Rescuing Chondrocyte Hypertrophic Differentiation Potential and Exploring Therapeutic Approaches for Enhancing Bone Formation in Mucopolysaccharidosis VII Dogs

Sun Peck, PhD
Jennifer Kang, BS
Justin Bendigo, BS
Patricia O'Donnell
Caitlin Fitzgerald
Jessica Bagel
Neil Malhotra, MD
Eileen Shore, PhD
Margret Casal, DVM, PhD
Lachlan Smith, PhD

Introduction

The mucopolysaccharidoses (MPS) are genetic, lysosomal storage diseases characterized by deficient activity of enzymes that degrade glycosaminoglycans (GAGs)[1]. MPS VII is characterized by mutations in the β-glucuronidase gene, leading to incomplete digestion and progressive accumulation of three GAG types[2]. MPS VII patients exhibit severe skeletal abnormalities, especially of the spine[3]. Persistent cartilaginous lesions are present in the vertebrae representing failed cartilage-to-bone conversion during postnatal development, which result in progressive kyphoscoliosis and spinal cord compression[4-7]. Previously, using the naturally-occurring MPS VII canine model, we established that impaired hypertrophic differentiation of epiphyseal chondrocytes contributes to failed bone formation during early postnatal development[8], which in turn is associated with decreased Wnt/β-catenin signaling[9]. We also showed that Wnt pathway activation resulted in normalization of chondrocyte differentiation *in vitro* in MPS VII epiphyseal cartilage[10]. GAGs perform crucial roles in controlling the distribution and availability of Wnts, which are critical positive regulators of chondrocyte differentiation during endochondral ossification. Thus, we hypothesized that aberrant GAG accumulation in MPS VII contributes directly to impaired chondrocyte function and that in the absence of abnormal GAGs, hypertrophic differential potential could be rescued. To test this hypothesis, we undertook *in vitro* studies to compare differentiation potential of MPS VII chondrocytes in the presence and absence of their GAG-rich environment. Furthermore, to explore therapeutic approaches to correct MPS VII bone disease, we undertook a preliminary *in vivo* study in our canine model to establish a dosing regimen and safety profile using lithium, a Wnt pathway agonist, which has been previously shown to enhance bone formation and is approved clinically for other indications[11].

Methods

For this study, we used the naturally-occurring MPS VII canine model that closely mimics the skeletal phenotype of human patients[12].

In Vitro Analysis of GAG Accumulation and Chondrocyte Hypertrophic Differentiation Potential

With IACUC approval, unaffected control and MPS VII dogs (n = 4 for each) were euthanized at 9 days-of-age, and lumbar vertebral epiphyseal cartilage was isolated. For monolayer cultures, cartilage was digested with collagenase until cells were released from the extracellular matrix. Isolated chondrocytes were expanded in basal medium (DMEM, 10% FBS, 1% PSF) then cultured in monolayer in either basal or osteogenic media. For explant cultures, epiphyseal cartilage was cultured as whole tissue explants in basal medium. Media was collected at 3, 7, and 14 days for monolayer cultures and at 5 days for explant cultures. Total media GAG content was measured using the dimethylmethylene blue assay and normalized to total cell count. Cells from monolayer cultures and explants were harvested, RNA extracted, and mRNA expression levels of chondrocyte differentiation markers (Sox9-proliferative; Runx2-prehypertrophic; Col10-hypertrophic) were measured using qPCR. Significant differences between groups ($p < 0.05$) were established using unpaired t-tests.

In Vivo Lithium Treatment

To establish dosage needs of lithium, normal control dogs (n = 2) were treated with twice daily doses of 5 mg/kg of powdered lithium carbonate packaged into gelatin capsules for 1 week for acclimation, then with twice daily doses of 10 mg/kg for 2 weeks, starting at 15 days-of-age. Dogs were monitored for side effects, and serum lithium levels were measured using a commercial assay (Crystal Chem).

Results

In Vitro Analysis of GAG Accumulation and Chondrocyte Hypertrophic Differentiation

In whole explant culture, MPS VII chondrocytes secreted significantly higher amounts of GAGs into the media compared to controls over time,

while isolated chondrocytes showed no differences over 14 days of culture (Figure 1). Likewise, in whole explant culture, MPS VII chondrocytes showed impaired differentiation over time compared to controls, but both control and MPS VII chondrocytes exhibited similar propensity to differentiate over time in isolated cell culture (Figure 2).

In Vivo Lithium Treatment

After the initial 1 week acclimation period, both dogs maintained serum lithium levels within in the desired therapeutic range (0.2-1.5mmol/L) over the following 2 weeks (Figure 3). Dogs exhibited a mild tremor which resolved within a few days. No significant adverse side effects from lithium treatments were observed.

Discussion

The results of this study show that MPS VII chondrocytes regain normal hypertrophic differentiation potential upon removal from their GAG-rich environment. Abnormal GAG accumulation in MPS VII epiphyseal cartilage may disrupt extracellular control of secreted growth factors, such as Wnts, which are necessary to initiate and sustain chondrocyte differentiation. We previously showed that activation of the Wnt pathway with exogenous factors can also normalize chondrocyte differentiation *in vitro*, and taken together, these results indicate that combinatorial therapies that normalize GAG accumulation and activate Wnt signaling may be able to rescue the differentiation potential of resident cells and ultimately normalize bone formation. As a preliminary step, we successfully treated neonatal dogs with lithium, establishing safety and optimizing an oral dosing regimen to sustain therapeutic serum levels. In ongoing *in vivo* studies, we are examining whether GAG reduction via exogenous enzyme replacement therapy (ERT) and Wnt/β-catenin pathway activation via lithium treatment are able to normalize chondrocyte function and bone formation in MPS VII dogs during postnatal growth.

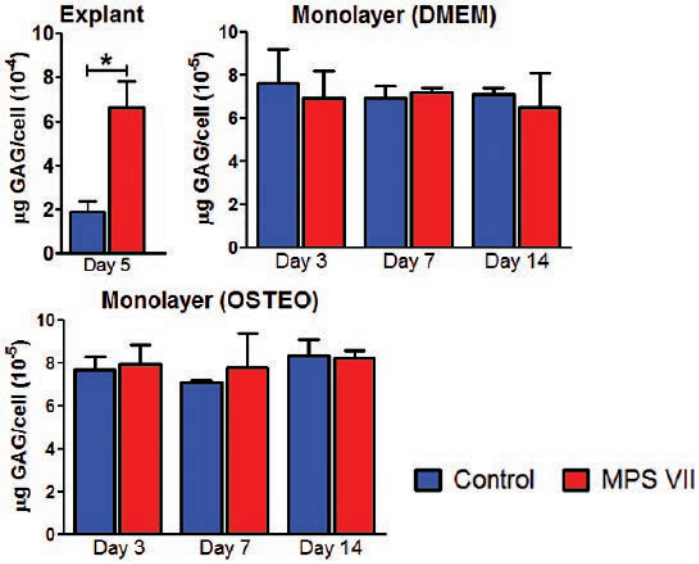

Figure 1. Culture media GAG content. Intact epiphyseal cartilage explants from MPS VII animals exhibited significantly higher GAG content secreted into the media compared to controls. Isolated chondrocytes in monolayer culture showed normalization of secreted GAG content regardless of media conditions. N = 4; *p < 0.05.

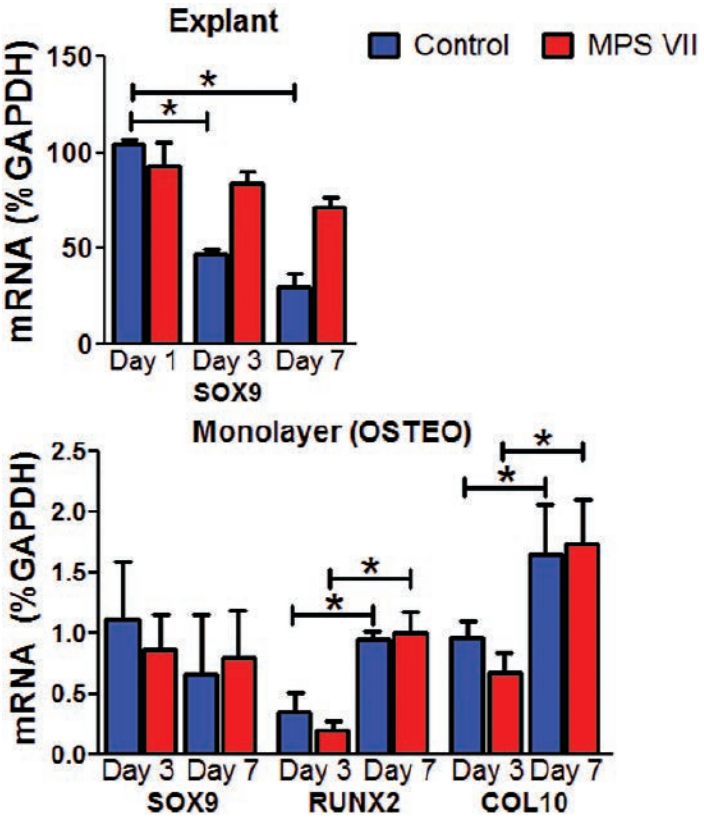

Figure 2. Chondrocyte differentiation potential. In culture, control chondrocytes in intact epiphyseal cartilage explants exhibited propensity to differentiate over time (decreasing SOX9 expression) while MPS VII chondrocytes did not (persistent SOX9 expression). In contrast, both control and MPS VII isolated chondrocytes grown in monolayer culture differentiated normally in the presence of osteogenic conditions. N = 4, *p < 0.05.

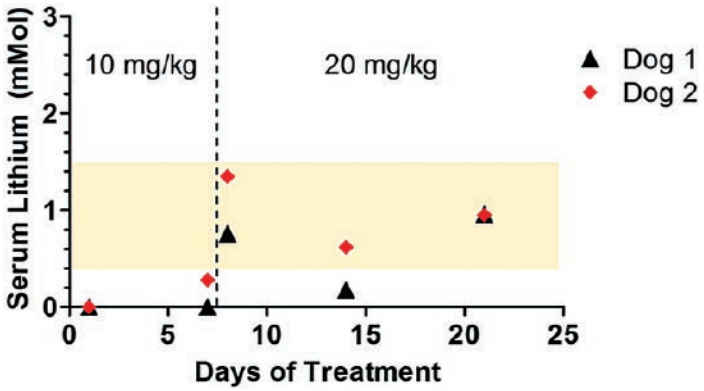

Figure 3. Serum lithium levels in treated dogs. 15-day old normal control animals were treated for 21 days with daily doses of lithium (10 mg/kg daily for 7 days, 20 mg/kg for following 14 days). Dashed line indicates day of increase in lithium dosage. Yellow shaded area indicates target non-toxic, therapeutic range for serum lithium concentration.

Significance

MPS VII is associated with debilitating skeletal disease for which there is no treatment. Our results suggest that therapeutic strategies combining GAG reduction (ERT)

and inducing endochondral bone formation (lithium) may effectively treat skeletal abnormalities in MPS VII patients.

Acknowledgements

Funding sources: NIH, Penn Orphan Diseases Center, National MPS Society and Penn Center for Musculoskeletal Disorders.The authors thank the staff and students at the Penn Vet School for animal care.

References:

1. Neufeld E, Muenzer, J. *The Mucopolysaccharidoses. The Online Metabolic and Molecular Bases of Inhertited Disease.* 2001.

2. Sly WS, Quinton BA, McAlister WH, Rimoin DL. Beta glucuronidase deficiency: report of clinical, radiologic, and biochemical features of a new mucopolysaccharidosis. *J Pediatr.* 1973;82(2):249-257.

3. Peck SH, Casal ML, Malhotra NR, Ficicioglu C, Smith LJ. Pathogenesis and treatment of spine disease in the mucopolysaccharidoses. *Mol Genet Metab.* 2016;118(4):232-243.

4. Pizzutillo PD, Hummer CD, 3rd. Nonoperative treatment for painful adolescent spondylolysis or spondylolisthesis. *J Pediatr Orthop.* 1989;9(5):538-540.

5. de Kremer RD, Givogri I, Argarana CE, *et al.* Mucopolysaccharidosis type VII (beta-glucuronidase deficiency): a chronic variant with an oligosymptomatic severe skeletal dysplasia. *Am J Med Genet.* 1992;44(2):145-152.

6. Smith LJ, Martin JT, Szczesny SE, Ponder KP, Haskins ME, Elliott DM. Altered lumbar spine structure, biochemistry, and biomechanical properties in a canine model of mucopolysaccharidosis type VII. *J Orthop Res.* 2010;28(5):616-622.

7. Yasin MN, Sacho R, Oxborrow NJ, Wraith JE, Williamson JB, Siddique I. Thoracolumbar kyphosis in treated mucopolysaccharidosis 1 (Hurler syndrome). *Spine (Phila Pa 1976).* 2014;39(5):381-387.

8. Peck SH, O'Donnell PJ, Kang JL, *et al.* Delayed hypertrophic differentiation of epiphyseal chondrocytes contributes to failed secondary ossification in mucopolysaccharidosis VII dogs. *Mol Genet Metab.* 2015;116(3):195-203.

9. Peck SH, Al et. Orthopaedic Research Society Annual Meeting.2016.

10. Peck SH. American Society of Bone and Mineral Research Annual Meeting.2016.

11. Clement-Lacroix P, Ai M, Morvan F, *et al.* Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice. *Proc Natl Acad Sci U S A.* 2005;102(48):17406-17411.

12. Haskins ME, Desnick RJ, DiFerrante N, Jezyk PF, Patterson DF. Beta-glucuronidase deficiency in a dog: a model of human mucopolysaccharidosis VII. *Pediatr Res.* 1984;18(10):980-984.