the complement system may directly result in elastin fragmentation, or may indirectly contribute by induction of signal transduction pathways that result in upregulation of elastases.

## 2. Materials and methods

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

### 2.1. Animals

National Institutes of Health (NIH) guidelines for the care and use of animals in research were followed. MPS VII [27], CtsS-deficient [36], and MMP12 deficient [37] mice were all in a C57BL/6 background. Some MPS VII mice were injected IV with $1\times10^{10}$ transducing units/kg of the RV designated hAAT-cGUSB-WPRE [38] at 2 to 3 days after birth to enable them to survive and breed. Genotyping for MPS VII mice used a SNP assay on tail DNA with a forward primer (5′ CCATAGTCATGATACCAAGAAAAGTAGCT-3′), a reverse primer (5′-TGACTATTCTGACCTCAGTGTGTGA-3′), a wild-type minor-groove binding probe labeled with Vic (5′-TTGTCTTAGGCCCCGTACGT-3′; the underlined C represents the position deleted in the MPS VII mouse), a mutant minor groove binding probe labeled with FAM (5′-TTGTCTTAGGCCC-GTACGT-3′) and a One-Step Plus PCR machine (Applied Biosystems; Foster City, CA). PCR for the wild-type CtsS allele used the primers 5′-CTTGAAGGGCAGCTGAAGCTG-3′ (forwards) and 5′-GTAGGAAGCGTCTGCCTCTAT-3′ (reverse), and PCR for the mutant CtsS allele used the primers 5′-CTCTGTGTAGCCTGGAATTC-3′ (forwards) and 5′-CTAAAGCGCATGCTCCAGACTGCC-3′ (reverse) [36] with analysis of the $C_T$ using SYBR green real-time PCR of DNA. MMP12 mice genotyping PCR used a forward primer common to the wild-type and mutant MMP12 alleles (5′-CCCTCGATGTGGA GTGCCCG-3′), a reverse primer specific for the PGK-neo cassette (5′-AAGAACGGAGCCGGTTGGCG-3′), and a reverse primer specific for the MMP12 wild-type allele (5′-ACTTGCCCTGAGCACCCCCT-3′), with gel electrophoresis used to identify wild-type (337 bp) or mutant (460 bp) alleles.

### 2.2. Measurement of aortic diameter and histopathology

Mice were anesthetized with 120 mg/kg ketamine/40 mg/kg xylazine in phosphate buffered saline (PBS) at pH 7.4. For some animals, aortic compliance was assessed, in which the outer diameter of isolated aortas was determined at different internal pressures [39]. For histopathology, ascending aortas obtained from 1 to 2 mm from the aortic valve were fixed with buffered formalin, embedded in paraffin, and 6 μm sections were stained with Verhoeff Van Gieson (VVG) stain. For biochemical analyses, animals were perfused with 20 ml of PBS, and the aorta from just above the aortic valve to just before the first branch was cleaned of surrounding fat. To test the effect of gene therapy, the width of dissected ascending aortas was measured after gently pressing it against a surface.

### 2.3. RNA analysis

Frozen ascending aortas were homogenized in Trizol for 30 s with a hand-held homogenizer (Kimble-Kontes; Vineland, NJ), and RNA was isolated using a Qiagen column. Reverse transcription (RT) 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 20 μl, followed by real-time PCR on 0.4 μl of each cDNA sample using SYBR green reagents from Applied Biosystems [10]. Primer sequences are in our previous publication [10] or in Supplementary Table 1. The percentage 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 assay using β-actin primers to determine the $\Delta C_T$, and the formula: percent β-actin $= (100)\times 2^{\Delta CT}$. The percent β-actin for MPS animals was divided by the percent β-actin in normal animals to determine the ratio of the gene in MPS to normal mice.

For microarray, RNA was reverse transcribed with primers with a T7 RNA polymerase binding site, amplified with T7 RNA polymerase with fluorescently-labeled deoxynucleotides, and hybridized to an Illumina bead microarray (Mouse8, version 2). Expression analysis was performed with ParTek software (St. Louis, MO). Pathway analysis was performed with GeneGo interactions software (https://portal.genego.com/cgi/data_manager.cgi; St. Joseph, MI).

### 2.4. Enzyme and GAG assays

For the GUSB, α-L-iduronidase (IDUA), and cathepsin assays, frozen aortas were homogenized with the hand-held homogenizer in 100 mM sodium acetate pH 5.5 containing 2.5 mM ethylenediaminetetraacetic acid, 0.1% Triton X-100, and 2.5 mM dithiothreitol, and centrifuged at 10,000 *g* for 5 min at 4 °C. The protein concentration was determined with the Bradford assay (BioRad Laboratories, Hercules CA). For the MMP12 and GAG assays, extracts were homogenized in the neutral buffer provided with the MMP12 kit with 0.1% Triton-X.

GUSB and IDUA assays were performed with the extracts prepared at pH 5.5 using the fluorogenic substrates 4-methylumbelliferyl-β-L-glucuronide (Sigma-Aldrich, St. Louis, MO) for GUSB and 4-methylumbelliferyl-α-L-iduronide (Toronto Research Chemicals, North York, Canada) for IDUA and a Fluoroskan Ascent microplate fluorometer (Thermo Electron, Milford, MA) as previously described [9]. One unit of enzyme converts 1 nmol of substrate to product per hour at 37 °C. GAG content was determined in the samples obtained