Studies on intervertebral disc degeneration in the chondrodystrophoid dog breed

Summary of Doctoral Thesis

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Introduction

Low back pain resulting from intervertebral disc (IVD) degeneration is a leading cause of incapacity in human and veterinary health. IVD degeneration leads to loss of proteoglycans and water content in the nucleus pulposus (NP), which contains large amounts of aggregating proteoglycans and type II collagen (Col2), typical of compression-resisting tissues. NP cells display a rounded, chondrocyte-like morphology and secrete extracellular matrix (ECM) macromolecules composing the hyaline cartilage.

Cells in the NP originate from the notochord. There is a significant difference in the lifespan of notochordal cells among different species, and their loss correlates with early disc degeneration. In pigs, rabbits, rodents, and non-chondrodystrophoid dogs, the notochordal cell population persists into late adult life. However, in humans, sheep, and chondrodystrophoid breeds (CDBs), such as the Beagle and the Dachshund, those cells disappear with age and are replaced by fibrochondrocyte-like cells. CDBs are affected by a profound degenerative disc disease with early onset, often developing within the first year of life; clinical symptoms derived from abnormal endochondral ossification develop between 3 and 7 years of age, with high incidence and high relative risk of developing disc herniation. Indeed, the relative risk for disc herniation is approximately 10–12 times higher in the Dachshund than in non-chondrodystrophoid breeds. It is thought that the chondrodystrophoid phenotype of CDB is similar to that of humans.

The mechanism underlying age-related IVD degeneration, however, is poorly understood. Many studies have shown an increase in the expression and activity of matrix metalloproteinases (MMPs) during IVD degeneration, and prominent ECM

components of the disc, including Col1 and Col2 and aggrecan, have been shown to be substrates of various MMPs.

It has been reported that, in osteoarthritic articular cartilage, there is increased accumulation of β-catenin and decreased expression of aggrecan and Col2a1. Several research groups have suggested that Wnt/β-catenin signal play an important role in IVD degeneration. Wnt signals typically involve a noncanonical pathway or a canonical pathway, and of these, the canonical Wnt/β-catenin pathway, which activates the transcription factors T-cell factor (TCF) and lymphoid enhancer factor (LEF) through β-catenin activity, is well known. When the Wnt ligand is absent, β-catenin undergoes glycogen synthase kinase 3β (GSK-3β)-mediated phosphorylation and proteasome-mediated degradation. When the Wnt ligand is present, it interacts with its receptor, low-density lipoprotein (LDL) receptor-related protein (LRD) 5/6, which recruits Axin to facilitate its decomposition. In addition, Dishevelled proteins facilitate the dissociation of the adenomatous polyposis coli/Axin/GSK-3β complex, whereas frequently rearranged in advanced T-cell lymphoma/GSK-3 binding protein (FRAT/GBP) directly inhibits GSK-3β phosphorylation activity. As a result, the phosphorylation of β-catenin by GSK-3β is inhibited and the β-catenin is stabilized. The stabilized β-catenin moves into the nucleus and, together with TCF and LEF, controls the formation of the body axis and somites, as well as cellular proliferation and differentiation. The quantitative changes of β-catenin are therefore an extremely important factor. However, the role of Wnt/β-catenin signals in IVD cells is not yet well understood.

Another important factor in the process of disc degeneration is Runt-related

transcription factor 2 (Runx2) expression. A previous report suggested that Runx2 is also implicated in the progression of intervertebral disk aging and calcification in CDBs. Runx2 is an essential transcription factor for osteoblast differentiation and chondrocyte maturation. Runx2 expression is also induced in the articular cartilage of wild-type mice in early stages of osteoarthritis, and this induction occurs prior to MMP-13 expression, indicating that Runx2 has an important role in the osteoarthritis disease process. In addition, Wnt signaling enhances Runx2 expression through the direct binding of TCF7 or LEF1/β-catenin on the Runx2 promoter and through DNA binding of small mothers against decapentaplegic (SMAD) proteins and TCF7L2/β-catenin to their cognate sequences as well as protein–protein interactions between them.

The objective of this study was to evaluate whether the Wnt/β-catenin signaling enhances NP cell degeneration and calcification. We hypothesized that Wnt/β-catenin signaling would enhance Runx2 expression in intervertebral degeneration and lead IVD calcification.

1. Quantitative evaluation of degeneration of NP tissue

To evaluate its degeneration, we graded the NP tissue based on the MR signal intensity that was measured using Image J software. NP tissue which exhibits signal intensity >86 is classified as grade 1, 45–85 as grade 2, and <45 as grade 3. The results showed that the average signal intensity (ASI) of grade 1 NP tissue was 112.3, grade 2 was 68.3, and grade 3 was 34.2. Moreover, the percentage of tissue occupied by the grade 1 was 61%,

grade 2 was 18%, and grade 3 was 21%. It should be noted that, although all NP tissue was derived from 12-month-old CDBs, grade 3 NP tissues were detected, indicating that CDBs are affected by a profound degenerative disc disease with early onset that often develops within the first year of life.

2. **Variations in Gene and Protein Expression in Canine Chondrodystrophic Nucleus Pulposus Cells Following Long-Term Three-Dimensional Culture**

Specifically, we evaluated the potential of a three-dimensional (3D) culture of healthy NP as an in vitro model system to investigate the mechanisms of IVD degeneration. Agarose hydrogels were populated with healthy NP cells from beagles after performing magnetic resonance imaging, and mRNA expression profiles and pericellular extracellular matrix (ECM) protein distribution were determined. After 25 days of 3D culture, there was a tendency for redifferentiation into the native NP phenotype, and mRNA levels of *Col2A1*, *COMP*, and *CK18* were not significantly different from those of freshly isolated cells. Our findings suggest that long-term 3D culture promoted chondrodystrophic NP redifferentiation through reconstruction of the pericellular microenvironment. Further, lipopolysaccharide (LPS) induced expression of *TNF-α*, *MMP3*, *MMP13*, *VEGF*, and *PGES* mRNA in the 3D cultures, creating a molecular

milieu that mimics that of degenerated NP. These results suggest that this in vitro model represents a reliable and cost-effective tool for evaluating new therapies for disc degeneration.

3. **Wnt/β-catenin signaling enhances intervertebral disc degeneration and calcification through Runx2 signaling**

Here, we demonstrate that Wnt/β-catenin signaling would enhance Runx2 expression in intervertebral degeneration and lead to IVD calcification.

NP tissue was obtained from 12-month-old male Beagle dogs after evaluation of the degeneration based on the magnetic resonance (MR) signal intensity. Histological analysis showed that lack of Safranin-O staining, calcified area, and MMP13-positive cells increased with progression of the degeneration. Furthermore, β-catenin- and Runx2-positive cells also increased with the progression of the degeneration. Real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis showed that the MRI signal intensity and mRNA expression levels of β-catenin and Runx2 are correlated in NP tissues.

Moreover, to evaluate the role of the Wnt/β-catenin pathway in the regulation of NP cells degeneration, we studied the effects of LiCl on cultured canine NP cells.

Using western blotting analysis, we found that the levels of β-catenin were consistently upregulated by LiCl. A supplementation of 20 mM LiCl induced β-catenin accumulation and Runx2 expression. In contrast, FH535, an inhibitor of β-catenin/TCF activity, inhibits the upregulation. These results indicate that Wnt/β-catenin signals have a significant role in degeneration and calcification in IVD through Runx2 signal.

In summary, our findings support a pivotal role for culture microenvironment on chondrodystrophic disc cell behavior and further suggest that the length is an important factor in 3D scaffolds. Because the phenotype of NP cells of CDBs is similar to that of humans, these results also suggest that the same basic mechanism of accelerated degeneration functions in human NP tissue.

In addition, our results suggest that Wnt/β-catenin signals may have a significant role in degeneration and calcification in IVD through Runx2 signal.

Our data support the possibility that Wnt/β-catenin induces Runx2 and MMP expression in disc cells of CDBs.