INTRODUCTION: Cartilage intermediate layer protein (CILP) has been implicated in several diseases that affect cartilage. In osteoarthritis, CILP is among the few cartilage matrix proteins whose expression is upregulated in early and late stages of the disease. CILP has been reported to be associated with the need for spinal surgery in a group of patients with lumbar disc disease (LDD) defined by the presence of a lumbar disc herniation and sciatica. The pathomechanism of CILP has been theorized to involve excessive CILP binding of TGF-β that leads to decreased proteoglycan synthesis. However, regulation of the CILP gene remains largely unknown. Using two different rabbit models, we evaluated the effects of annulotomy induced degeneration in young rabbits, natural aging, and BMP-2 on CILP expression in order to better understand the regulation of CILP expression in the nucleus pulposus.

METHODS: Animal Models: To study the effect of disc degeneration on CILP expression, four six-month-old rabbit’s discs were punctured to a depth of 5 mm using an 18-gauge needle at L2-3 and L4-5. Four weeks after the puncture nucleus pulposi were harvested. Control discs consisted of L1-2, L3-4, and L5-6 from the same animal. To study the effect of aging, twelve New Zealand White rabbits were used: four six-months old, four three-years old, and four five-years old. From each rabbit, the six lumbar discs (L1–L2, L2–L3, L3–L4, L4–L5, L5–L6, and L6–L7) nucleus pulposi were harvested under sterile conditions. The disc tissues from each animal were pooled to make a single sample.

Cell Culture and treatment: The cells from the disc tissue were cultured in DMEM/F-12 medium and treated for two days with either: 1) rhBMP-2; 2) Noggin; 3) siRNAs; 4) mock controls. After treatment, the cells were harvested for analysis of Western immunoblot, or Real-time PCR.

Real-time PCR and Western blots: Total RNA was isolated using TRIzol reagent and reverse-transcribed using SuperScript RNase H Reverse Transcriptase. Quantitative PCR (QPCR) was run in triplicate using Brilliant® SYBR® Green QPCR Core Reagent Kit. Gene expression profiling was completed using the comparative Ct method of relative quantification. Relative RNA quantities were normalized to endogenous 18S ribosomal RNA (18S rRNA). The results are expressed as a ratio to untreated control. Western blots were performed to examine CILP expression by using CILP specific antibody (from Dr. Shoji Seki). The promoter region of human CILP was amplified from the human disc genomic DNA, and was cloned into pGEL3 Basic. The CILP promoter luciferase reporters were co-transfected into the rabbit disc cells with a pRTK-Luc vector, which expresses the Renilla Luciferase (RLuc) in order to normalize transfection efficiency. Three days after transfection, Firefly luciferase (Luc) and RLuc activities were measured by using the Dual Luciferase Reporter Assay System, according to the manufacturer’s instructions. Firefly luciferase was always normalized with RLuc activity.

RESULTS:

Figure 1: To study the effect of disc degeneration in an injury model in young animals, four six-month-old rabbit’s discs were punctured to a depth of 5 mm using an 18-gauge needle. At four weeks after the puncture, the discs were degenerated based on MRI (not shown) and decrease in aggrecan and collagen II mRNA levels. In contrast, CILP level was increased in the tissue from the punctured discs as compared to that from the un-punctured discs based on Western blot analysis. This showed that CILP expression rises with degeneration even in young animals.

Figure 2: To study the effect of age related degeneration on CILP expression, we used twelve different New Zealand White rabbits as a model: four six-months old, four three-years old, and four five-years old. The cells from the disc tissue were obtained and pooled (in a similar fashion as explained above). As expected, Real-time PCR shows that the mRNA levels of aggrecan and collagen II were decreased with age, and Western blot demonstrates that CILP expression increases substantially with age in the rabbit discs.

DISCUSSION: The major findings to come from this study using rabbit lumbar intervertebral discs are: 1) rabbit discs express CILP, and CILP expression increases substantially with an annulotomy induced disc degeneration and with natural ageing; 2) the stimulatory effect of BMP-2 on CILP expression increases with age; 3) Smad is involved in BMP-2 induced CILP expression. Taken together, these findings demonstrate that BMP-2, degeneration, and age are regulators of CILP gene expression. Our previously published data indicated that BMP-2 levels increase with age raising the possibility of a mechanism involving age related BMP-2 upregulation that could then contribute to age related CILP upregulation. Our findings provide better understanding of how CILP is controlled. As excessive CILP may be deleterious to proteoglycan synthesis our findings may provide some targets for modulating the process of disc degeneration.
