effects of short and long-term exposure of leptin on both normal and osteoarthritic chondrocyte metabolism.

Methods: Human normal and OA cartilage tissues were obtained following autopsy or joint surgery respectively. Chondrocytes were isolated from cartilage by enzymatic digestion and resuspended in alginate beads at 2 x 10⁵/mL. Chondrocytes were permitted to stabilize in 5% FBS for 72 hrs, and transferred to serum free media supplemented with "mini-ITS" for 3 days prior to experimental treatments. [³⁵S] was added for the final 18 hr of culture and sulphate incorporation was measured by liquid scintillation. Cytokine release in culture supernatants was measured using a custom multiplex bead immunoassay.

Results: Short-term leptin exposure resulted in an increase in proteoglycan synthesis in normal cartilage, and no change in response from OA cartilage. Continued treatment with leptin induced a decrease in proteoglycan synthesis from normal and OA cartilage. Only modest increases in cytokine production were detected from normal chondrocytes following leptin treatment and varied among donors.

Conclusions: Prolonged exposure to leptin induces a catabolic response in both normal and OA cartilage, independent of cytokine production. These observations indicate that the chronic increased levels of leptin present in obesity may contribute to the development and progression of OA. Future studies are planned to determine if inhibition of leptin signaling provides a protective effect on cartilage.

![Graph showing the effect of leptin on normal chondrocytes over 2 days.](image)

Normal chondrocytes, 2 day treatment.

![Graph showing the effect of leptin on normal chondrocytes over 4 days.](image)

Normal chondrocytes, 4 day treatment.

[236] RECIPROCAL REGULATION OF ADAMTS BY II-1 AND TGF-β IN CHONDROCYTES: MODULATION BY SELECTIVE PPAR AGONISTS

S. Bultel, A. Poinsard, N. Gambier, S. Sebillaud, A. Bianchi, P. Netter, J-Y. Jouzeau. LPPA UMR 7561 Nancy Université CNRS, Vandoeuvre les Nancy, FRANCE

Purpose: Recent findings suggest that ADAMTS (A Desintegrin And Metalloprotease with Thrombospondin motifs) play a key role in cartilage destruction in osteoarthritis (OA) and many ADAMTS are able to cleave aggrecan. II-1 and TGF-β, which contribute to OA by promoting cartilage destruction and osteophytosis respectively, are key modulators of some ADAMTS. Peroxisome Proliferators-Activated Receptors (PPAR) are nuclear transcription factors able to suppress II-1-induced inflammatory responses as well as TGF-β-induced synthesis of extracellular matrix components in joint cells. To investigate the regulation of several ADAMTS by II-1 and TGF-β, used alone or in combination, in rat or human OA chondrocytes and to determine the modulating potency of selective PPAR agonists.

Methods: Rat and human OA chondrocytes cultured as monolayers were stimulated with 10 ng/ml of recombinant homolog II-1 or 10 ng/ml of recombinant TGF-β used alone or as costimulators. Levels of mRNA for ADAMTS-1, 4, 5, 8, 9, 15 were assessed by real-time quantitative PCR. Aggrecanase activity was measured by a colorimetric assay and a commercial ELISA-based kit (InvLISA®, Invitex). In some experiments, selective PPAR agonists, Wy14643 (100 μM) or GW7647 (250 μM) for PPARα, GW501516 (100 μM) for PPARγ, pioglitazone (Pi, 30 μM) or rosiglitazone (Rosi, 10 μM) for PPARγ, were added to culture medium 2 hours before cytokine stimulation. These concentrations were shown previously to activate PPAR-target genes in a subtype selective manner in rat chondrocytes.

Results: A preliminary experiment showed that the inducing effect of II-1 on ADAMTS-4 expression was higher in human chondrocytes obtained from monolayers than from alginate beads. In human chondrocytes, II-1 reduced ADAMTS-1 and -15 mRNA levels by 3.8- & 3.3-fold respectively, increased ADAMTS-4 and -9 mRNA levels by 175- & 19-fold, without affecting ADAMTS-5. In contrast, II-1 increased ADAMTS-1, -3 and -8 mRNA levels by 12.4-, 5.9- & 90-fold respectively, without modifying ADAMTS-4, -9 and -15 expression in rat chondrocytes. In human chondrocytes TGF-β increased ADAMTS-4 mRNA level by 20-fold but decreased ADAMTS-1, -5, -9 and -15 mRNAs by 15-, 19-, 4.5- and 3.6-fold respectively. In contrast, TGF-β increased ADAMTS-5 mRNA level by 16.4-fold and decreased ADAMTS-9 and -15 mRNAs by 3- and 3.5-fold respectively without affecting ADAMTS-1, -4 and -9 expression in rat chondrocytes.

Conclusions: Our data show that: (i) the regulation of ADAMTS expression by II-1 and TGF-β and II-1 differed between rat and human chondrocytes; (ii) the effect of II-1 depended on the ADAMTS considered whereas TGF-β was globally inhibitory; (iii) TGF-β counteracted the stimulating effect of II-1 on ADAMTS-9 and its inhibitory potency on ADAMTS-15. In rat chondrocytes, TGF-β counteracted the stimulating effect of II-1 on ADAMTS-1, -5 and -8 mRNA levels. In human chondrocytes, the global aggrecanase activity was increased by II-1, decreased by TGF-β which was also able to antagonize II-1 effect. When added before II-1 or TGF-β stimulation, PPAR agonists failed to modulate ADAMTS mRNA levels in human chondrocytes as well as II-1-induced changes in ADAMTS expression in rat chondrocytes. However, Pi and Rosi decreased the basal expression of ADAMTS-5 in human chondrocytes but this did not translate into significant reduction of aggrecanase activity.

[237] THE IMPORTANCE OF AN EXTRACELLULAR MATRIX IN APOPTOSIS IN CHONDROCYTES

G.R. Dodge¹, K. Copeland¹, A. Callaway¹, C. Farach-Carson².

¹A I duPont Hospital for Children, Wilmington, DE, USA; ²University of Delaware, Newark, DE, USA

Purpose: One of the mechanisms for the pathogenesis of osteoarthritis is chondrocyte apoptosis, which results in a disturbance of cell function, impacts extracellular matrix (ECM) production, and leads to cartilage failure. The presence of ECM is crucial to the integrity of chondrocyte/cartilage function. The purpose of our study was to determine the importance of the extracellular matrix to the homeostasis of chondrocytes and its importance in protecting chondrocytes from apoptosis. Since chondrocytes grow in vivo in such a specialized micro-environment our goal was to mimic aspects of this in vitro and test cell's response Fas-mediated apoptosis.

Methods: Equine cartilage was obtained from the stifle joints of young adult horses and placed in DMEM with 10% FBS (DMEM-FBS) to determine the effect of II-1 and TGF-β on OA chondrocytes isolated by collagenase digestion. The cells were cultured in DMEM-FBS at 100,000 cells/ml established in chamber slides or culture dishes with either fibronectin (FN), perlecan, Matrigel, or growth factor reduced Matrigel (Matrigel-1) (BD). Some cultures were left to adhere to plates and others were grown in suspension using polyHEMA. In all cases cultures were allowed to equilibrate with the environment for 24 hr prior to treatment for an additional 24-48 hr with Fas antibody (500 ng/ml) (Axxora). Media and cells were collected. Slides were prepared and TUNEL assay was used to identify nick end-labeling of DNA. Where
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%±3.48. 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 Fas (increase 224% over CTL) and to cells grown as adherent monolayer without matrix (897% over CTL). Chondrocytes in a hypocellular 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 phenotype-dependent environment also protects or prevents them from 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


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 in chondrocyte phenotype, notably in the dedifferentiation process mediated by interleukin-1β (II-1β) and Wnt-5a in osteoarthritis. Inorganic pyrophosphatase has also been shown to influence osteo-articular cells such as osteoblasts. Moreover, we demonstrate 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 II-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. Gene 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 continuously decreasing at the same time. II-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 II-1β 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 of 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 II-1 AND TNF-α INDUCED INOS EXPRESSION VIA S1P1, AND S1P3 IN HUMAN ARTICULAR CHONDROCYTES

M.H. Stradner1, G. Gruber2, H. Angerer1, F.C. Fürst1, V. Huber1, J. Hermann1, R. Windhager2, W.B. Graninger1.

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 (II-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 S1P1, S1P2 and S1P3 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 II-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 S1P2 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 II-1β and TNF-α was dose dependently reduced by S1P by 47.0% and 58.2% 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 S1P1 (pertussis toxin) and S1P2 (suramin) we found that inhibition of S1P1, but not S1P3 partly reversed iNOS and ADAMTS-4 inhibition.

Conclusions: Our results suggest that S1P reduces II-1 and TNF-α induced mRNA transcription of ADAMTS-4 and iNOS in human articular chondrocytes via S1P1 and S1P2. 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 II-1β and TNF-α.

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

V. Ullic, 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...