3. TGase 2 assay. The expression and enzyme activity of TGase 2 was examined with western blot and immunocytochemistry. Total cellular proteins were isolated and western blotted as described previously. Enzyme activity was evaluated by determining the incorporated biotinylated pentylamine using horseradish peroxidase-conjugated streptavidin and measuring the absorbance at 492 nm using a microplate spectrophotometer.

Results: 1. RA up-regulated TGase 2 expression and enzyme activity in human chondrocytes. Human chondrocytes treated with RA resulted in up-regulation of TGase 2 protein as shown by the western blot and immunocytochemistry. RA-induced TGase 2 expression increased time dependently. The enzyme activity of TGase 2 was also increased in RA treated cells compared with normal human chondrocyte cells.

2. TGase 2 induced by RA results in decreased apoptosis in human chondrocytes. Apoptosis in RA-treated chondrocytes was decreased, measured by Annexin-V FACS analysis, when compared with H2O2-treated cells. Similar patterns were observed in 3 independent experiments using chondrocytes obtained from different patients.

Conclusions: We have previously reported that endogenous TGase 2 expression was increased in normal human chondrocytes undergoing apoptosis. Inhibition of TGase 2 by MDC and TGase 2 siRNA was also shown to increase apoptosis and suggest a possible protective role of TGase 2 in chondrocyte apoptosis. The protective role of TGase 2 was further validated in this study as the RA-induced overexpression of TGase 2 decreased apoptosis of human chondrocytes. These results implicate a protective role of TGase 2 against apoptosis in human chondrocytes and the possibility of TGase 2 as a potential modulator of osteoarthritis.

192 VISFATIN/NAMPT: A POTENTIAL TARGET FOR NGF-TRIGGERED PAIN IN OSTEOARTHRITIS

S. Priam, E. Pecchi, M. Gosset, X. Hourd, F. Berenbaum
Univ. Pierre et Marie Curie, Paris, France

Purpose: Obesity is the main risk factor for knee OA. The two main features of the pathophysiology of obesity-induced OA are based on a local component (mechanical stress) and a systemic component (pro-inflammatory adipokines). Nerve growth factor (NGF) is present within OA synovial fluid and may be involved in pain associated with OA. We previously showed that visfatin/NAMPT, an adipokine, has pro-degradative effects. However, its role in OA pain has not been evaluated yet.

Methods: Primary cultures of newborn mouse articular chondrocytes or cartilage explants were stimulated by increasing amounts of visfatin/NAMPT, IL-1 beta, prostaglandin E2 (PG_E2) or by cyclic mechanical compression (0.5 Hz, 1 Mpa). mRNA levels were assessed by real-time quantitative PCR and NGF released into media was determined by ELISA.

Results: Unstimulated articular chondrocytes expressed low levels of NGF. Mechanical stress induced NGF mRNA expression and release in conditioned media. Visfatin/NAMPT, a pro-inflammatory adipokine produced by chondrocytes in response to IL-1 beta, stimulated NGF expression (2 fold) and release (3.7 fold). When stimulated by IL-1 beta, a dose-dependent increase in NGF mRNA expression (5.7 fold increase with 10 ng/ml IL-1) and NGF release (14 ng/ml, 3.8 fold increase with 10 ng/ml) in chondrocyte conditioned media was observed. Neither siRNA visfatin/NAMPT nor APO 866, an inhibitor of NAMPT enzymatic activity, prevented the production of NGF induced by IL-1 beta. Interestingly, PGE2, which is produced by chondrocytes in response to IL-1 beta and visfatin/NAMPT, did not stimulate NGF production. Consistently, indomethacin, a cyclooxygenase inhibitor, did not counteract IL-1-induced NGF production.

Conclusions: These results suggest that obesity-induced OA pain may involve NGF mediated by the overexpression of visfatin/NAMPT and mechanical stress. These effects seem to be independent of the well-known pro-inflammatory mediators involved in OA pain, IL-1b and PGE2. Thus, along with reduction of weight, visfatin/NAMPT could be an interesting target for pain in OA

193 HUMAN OSTEOARTHRITIS CHONDROCYTES EXPRESS AND RESPOND TO THE PARATHYROID HORMONE RECEPTOR 1

T. Segovia-Silvestre, B.C. Sonderegard, H. Roosan, A. Goetrup, M.A. Karsdal, A.-C. Bay-Jensen
Nordic BioSci. A/S, Herlev, Denmark

Purpose: Parathyroid hormone (PTH) is an endogenous peptide hormone that elevates calcium levels by releasing calcium salts stored in bone and preventing their renal excretion. Although its role in bone metabolism has been thoroughly studied, little is known about its effects on cartilage. In this study, we sought to test the hypothesis that human chondrocytes do express the PTH 1 receptor (PTH1R) and are responsive to PTH ex vivo.

Methods: RNA was purified from freshly isolated, primary chondrocyte samples from OA knee cartilage. A dedicated reverse transcription polymerase chain reaction (RT-PCR) to amplify the coding region of the parathyroid hormone 1 receptor (PTH1R) mRNA was run. Similarly, western blotting was done with cell lysate; specific antibodies for the PTH1R protein were used to detect the presence of the receptor on human chondrocytes. In addition immune-cytochemistry as well as immune-histochemistry was done to identify the receptor in situ. Human chondrocytes were isolated and maintained serum-free for 1h in the presence of 100nM IBMX (PDE inhibitor), and subsequently stimulated with PTH 1nM-100nM + IBMX and the cAMP levels were quantified by ELISA. In addition, articular cartilage explants were cultured in 6 replicates for 17 days, with or without 10nM PTH treatment, and 5 μM 32 sulphate was added for the last 24 hours. Soluble proteoglycans were released by 4M GuHCl and incorporated sulfate was measured. Neo-epitopes of pro-peptides of collagen type II (PIINP) were quantified as a measure of formation in the conditioned medium.

Results: We identified an approx. 2 kb band after RT-PCR, which was sequenced and aligned with the coding region of PTH1R mRNA. Furthermore, we detected a protein reacting against PTH1R antibody consistent with the transcript sequence in chondrocyte samples from osteoarthritis patients. PTH1R immunoreactivity was primarily localized in the cell membrane of chondrocytes, observed by immunochemistry. When stimulated with PTH, the cultured chondrocytes accumulated intracellular cAMP levels significantly (P <0.003) in a dose-depend manner. The maximum concentration of PTH (100 nm) resulted in a 23-fold increase compared with baseline. In the explant cultures of OA articular cartilage, a two-fold increase of PIINP was observed in the supernatant after PTH stimulation when compared to non-stimulated cartilage samples. Furthermore, 10 nM PTH increased incorporation of 35 sulphate by 40% (p<0.002).

Conclusions: The current data strongly suggest that PTH, in addition to osteoblasts and bone turnover, also has direct anabolic effects on chondrocytes and cartilage. Human articular cartilage chondrocytes express both the mRNA and protein molecules of the parathyroid hormone 1 receptor. We have shown that PTH can not only avert but also facilitate cartilage generation in both in vitro and in vivo situations. Presented data indicate the potency of PTH and intrigues further investigation of PTH as a potential DMOAD.

194 REVERSIBILITY OF IMMOBILIZATION-INDUCED ARTICULAR CARTILAGE DEGENERATION AFTER REMOBILIZATION IN RAT KNEE JOINTS

A. Ando¹, H. Suda¹, Y. Hagiwara², Y. Onoda¹, E. Chimoto¹, Y. Saji¹, E. Itoi¹
¹Tohoku Univ., Sendai, Japan; ²Takeda Gen. Hosp., Aizuawakamatsu, Japan; ³Tohoku Univ. of Biomed. Engineering, Sendai, Japan

Purpose: Joint immobilization (Im) causes articular cartilage degeneration. Im-induced cartilage degeneration is generally recognized as disuse atrophy caused by decreased chondrocytes activity. In our rat immobilized knee model with a plate and screws, the changes in the non-contact (NC) area was similar to disuse atrophy, but the changes in the transitional (T) area and contact (C) area were quite different. Reversibility of Im-induced cartilage degeneration is still controversial. The differences may be originated from the methods of Im and measurement sites. The purpose of this study was to clarify the reversibility of Im-induced cartilage degeneration after remobilization.

Methods: Unilateral knee joints of adult male rats were rigidly immobilized at 150° of flexion with a plate and screws for 1, 2, and 4 weeks. After the experimental periods, the fixation devices were removed and the rats were allowed to move freely in standard cages for 16 weeks. Only screws were
inserted in sham-operated rats. The immobilized rats and sham-operated rats made up the immobilized-remobilized (Im-Re) group and control group, respectively. Five μm sections at the medial midcondylar region in sagittal plane were obtained and stained with H-E and Safranin-O (S-O). Six areas (NC area, T area, C area in the femur and tibia) were set and modified Mankin’s score, thickness of the articular cartilage, and number of chondrocytes were evaluated at each area. Mechanical properties of the articular cartilage were assessed by the scanning acoustic microscope (SAM).

**Results:** [C Area] Chondrocytes decreased and disappeared after 1W Im-Re group (Figs. 1, 2). A marked reduction of S-O staining was observed (Figs. 4, 5). Mankin’s score was significantly higher after 1W Im-Re group (Fig. 7). Number of chondrocytes was significantly smaller after 2W Im-Re group (Fig. 9). The articular cartilage in the Im-Re group was almost blue (low sound speed) compared to the control (Figs. 10, 11). [T Area] Hypertrophy and cloning of chondrocytes were observed after 2W Im-Re group (Fig. 3). Thickness of the cartilage was significantly higher at 4W Im-Re group (Figs 8). [NC Area] Reduction of S-O staining in the non-calcified cartilage was almost restored but it was not restored around the tidemark (Fig. 6).

**Conclusions:** These results have indicated that atrophic changes (decrease of proteoglycans) through decreased mechanical stress in the NC area were reversible, but chondrocytes death and hypertrophy of chondrocytes in the C and T areas through increased mechanical stress by rigid immobilization were irreversible after remobilization. Clinicians should be aware that even a short-term rigid immobilization could cause irreversible articular cartilage damage.

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**ROLE OF HIGH EXTRACELLULAR GLUCOSE CONCENTRATIONS IN MODULATING ANABOLIC AND CATABOLIC GENE EXPRESSION IN NORMAL AND OSTEOARTHRITIC HUMAN ChONDROCYTES**

S.C. Rosa1,2, A.T. Rufino1,2, F.M. Judas3,4, M.C. Lopes1,2, A.F. Mendes1,2

1Ctr. for NeuroSci, and Cell Biology, Coimbra, Portugal; 2Faculty of Pharmacy, Univ. of Coimbra, Coimbra, Portugal; 3Univ. Hosp. of Coimbra, Coimbra, Portugal; 4Faculty of Med., Univ. of Coimbra, Coimbra, Portugal

**Purpose:** Articular chondrocytes require a steady supply of glucose for optimal energy production and cell homeostasis, as well as for anabolic functions, namely the synthesis of cartilage matrix molecules. As such, articular chondrocytes may be especially sensitive to alterations in the synovial fluid glucose concentration due to disorders that affect glucose metabolism, namely Diabetes Mellitus (DM). Our previous study showed that hyperglycemia-like glucose concentrations reduce glucose transport into normal but not into osteoarthritic (OA) chondrocytes, in which it leads to intracellular glucose accumulation with prolonged production of Reactive Oxygen Species (ROS) and oxidative stress. Since ROS are known to contribute to OA pathogenesis, this study aimed at elucidating the role of high extracellular glucose in modulating anabolic and catabolic gene expression in normal and OA human chondrocytes. For this, we examined the effects of culturing normal and OA human chondrocytes under elevated glucose concentrations, using real time RT-PCR (qRT-PCR) to evaluate the expression of several genes important in cartilage homeostasis and OA pathogenesis, namely TIMPs and -2, MMPs-1 and -13 and collagen types I and II.

**Methods:** Normal (N=7) and OA (N=11) human chondrocytes were obtained from multi-organ donors or patients undergoing total knee replacement surgery, respectively, at the University Hospitals of Coimbra. Non-proliferating non-pooled chondrocyte cultures were maintained for 24, 48 or 72 h in Ham’s F-12 containing 10 (regular glucose) or 30 mM (high glucose) D-glucose. Gene expression was assessed by qRT-PCR.

**Results:** Basal MMP-1 and -13 mRNA levels were approximately 5 and 8 fold higher in OA than in normal chondrocytes, respectively. A trend towards increased expression of TIMP-1 and collagen I and decreased expression of collagen II was found in OA relative to normal chondrocytes, although it didn’t reach statistical significance. Culture of OA chondrocytes in high glucose for 24 or 48 h increased MMP-1 (1.5±0.2 and 1.4±0.3) and -13 (1.7±0.2 and 1.4±0.2) mRNA levels relative to cells maintained in regular glucose, whereas no changes were observed in normal chondrocytes. TIMP-1 and -2 gene expression was not affected by culturing of either normal or OA chondrocytes in high glucose. Culture in high glucose for 24 h modest and similarly increased collagen II expression in normal (1.28±0.07) and OA chondrocyte cultures (1.34±0.13).

**Conclusions:** Acute exposure of OA, but not normal chondrocytes to high glucose increased MMP gene expression which was not compensated by concomitant increases in the expression of their tissue inhibitors, but was accompanied by a modest transient increase in collagen II expression. These results indicate that OA chondrocytes are more sensitive to high glucose-induced deleterious effects than normal ones. This may constitute a pathogenic mechanism by which conditions characterized by hyperglycaemia, like DM, can promote changes in chondrocytes that facilitate the development and/or progression of OA. Besides, the possibility that more prolonged and/or repeated exposure to high glucose can also induce uncompensated catabolic gene expression in normal chondrocytes, favoring OA development, deserves to be further investigated.

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**METHYLATION SPECIFIC MICROARRAY ON HUMAN CHONDROCYTES**

G. Gruber1, F.C. Fuerst2, M.H. Stradner2, A. Weihaeusel1, N. Kastner1, A. Leithner1, W.B. Graninger2

1Univ. Clin. for Orthopedic Surgery, Graz, Austria; 2Med. Univ. Graz, Graz, Austria; 3AT Vorarlberg, Austria

**Purpose:** Osteoarthritis (OA) is an increasing, multifactorial disease affecting millions of people worldwide. Methylation, the most common eukaryotic DNA modification, is an epigenetic event and has been intensively studied in embryogenesis, aging and carcinogenesis. Up to now very few studies have examined the DNA methylation status of human cartilage genes. The aim of our study was to perform a whole genome methylation specific array on human chondrocytes for the first time to display molecular patterns of OA that may lead to a better understanding of the disease.