REGULATION OF CD44 PHOSPHORYLATION IN HUMAN ARTICULAR CARTILAGE

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Introduction: Under osteoarthritic conditions, chondrocytes exhibit a decreased capacity to retain extracellular matrix components at the cell surface. Phosphorylation of the transmembrane receptor CD44 is thought to directly regulate matrix assembly and retention in articular cartilage (1). Phosphorylation of the CD44 cytoplasmic domain appears to be hyaluronan dependent and therefore may alter cytoskeletal protein interactions or serve other intracellular signaling functions (2). Results from previous work provide strong evidence that casein kinase II (CKII) is involved in articular cartilage metabolism, as working through CD44 phosphorylation. Studies have shown that CKII protein levels decrease in damaged articular cartilage, and that the ability of CD44 to form a pericellular matrix in chondrocytes is diminished following CKII antisense oligonucleotide treatment (3). The goals of the present study are twofold: (1) to determine changes in CKII mRNA in damaged tissue as well as osteoarthritic tissue obtained following joint replacement surgery, and (2) to over-express the cytoplasmic tail of CD44, which presumably contains the critical serine residues for phosphorylation, to gain a better understanding of how CD44 phosphorylation affects chondrocyte behavior.

Methods: Human knee osteoarthritic (OA) tissue was obtained following total joint replacement surgery and human knee articular cartilage was obtained within 24 h of death from the Regional Organ Bank of Illinois in accordance with institutional IRB guidelines. Following assignment of a Collin’s Grade score (0-4) to assess damage, total RNA was isolated from the damaged or OA cartilage and semi-quantitative RT-PCR performed at low template concentration and sub-saturating cycle number (28) for GAPDH, CD44, casein kinase and aggrecan (only for OA and grade 0 samples). Band intensity was quantitated using a fluorimaging system and GAPDH normalized. Using PCR methodologies and the cDNA for full-length CD44, the cytoplasmic tail of CD44 was inserted into pcDNA3.1 (Invitrogen) that contains an amino-terminal Xpress™ epitope. COS-7 or HeLa cells were transfected (15µg) with the pcDNA3.1 vector alone, or with the cytotail/Xpress construct either alone or in combination (7.5ug each) with pTracer empty vector in the presence of Lipofectamine 2000. After 48 h, total RNA and protein were harvested, or the cells were fixed for immunostaining. Equivalent amounts of COS-7 or HeLa cell RNA were reverse transcribed and amplified using specific primers designed against pcDNA3.1, the cytotail insert and GAPDH, and the cDNAs amplified by PCR. Immunochemical studies were conducted on 2% paraformaldehyde-fixed HeLa cells using an anti-Xpress mouse monoclonal antibody. Western blot techniques were employed using the same antibody following 18% SDS-PAGE using COS-7 protein lysates.

Results: Fig. 1 shows ongoing comparisons of (A) normal donor knee cartilage to knee OA cartilage and, (B) human donor articular cartilage derived from the knee from donors with varying Collin’s grade changes in tissue integrity (grades 1 to 3). As expected, CKII mRNA levels were decreased in chondrocytes taken from damaged tissue or following total joint replacement compared to normal donor tissue. Levels of CD44 and aggrecan mRNAs were increased in these tissues, possibly indicative of a repair attempt by the chondrocytes. Bars show the ratio of mRNA from the OA or Collin’s grades 1 to 3 cartilage to the mRNA from Collin’s grade 0 samples.

To visualize expression of the protein, HeLa cells were co-transfected with pTracer empty vector (with GFP expression as a marker) and the cytotail/Xpress construct. As shown in Fig. 4, only the GFP-positive HeLa cells (A) were positive for anti-Xpress antibody red immunofluorescence (B). The GFP/Anti-Xpress overlay is shown in panel C. As a control, HeLa cells co-transfected with pTracer and pcDNA3.1 empty vectors exhibited GFP (D) but no red anti-Xpress fluorescence (E). The overlay of panels D and E is shown in panel F. Non-transfected cells were negative for GFP and anti-Xpress.

Discussion: Results from this study further implicate CKII in normal and damaged chondrocyte metabolism. Current experiments focus on investigating the effects of specific casein kinase inhibitors on hyaluronan binding and CD44 phosphorylation in cultured COS-7 cells that have been transfected with the full-length CD44 or the cytotail/Xpress. Over-expression of the cytotail domain of CD44 is one approach being taken to perturb CD44 phosphorylation. The tail domain has successfully been transfected into COS-7 and HeLa cells, as shown by RT-PCR, Western Blot, and immunostaining. Experiments underway will determine in HeLa, COS-7 and most importantly, human or bovine articular chondrocytes, the effect of over-expression of this cytotail/Xpress on CD44 receptor phosphorylation. Additional CD44 tail fusion proteins are currently under construction and will aid in determining the role of CKII in CD44 phosphorylation. Fusion proteins have been created by introducing CD44 into vectors that contain green and red fluorescent, and V5 and Xpress tags. These flags have and will continue to provide valuable information concerning modulation of chondrocyte behavior through CD44. The critical serine residues within the cytoplasmic tail of CD44 have been mutated and inserted into the above-mentioned vectors in preparation for transfection into chondrocytes. It is hypothesized that loss of these critical residues will interfere with CD44 phosphorylation and potentially lead to altered HA binding, matrix assembly and downstream signaling. Results from these studies will delineate the mechanisms of CD44 phosphorylation in chondrocytes and may lead to identification of cytoplasmic proteins in addition to CKII that may partner with CD44. Knowledge of these interactions may prove helpful in the understanding of the signaling and regulatory pathways of CD44 in both normal and osteoarthritic cartilage.