necessary supernatants were analyzed with the cell death ELISA (Roche) measuring photometrically the % increase of apoptosis over control (no Fas).
Results: TUNEL assay identified the number of live cells as compared apoptotic cells that were induced by Fas activation. The number of TUNEL positive cells was 40.7%±19.4 as compared to the control 0.84%±1.01 in the chondrocytes grown in suspension on a poly-HEMA coated slide chambers. The amount apoptosis found in chondrocytes was significantly reduced when cells were grown on Matrigel 1.03%±0.91 as compared to the Matrigel− as compared to 93.9%±3.46. The control group grown in suspension, no matrix was increased by 40.7%±19.4. The presence of perlecan resulted in an ~50% reduction in the number of apoptotic cells as compared to the control (21.52±7.06) over no matrix addition control. Cells grown on Matrigel (increased only 21.4% over control no Fas (CTL)) clearly indicating protection from apoptosis; compared to FN (increase 224% over CTL) and to cells grown as adherent monolayer without matrix (897% over CTL). Chondrocytes in a phenotypic correct condition were very unresponsive to Fas-apoptosis (90.8% increase over CTL) as compared to monolayer culture (897% increase over control).
Conclusions: Since cartilage chondrocytes live within a unique ECM environment it is important to understand functions like apoptosis within a matrix-rich environment. Perlecan is found in the chondrocyte's pericellular matrix and is important in development and chondrogenesis. In this study we show that certain ECM components can alter the response to an inducer of apoptosis. Also, growing chondrocytes in a phenotypically-correct environment also protects or prevents them form undergoing apoptosis. Specifically matrix containing perlecan can protect or alter the response of chondrocytes to stimuli of apoptosis. The data suggest that perlecan is involved, but it may be indirect, since many growth factors and molecules bind to perlecan. The importance of maintaining the integrity of the ECM of chondrocytes to maintain their "well-being" in vivo is likely a factor in the integrity and function of articular cartilage. Further understanding of the precise molecules involved may provide a viable target for therapeutic intervention in a variety of cartilage diseases.

238 IMPLICATION OF THE INORGANIC PYROPHOSPHATE TRANSPORTER ANK IN ARTICULAR CHONDROCYTE PHENOTYPE SUSTAIN

F. Cailotto, S. Sebillaud, D. Moulin, J-Y. Jouzeau, P. Netter, A. Bianchi. *LPPA UMR 7561 Nancy Université CNRS, Vandoeuvre les Nancy, FRANCE*

Purpose: Articular chondrocyte phenotype is mainly characterized by an expression pattern of genes coding for the extracellular matrix, in particular type II collagen and aggrecan, and the absence of type I and X collagens. The wnt genes family has been described to play a major role on chondrocyte phenotype, notably in the dedifferentiation process mediated by interleukin-1β (Il-1β) and Wnt-5a in osteoarthritis. Inorganic pyrophosphate has also been shown to influence osteo-articular cells phenotype, like osteoblasts. Moreover, we demonstrated that ANK is mainly responsible for extracellular inorganic pyrophosphate (ePPi) generation. In the present study, we focused on the role of ANK and ePPi in the maintenance of articular chondrocyte phenotype. We pointed out the implication of Wnt signaling in this process.
Methods: Firstly, we characterized chondrocyte phenotype in a cell culture dedifferentiation model, using explants, primary monolayer culture, second and fourth passage monolayer culture. Secondly, we defined the Il-1β-induced dedifferentiation model, in cells transfected with either empty vector or ANK overexpression plasmid. To further analyze the role of ANK, we analyzed the phenotype of cells transfected with Ank siRNA. Genes expression was measured by quantitative PCR, protein expression was assessed by Western blot analyses or immunocytochemistry. ePPi was quantified by a radiometric assay in cell supernatants. We also used conditioned supernatant from cells transfected with Ank siRNA, supposed to contain soluble factors, to explore the contribution of the canonical (Tcf/Lef reporter plasmid) and non-canonical (JNK phosphorylation) Wnt pathways to chondrocyte dedifferentiation. Finally, the effect of exogenous PPi in cell culture medium was assessed on siRNA-induced dedifferentiation.
Results: Chondrocytes exhibited a progressive increase in Wnt-5a expression during culture dedifferentiation, whereas type II collagen and Ank expression, as well as ePPi production, were continually decreasing at the same time. Il-1β induced Wnt-5a mRNA expression by 2-fold and strongly reduced type II collagen expression (90%) in control cells, whereas in cells overexpressing ANK, Wnt-5a induction by Il-1 β was suppressed and type II collagen expression was only reduced for 50%. These observations suggested a role of Ank in articular chondrocyte phenotype sustain. Ank knock-down led to chondrocyte dedifferentiation, as it reduced type II collagen and Sox-9 expression respectively by 50% and 30%, whereas Wnt-5a expression was induced 2-fold. Then, we showed that conditioned supernatant from these cells induced a 2.5-fold activation of Tcf/Lef reporter plasmid, suggesting the involvement of Wnt canonical pathway in the dedifferentiation process. Moreover, neutralization of conditioned supernatant using Wnt-5a antibody inhibited by 95% the Tcf/Lef activation, demonstrating the role of Wnt5a in Wnt canonical pathway induction, whereas no significant activation of JNK was found. We also showed that addition of exogenous PPi contrasted the Ank siRNA-induced dedifferentiation process, as type II collagen expression was only reduced by 30%, and Wnt-5a induction was suppressed. Moreover, Tcf/Lef activation was reduced by 80%, suggesting a potent role for ePPi in chondrocyte phenotype sustain.
Conclusions: These results indicate that ANK, and its by-product ePPi are implicated in articular chondrocyte phenotype sustain, markedly resulting from suppression of Wnt canonical pathway activation. Our results underline a new and original role for Ank, and could open new therapeutic insights in chondrocyte dedifferentiation process associated to osteoarthritis.

239 SPHINGOSINE-1-PHOSPHATE INHIBITS Il-1 AND TNF-α INDUCED INOS EXPRESSION VIA $S1P_1$ AND $S1P_2$ IN HUMAN ARTICULAR CHONDROCYTES

M.H. Stradner[1], G. Gruber[2], H. Angerer[1], F.C. Fürst[1], V. Huber[1], J. Hermann[1], R. Windhager[2], W.B. Graninger[1]. [1]*Department of Rheumatology, Medical University of Graz, Graz, AUSTRIA,* [2]*Department of Orthopedic Surgery, Medical University Graz, Graz, AUSTRIA*

Purpose: We have prior identified the bioactive Sphingolipid Sphingosine-1-Phosphate (S1P) as a potent inhibitor of Interleukin-1 (Il-1) induced cartilage degradation in bovine cartilage. As micro-molar concentrations of S1P have been found in the synovial fluid of osteoarthritis and rheumatoid arthritis joints we investigated the role of S1P and its receptors in human cartilage.
Methods: Human cartilage specimens were obtained from patients undergoing total knee joint replacement. Specimens were formalin fixed and paraffin embedded and S1P receptor iso-types $S1P_1$, $S1P_2$ and $S1P_3$ were detected by immunohistochemistry. For cell culture human Chondrocytes were isolated using collagenase B. Cells grown in monolayer were cultured in Ham's F-12/DMEM (1:1) and 10% FCS over 3 passages. Cultured chondrocytes were serum starved for 24 hours and incubated with 10 ng/ml Il-1β or 100 ng/ml TNF-α alone or in combination with 0.1 μM up to 3 μM S1P. Expression of iNOS, ADAMTS-4 and MMP-13, was evaluated using real-time PCR.
Results: All three investigated S1P-receptor iso-types were detected in human cartilage; however $S1P_2$ was the most prominent subtype. Interestingly, the expression of the receptors was confined to zones of cartilage damage and proliferating chondrocytes. Furthermore, expression of iNOS mRNA induced by Il-1β and TNF-α was dose dependently reduced by S1P by 47.0% and 56.2% respectively ($P < 0.05$). Cytokine induced ADAMTS-4 mRNA was diminished in the presence of S1P by 35.6% and 41.8% respectively ($P < 0.05$). In contrast to our previous observations in bovine cartilage MMP-13 mRNA expression was not affected by co-incubation with S1P in human chondrocytes. Using specific inhibitors for $S1P_1$ (pertussis toxin) and $S1P_2$ (suramin) we found that inhibition of $S1P_1$ but not $S1P_3$ partly reversed iNOS and ADAMTS-4 inhibition.
Conclusions: Our results suggest that S1P reduces Il-1 and TNF-α induced mRNA transcription of ADAMTS-4 and iNOS in human articular chondrocytes via $S1P_1$ and $S1P_2$. Therefore expression of these S1P receptor subtypes in damaged areas of human cartilage can be interpreted as a counter regulation of chondrocytes to inhibit further tissue degradation by Il-1β and TNF-α.

240 GENES COMMONLY REGULATED BY THE P38 MAPK AND PI3K SIGNALING PATHWAYS IN HYPERTROPHIC GROWTH PLATE CHONDROCYTES

V. Ulici, C.G. James, L-A. Stanton, F. Beier. *University of Western Ontario, London, ON, CANADA*

Purpose: Endochondral ossification, the process through which long bones are formed, involves chondrocyte proliferation and hypertrophic differentiation in the cartilage growth plate. Numerous growth factors