Osteoarthritis and Cartilage Vol. 15, Supplement C

ANK EXPRESSION MODULATES BOTH THE EXTRACELLULAR PYROPHOSPHATE CONCENTRATION, AND THE PHENOTYPE OF HUMAN OSTEOARTHRITIC CHONDROCYTES

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Purpose: Chondrocyte extracellular pyrophosphate (ePPi) plays a role in the pathology of calcium deposition diseases and, potentially, primary osteoarthritis. [ePPi] is determined via a number of mechanisms that regulate its generation and transport. The purpose of the current work was to determine the effect on ePPi and cell phenotype, after either knockdown or over expression of the PPI transporter Ank in primary chondrocytes.

Methods: Human articular chondrocytes were isolated from cartilage obtained from consenting donors undergoing joint replacement surgery. Bovine articular chondrocytes were isolated from cartilage obtained from the metacarpophalangeal joint of adult bovines. Chondrocytes were isolated using sequential digestion with pronase and bacterial collagenase. Cell lines stably expressing human Ank (native or C-terminal His tag) were created via the enzymatic procedure of Lust and Seegmiller. siRNA transfections were carried out using 1.25ul/cm2 of siRNA effectively knocked-down Ank expression (>80%) in human OA chondrocytes and this was accompanied by > 90%-inhibition of [ePPi]. In human OA chondrocytes, siRNA knock-down of TNAP, NPP1 or Ank mRNA, was achieved either individually or in combination with each of the other two genes. Knock-down of Ank or NPP-1, either alone or in any combination with the other two genes, blocked the accumulation of ePPi. Knock-down of TNAP alone had no effect on [ePPi]. Overexpression of Ank in normal bovine chondrocytes via electroporation led to efficient expression of human Ank and this was accompanied by the upregulation of genes related to both catabolism (catechisin K, MMP-13), and hypertrophy (type X collagen, tissue transglutaminase), and the down-regulation of genes related to anabolism (type II collagen and aggrecan).

Conclusions: Overexpression of Ank in HEK 293 cells leads to increased accumulation of ePPi, presumably through Ank’s transmembrane transport of intracellular PPI (iPPi) to the extracellular medium. Consistent with this concept, knock-down of Ank expression in human OA chondrocytes blocked the accumulation of ePPi. The mechanism of generation of iPPi for Ank transport is unclear; however, the inhibition of ePPi by knock-down of either Ank or NPP1 suggests that NPP1 hydrolysis of ATP to AMP and PPI may play a role in providing substrate for Ank-mediated PPI transport. TNAP knock-down had no effect on [ePPi], suggesting that TNAP activity does not play a major role in either generating ePPi or hydrolyzing ePPi to inorganic phosphate in human OA chondrocytes. Overexpression of Ank in chondrocytes drives changes in cell phenotype that are consistent with those observed in OA i.e. increased expression of catabolic and hypertrophic genes, and decreased expression of anabolic genes. These data suggest that increased Ank expression or function may play a role in the progression of degenerative joint diseases.

Investigating Wnt1 Inducible Secreted Protein 3 in Osteoarthritis and Chondrocyte Biology

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Purpose: Progressive pseudorheumatoid dysplasia (PPRD) is a rare childhood disease of articular cartilage. Mutations in the gene encoding WISP-3 (Wnt1 inducible secreted protein 3), a member of the CCN (Connective Tissue Growth Factor/Cysteine-Rich 61/Nephroblastoma Over-expressed) family, have been identified in several cases of PPRD. Previous work in another laboratory suggests WISP-3 may regulate expression of type II collagen and aggrecan in immortalised chondrocytic cell lines. IGF-1 (Insulin-like growth factor 1) activates different signalling pathways in chondrocytic cells to encourage proliferation and differentiation. IGF-1 activity in cartilage depends on its bioavailability and the responsiveness of chondrocytes. WISP-3 has an IGFFBP (Insulin-like growth factor binding protein) domain. Therefore, WISP-3 may alter chondrocyte activity by modulating IGF-1 availability and signalling.

The aim of the current project is to further examine the role of WISP-3 in chondrocyte biology and cartilage matrix turnover.

Methods: The expression of WISP-3 mRNA in osteoarthritic and normal post mortem cartilage was determined by real time PCR. Expression of WISP-3 protein in osteoarthritic and normal post mortem cartilage sections was assessed by immunohistochemical staining.
The WISP-3 gene was stably expressed in an immortalised chondrocytic cell line (C-28/I2) to investigate the effects of WISP-3 on chondrocyte biology. The expression of cartilage-specific genes was compared by real-time PCR in clonal cells stably transfected with WISP-3 or the empty vector alone. Results in the WISP-3 stably expressing cells were verified by treating untransfected parental C-28/I2 cells with recombinant human WISP-3 protein.

The potential role of WISP-3 in cell signalling was investigated by treating C-28/I2 cells with WISP-3 alone, IGF-1 alone or IGF-1 and WISP-3 together. Erk1/2 phosphorylation was then measured by western blotting after treatment for 10 minutes or 1 hour.

**Results:** Real-time PCR revealed WISP-3 mRNA was upregulated in osteoarthritic cartilage compared to normal post mortem cartilage. Immunohistochemical staining of osteoarthritic and post mortem cartilage sections showed WISP-3 protein was present in damaged areas. In severely damaged cartilage sections, WISP-3 often appeared as strong halos around chondrocytes.

Stable over-expression of WISP-3 in C-28/I2 cells led to a down-regulation of Col2α1 mRNA. Preliminary results suggest this effect can also be produced by treatment of parental C-28/I2 cells with recombinant WISP-3 protein.

Treatment of C-28/I2 cells with IGF-1 induced Erk1/2 phosphorylation after 10 minutes. WISP-3 could also induce Erk1/2 phosphorylation, although when in combination with IGF-1 it did not appear to enhance or prolong Erk1/2 phosphorylation.

**Conclusions:** Our data suggest that WISP-3 may be a marker for damaged cartilage and may exert effects on matrix synthesis and chondrocyte signalling. Further work is required to determine the role of WISP-3 in cartilage matrix metabolism. We intend to assess the effects of WISP-3 on metalloproteinase expression, proliferation and IGF-1 induction of Col2α1 in C-28/I2 cells.

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**IKKα AND IKKβ ARE BOTH REQUIRED FOR THE CHEMOTACTIC ACTIVITY OF MONOCYTES TOWARDS PRIMARY OSTEARTHROITIC CHONDROCYTES**

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**Purpose:** IKKα and IKKβ are essential kinases for activating NF-κB transcription factors that regulate cellular differentiation and inflammation. By virtue of their small size and ECM diffusibility chemokines could invoke cross-talk between osteoarthritic (OA) chondrocytes with other joint compartments and also contribute to immune cell chemotaxis into the synovial space. Because many chemokines are known to be direct NF-κB targets, here we employed shRNA retroviruses to ablate the expression of each IKK to determine their individual contributions for monocyte chemotaxis in response to chondrocyte conditioned media.

**Methods:** Primary chondrocytes were derived from 6 OA patients undergoing joint arthroplasty. IKKα or IKKβ shRNAs were stably expressed by pSuper retroviral transduction of IKKα or IKKβ specific shOligos followed by selection of puromycin resistant cells and KD efficiencies were verified by immunoblotting. High density monolayer or micromass cultures were established for each patient. Conditioned media was collected, with or without 72 h of prior exposure to IL-1β. Chemotaxis of human monocytes towards chondrocyte conditioned media was assessed with boyden chambers. The chemokine expression repertoires under basal and stimulated conditions in wild type and IKK KD chondrocytes were evaluated by focused Oligo GEarrays (SuperArray) and the concentrations of monocyte active chemokines (CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES) were also quantified by Multiplex fluorescence-based assays.

**Results:** Penetrant IKKα or IKKβ KDs of 80-90% blunted the monocyte chemotactic potential of chondrocyte conditioned media. Under basal conditions CCL2/MCP-1 was the chemokine of highest concentration (nanogram levels) in wild type chondrocyte conditioned media, which presented the strongest association with monocyte chemotaxis (Spearman=0.943, p<0.05). Interestingly CCL2/MCP-1 production was co-dependent on IKKα and IKKβ, while stimulus induced expression of CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES appeared to be largely dependent on IKKα and not IKKβ. Similar results were obtained for CCL5/RANTES at the RNA level after IL-1 stimulation for either 2 or 8 hours.

**Conclusions:** Results of targeted retroviral mediated RNA interference revealed a co-dependency on IKKα and IKKβ for monocyte chemotaxis in response to chondrocyte conditioned media, even in the absence of an extracellular pro-inflammatory stimulus (i.e., IL-1β). Interestingly at least part of the dual IKK dependency for this inflammatory-like migration response was associated with the magnitude of MCP-1 production. Moreover IKKα and IKKβ appear to be constitutively active in OA chondrocytes, because chondrocyte conditioned media induced a strong migration response by primary monocytes under basal conditions, likely reflecting the synovitis often complicating OA. Our findings reveal important roles of both NF-κB activating kinases in driving a key inflammatory process underlying this debilitating disease.

**Acknowledgements:** Supported by the CARISBO Foundation (Grant number: 2005-1059); the University of Bologna and the CRBA laboratory; Ricerca Corrente Istituti Ortopedici Rizzoli and the EU FP6 IP (INNOCHEM, grant number LSHB-CT-2005-518167).