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### ARTICULAR LEVELS OF ADIPONECTIN ARE NOT CHONDROPROTECTIVE IN 3D CULTURES OF HUMAN CHONDROCYTES

A. Abot, V. Olive, A. Bianchi, S. Sebillaud, **P. Netter**, D. Moulin, J-Y. Jouzeau
*Laboratoire de Physiopathologie et Pharmacologie Articulaires, Vandoeuvre les Nancy, France*

**Purpose:** Adipokines are found in synovial fluid of OA patients and circulating levels of adiponectin (Apn) are reduced in obesity. As obese patients are at increasing risk of developing osteoarthritis (OA), even in non-weight bearing joints, we tested the hypothesis that low (physiological) concentrations of Apn could impair chondrocytes functions towards an "OA-like" phenotype.
**Methods:** Firstly, the expression of Apn and Apn receptors (AdipoR) was characterized by quantitative RT-PCR and western blotting. Secondly, a dose-ranging comparative study of physiological concentrations (0.2, 1 or 5$\mu$g/ml) of globular (gApn) and full length (flApn) forms of adiponectin was performed on chondrocytes cultured in alginate beads. The parameters measured were: proteoglycans (PGs) synthesis (by radiolabelled sulfate incorporation), pro-inflammatory mediators release (NOx by Griess method, PGE2 and TNF$\alpha$ by ELISA) and MMP-13 activity (by fluorimetric assay). Thirdly, in co-stimulation experiments, the ability of gApn or flApn to modify chondrocytes responses to the pro-catabolic factor, IL-1beta (10ng/ml) or the pro-anabolic factor, IGF-1 (50ng/ml) was studied.
**Results:** We demonstrated that chondrocytes expressed Apn (mRNA & protein) in monolayers or alginate beads as well as AdipoR1 (mRNA & Protein) and AdipoR2 (mRNA). We found that, in the concentration range tested, gApn was inactive on all parameters except a weak inhibitory effect on MMP-13 at 5$\mu$g/mL. In contrast, the highest concentration (5$\mu$g/ml) of flApn was able to decrease PGs synthesis by 27%, to induce NO and PGE2 release, and to increase MMP-13 activity. A weak effect was seen for lower concentrations of flApn on PGs synthesis and MMP-13 activity. Neither gApn nor flApn reduced the stimulating effect of IGF-1 on PGs synthesis, but flApn weakly potentiated the inducing effect of IL-1beta on PGE2 release and MMP-13 activity.
**Conclusions:** This preliminary study shows that, in human chondrocytes: i) gApn was less active than flApn; ii) flApn was active in the upper range of Apn joint levels found in OA patients; iii) flApn failed to protect from the deleterious effect of IL-1. Our data do neither support the meaning that Apn could be chondroprotective in OA nor that a reduced articular level could be deleterious for cartilage.

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### CHONDROCYTE APOPTOSIS IN ARTICULAR CARTILAGE: HOW LONG DOES IT TAKE?

**A. Tyler**, K. Nowlan, C. Thomas, C. Whittles, M. Sharif
*University of Bristol, Bristol, United Kingdom*

**Purpose:** Osteoarthritis (OA) is a prevalent degenerative disorder, especially in ageing individuals. Loss of articular cartilage (AC) is a key feature of the disease and recent studies suggest that a reduction in cell viability may be a trigger for both initiation and progression of AC damage in OA. Our previous studies have shown that hypocellularity in OA cartilage can be explained by apoptosis which is on average 3 times higher in OA cartilage compared to normal (Sharif *et al* 2004). The aim of this study was to determine the time course for chondrocyte apoptosis in equine AC.
**Methods:** Equine AC obtained from the metatarsophalangeal (MTP) joint of 9 horses were incubated with tumour necrosis factor-alpha (TNF-$\alpha$) and Actinomycin-D (ActD) for 0, 6, 12, 18, 24 and 48 hours. At each time point cartilage was assessed for cell viability using fluorescein diacetate (stains live cells green) and propidium iodide (stains dead cells red) and apoptosis using indirect immunohistochemistry for active caspase-3 expression.
**Results:** Cell viability was 89.9 $\pm$ 1.3% (Mean $\pm$ SEM) at baseline and decreased linearly to 69.9 $\pm$ 5.9% after 24 hours. Overall levels of cell death after 18 ($P<0.05$), 24 and 48 hours ($P<0.01$) were found to be statistically significant. Cell death by apoptosis (expression of caspase-3) also reached levels of significance after these time points. Apoptosis increased linearly from 27.1 $\pm$ 1.3% at baseline to 58.8 $\pm$ 5.1% after 24 hours. In addition, a significant positive correlation ($r = 0.53$, $p<0.001$) was found between the percentages of dead cells and apoptotic cells.
**Conclusions:** Our data demonstrated that TNF-$\alpha$ plus ActD treatment of equine AC over 48 hours results in peak apoptosis at 24 hours, and that apoptosis contributes significantly to the overall increase in cell death over the 48 hours. These findings support the role of chondrocyte apoptosis in the initiation of AC damage in OA.

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### INVESTIGATING SUPEROXIDE DISMUTASE EXPRESSION IN CARTILAGE AND A POTENTIAL ROLE IN OSTEOARTHRITIS

**J.L. Lindop**[1], R.K. Davidson[2], I.M. Clark[2], T.B. Kirkwood[1], D.A. Young[1]
[1]*Newcastle University, Newcastle Upon Tyne, United Kingdom;*
[2]*University of East Anglia, Norwich, United Kingdom*

**Purpose:** To characterise the expression of the superoxide dismutase (SOD) family of antioxidant enzymes in normal and osteoarthritic cartilage and investigate their potential role in the development of osteoarthritis (OA).
**Methods:** SOD gene expression was determined in RNA samples extracted from the cartilage of 12 normal fracture (NOF) and 12 OA hips using real time PCR. Immunohistochemistry was then used to assess the levels of SOD2 protein expression in NOF and OA hip cartilage sections. Histochemical stains for the electron transport chain proteins cytochrome c oxidase (COX) and succinate dehydrogenase (SDH), which are mitochondrially and nuclear encoded respectively, were used to identify any OA chondrocytes exhibiting mitochondrial dysfunction as a potential consequence of damage by reactive oxygen species (ROS). The mitochondrial genome of individual COX deficient cells was amplified by PCR and the mutational status determined by DNA sequencing.
**Results:** The expression of all SOD genes was significantly downregulated at the mRNA level. The downregulation of SOD2 appeared to be the most dramatic and this has been confirmed at the protein level. COX-SDH histochemistry identified a proportion of OA chondrocytes having deficient expression of the mitochondrially encoded COX enzyme.
**Conclusions:** SOD2 expression has been shown to be downregulated in OA cartilage compared to NOF cartilage. Subsequently, OA chondrocytes which contain potentially dysfunctional mitochondria have been identified histochemically and further work is being carried out to identify whether this is a consequence of increased levels of mitochondrial DNA (mtDNA) mutation. Mitochondrial dysfunction and mtDNA mutation may arise as a result of the downregulation of SOD2 expression and consequent increased levels of ROS. Future work will examine mtDNA mutation rates between age-matched OA and normal chondrocytes. We propose that re-instigation of normal levels of SOD2 expression, or antioxidant therapy, will reduce oxidative stress and damage seen in OA and may represent a potential therapy. In certain

cancers SOD2 expression is partially controlled epigenetically and our future work will determine the mechanism of repression of the gene in OA chondrocytes.

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### EXPRESSION OF ANK (*progressive ankylosis*) IS SENSITVE TO OXYGEN

**R. Zaka**, A.S. Dion, A. Kusnierz, J. Bohensky, V. Srinivas, C.J. Williams
*Thomas Jefferson University, Philadelphia, PA*

**Purpose:** The proximal promoter region of Ank, a gene that codes for a transmembrane protein that regulates the transport of inorganic pyrophosphate, contains a hypoxia responsive element (HRE). These studies were undertaken to investigate the expression and function of Ank in response to varying oxygen tensions.
**Methods:** ATDC5 and N1511 clonal chondrocytic cell lines were subjected to culture in either hypoxic (2% $O_2$) or normoxic (21% $O_2$) conditions for 24 and 48 hours. Transcript levels of Ank were determined using real time PCR and protein levels were evaluated by Western blot analyses. As control, levels of expression of the hypoxia inducible factor 1 (HIF-1)- regulated transcript Vegf were simultaneously evaluated under varying oxygen tensions. Transcript stability at the two oxygen tensions was determined by treatment of cells with the transcription inhibitor $\alpha$-amanitin. N1511 cells were also subjected to differentiation with the addition of BMP2 and the effect of varying oxygen tensions on the cells at hypertrophy was investigated. To determine the possible role of HIF-1 in the observed hypoxic response, N1511 cells in which Hif-1$\alpha$ was knocked down were exposed to varying oxygen conditions and levels of Ank expression were determined. Finally, the effect of varying oxygen tensions on the elaboration of extracellular pyrophosphate in ATDC5 cells was explored.
**Results:** At 24 and 48 hours, a relative fourfold and eight-fold increase, respectively, in Ank mRNA expression was observed at high oxygen tension in both cell lines. To exclude the possibility that these results were due to differential stability of Ank mRNA in normoxic vs. hypoxic conditions, steady-state levels of mRNA expression were explored after treating ATDC5 cells with $\alpha$-amanitin. No significant differences in the decay of Ank mRNA levels in hypoxia vs. normoxia were observed over a period of 40 hours in the presence of $\alpha$-amanitin. To determine if the oxemic response was conserved when N1511 cells were differentiated, BMP2-treated cells at day 5 of differentiation (hypertrophy) were exposed to varying oxygen tensions and a similar relative increase in the expression of Ank mRNA was observed in normoxia, with concomitant increase in Ank protein levels as determined by Western blot analyses. When N1511 cells in which Hif-1$\alpha$ was knocked down by siRNA were subjected to varying oxygen tensions, the relative expression of Ank mRNA in normoxic conditions was dramatically reduced, suggesting that the oxemic regulation of Ank expression is mediated by HIF-1. Finally, consistent with the relative increase in Ank expression in normoxia after 24 hours, levels of extracellular pyrophosphate were also increased in normoxia in the ATDC5 cell line.
**Conclusions:** These data suggest that Ank expression is up-regulated in normoxic environments and that the up-regulation is not due to differential mRNA stability. The results further suggest that the observed effect is regulated by Hif-1$\alpha$, the oxygen responsive subunit of HIF-1, and that the oxemic response also affects the pyrophosphate transport activity of Ank. Although many transcripts respond to oxygen by up-regulating their expression at low oxygen tensions, there are several examples of transcripts whose expression is down-regulated at low oxygen tensions, including $\alpha$-fetoprotein and heme 1 oxygenase. The general mechanism of hypoxia repression often involves the elaboration of a negative response element that mediates transcriptional activation by HIF-1. Future studies will explore such a mechanism in the oxemic control of Ank expression.

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### PPAR$\gamma$1 EXPRESSION IS DIMINISHED IN HUMAN OA CARTILAGE AND IS DOWN-REGULATED BY IL-1$\beta$ IN ARTICULAR CHONDROCYTES

**N. Chabane**, N. Zayed, M. Benderdour, J. Martel-Pelletier, J-P. Pelletier, H. Fahmi
*University of Montreal, Montreal, PQ, Canada*

**Purpose:** Peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) is a nuclear receptor involved in the regulation of many cellular processes. We and others have previously shown that PPAR$\gamma$ activators display anti-inflammatory and chondroprotective properties *in vitro* and improve the clinical course and histopathological features in an experimental animal model of osteoarthritis (OA). However, the expression and regulation of PPAR$\gamma$ in cartilage are poorly defined. This study was undertaken to investigate the quantitative expression and distribution of PPAR$\gamma$ in normal and OA cartilage and to evaluate the effect of interleukin-1$\beta$ (IL-1), a prominent cytokine in OA, on PPAR$\gamma$ expression in cultured chondrocytes.
**Methods:** Expression of PPAR$\gamma$ protein and mRNA in cartilage were determined by immunohistochemistry and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), respectively. Cultured chondrocytes were stimulated with IL-1 alone or in combination with selective inhibitors of signal transduction pathways, and PPAR$\gamma$1 expression was analyzed by Western blot. Activation of the PPAR$\gamma$1 promoter was assessed in transient transfection experiments.
**Results:** Immunohistochemical analysis revealed that the levels of PPAR$\gamma$ protein expression were significantly lower in OA cartilage compared to normal cartilage. Using RT-PCR, we demonstrated that PPAR$\gamma$1 mRNA levels were $\sim$10-fold higher than PPAR$\gamma$2 mRNA levels; and that only PPAR$\gamma$1 was differentially expressed, its levels in OA cartilage being 2.4-fold lower than in normal cartilage ($P<0.001$). IL-1 treatment of OA chondrocytes down-regulated PPAR$\gamma$1 expression in a dose- and time-dependent manner. This effect probably occurred at the transcriptional level, since IL-1 decreases both PPAR$\gamma$1 mRNA expression and PPAR$\gamma$1 promoter activity. Tumor necrosis factor (TNF-$\alpha$), IL-17, and prostaglandin $E_2$ ($PGE_2$), which are involved in the pathogenesis of OA, also down-regulated PPAR$\gamma$1 expression. Specific inhibitors of the mitogen-activated protein kinase (MAPK) p38 (SB203580) and JNK (SP600125), but not Erk (PD98059) prevented IL-1-induced down-regulation of PPAR$\gamma$1 expression. Similarly, inhibitors of NF-$\kappa$B signaling (PDTC, MG-132, and SN-50) abolished the suppressive effect of IL-1.
**Conclusions:** Our study has demonstrated for the first time that PPAR$\gamma$1 is down-regulated in OA cartilage. The pro-inflammatory cytokine IL-1 may be responsible for this down-regulation via a mechanism involving activation of the MAPKs (p38 and JNK) and NF-$\kappa$B signaling pathways. The IL-1-induced down-regulation of PPAR$\gamma$ expression may be an important new process by which IL-1 promotes articular inflammation and cartilage degradation.