bP0 increased 10-fold by 48 hrs., gradually increasing to 100-fold by week 3 and then did not change significantly. In the co-cultures Sox9 expression increased 10-fold by 48 hrs. and remained stable up to 4 weeks.

Conclusions: Redifferentiation of passaged cells occurs within the first two weeks of co-culture with primary chondrocytes. The primary cells cultured alone, formed cartilage tissue one week earlier than the co-cultured cells. This difference may be due to the time needed for the passaged cells to undergo re-differentiation similar to that seen for mesenchymal stem cells undergoing differentiation to chondrocytes. Interestingly, lower levels of Sox9 gene expression in co-cultures was sufficient to result in CoO2 expression similar to bP0. The data suggests that implantation of tissue must occur after 2 weeks of co-culture.

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IMPLICATION OF INORGANIC PYROPHOSPHATE AND ITS TRANSPORTER ANK IN THE MAINTENANCE OF ARTICULAR CHONDROCYTE PHENOTYPE. ROLE OF WNT-5A

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Purpose: Articular chondrocyte phenotype is characterized by an expression pattern of genes coding for the extracellular matrix. Various wnt genes were described to induce a chondrocyte dedifferentiation process mediated by interleukin-1β (IL-1β) in osteoarthritis. Inorganic pyrophosphate (PPI) was shown to influence osteo-articular cells phenotype. Moreover, we previously demonstrated that ANK was mainly responsible for extracellular PPI (ePPi) generation. In the present work, we studied the role of ANK and ePPi in the maintenance of articular chondrocyte phenotype, known to be dedifferentiated in osteoarthritis. We focused on the implication of the Wnt signaling in this process.

Methods: We characterized the dedifferentiating effect of IL-1β (10 ng/ml) on the articular chondrocytes. To investigate the role of Ank in phenotype maintenance, we either transfected chondrocytes with a plasmid expressing Ank, or with siRNA directed against Ank. We analyzed genes expression by real-time quantitative RT-PCR, and performed immunocytochemistry analyses. We also harvested the supernatant from cells transfected with Ank siRNA, and used it on chondrocytes to check for the activation of canonical (Tcf/Lef reporter plasmids, western blot of β-catenin nuclear translocation) and non-canonical Wnt pathway (western blot of JNK phosphorylation). These experiments were repeated with neutralizing Wnt-5a antibodies to evaluate the contribution of Wnt-5a. In another set of experiment, we stimulated chondrocytes with supernatant from cells transfected with Ank siRNA and challenged with 0.1 mM of exogenous PPI. Type II collagen and Sox-9 expression were assessed, as well as the activation of Tcf/Lef. Lastly, we controlled whether the effect of IL-1β on the activation of JNK and on the expression of genes specific of chondrocyte phenotype were modulated by exogenous PPI.

Results: IL-1β induced chondrocyte dedifferentiation, as Wnt-5a mRNA expression was up-regulated by 2.5-fold, while Ank and type II collagen expression were reduced respectively by 3-fold and 4-fold. In cells overexpressing Ank, IL-1β induced no more Wnt-5a expression and type II collagen expression was only reduced for 55%. Transient Ank knock-down also led to dedifferentiation, as type II collagen and Sox-9 expression were reduced respectively by 50% and 35%, while Wnt-5a expression was induced 2.5-fold. Immunocytochemistry confirmed these tendencies. This suggested a role of Ank in articular chondrocyte phenotype maintenance. Supernatant from cells transfected with Ank siRNA induced a 2-fold activation of Tcf/Lef plasmid and an increase in nuclear β-catenin level, but no phosphorylation of JNK was detected. This showed the involvement of only Wnt canonical pathway in the process. Moreover, neutralization of supernatant using Wnt-5a antibody ensured a complete suppression of Tcf/Lef activation, demonstrating the crucial role of Wnt-5a in the dedifferentiation process. Exogenous PPI diminished the effect of Ank siRNA, as expression of type II collagen was less reduced (25% instead of 50%), and likely, Tcf/Lef activation was reduced by 50%. Finally, exogenous PPI did not modify JNK activation by IL-1β, but reduced its dedifferentiating effect, as type II collagen expression was only reduced by 2-fold (instead of 4-fold), suggesting that PPI only countered the canonical Wnt pathway-induced dedifferentiation process.

Conclusions: ANK and ePPi are implicated in articular chondrocyte phenotype maintenance, markedly resulting from suppression of Wnt canonical pathway activation. This opens new insights in the understanding of the Wnt signaling mechanisms in osteoarthritis.

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DIFFERENTIAL ROLES OF THE IR AND THE IGFR IN MEDIATING INSULIN-INDUCED GLUCOSE TRANSPORT IN NORMAL AND OSTEOARTHRITIC HUMAN CHONDROCYTES

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Purpose: Some studies indicate that insulin can be an important anabolic factor for chondrocytes, namely due to its ability to promote proteoglycan and type II collagen synthesis. However, little attention has been devoted to the mechanisms that mediate the actions of insulin in chondrocytes, particularly whether the specific insulin receptor (IR) or the structural- and functionally-related insulin-like growth factor receptor (IGFR) is involved. Glucose is essential for glycosaminoglycan synthesis and one of the major actions of insulin is to increase glucose transport, namely in some cells by promoting the plasma membrane incorporation of the Glucose Transporter (GLUT)-1, which is expressed by chondrocytes and regulated by anabolic and catabolic stimuli. Thus, the aim of this study was to determine whether 1) adult human chondrocytes express the IR, 2) physiological concentrations of insulin are able to regulate glucose transport and GLUT-1 protein content in normal and osteoarthritic (OA) human chondrocytes, and 3) the effects of insulin on glucose transport are mediated by the IR or the IGFR.

Methods: Normal human chondrocytes were isolated from the femoral condyles of multi-organ donors without macroscopic signs of arthritic lesions. OA chondrocytes were isolated from patients undergoing total knee replacement surgery. Serum-starved subconfluent chondrocyte cultures were treated with 1 or 10 nM insulin for 48h or 30 minutes in the absence or presence of 0.05 or 0.5 μM Picropodophyllotoxin (PPP), an IGFR kinase inhibitor. Glucose uptake was measured as the amount of non-metabolizable 2-Deoxy-D-2-[2,6-3H]glucose (2-DG uptake) transported into chondrocytes for 30 minutes at 37°C. Phosphorylation of Akt and total GLUT-1 content were evaluated by Western blot in whole cell lysates. IR mRNA expression was evaluated in normal and OA chondrocytes by Real time RT-PCR.

Results: Similar levels of IR mRNA were detected in both normal and OA chondrocytes. Treatment with 1 or 10 nM insulin for 48h significantly increased 2-DG uptake (125±3.6% and 141±3.5%, respectively) in normal chondrocytes. The presence of PPP caused an 8 and 20% reduction in 2-DG uptake induced by
1 and 10 nM insulin, respectively. The effect of PPP was identical with both concentrations used (0.05 μM and 0.5 μM). 2-DG uptake in OA chondrocytes was increased to 112±2.1% and 123±2.5% relative to the respective control cells, by treatment with 1 or 10 nM insulin, respectively, but pretreatment with either concentration of PPP had no effect on insulin-induced 2-DG uptake. Similarly, treatment with PPP decreased insulin-induced Akt phosphorylation in normal but not in OA chondrocytes Total GLUT-1 protein content was increased by treatment with insulin 10 nM in normal, but not in OA chondrocytes, whereas 1 nM had no effect either in normal or OA chondrocytes.

Conclusions: Adult human chondrocytes express functional IR. Insulin increases glucose transport in normal and OA chondrocytes, but in the latter its effects seem to be mediated by the IR alone, as indicated by the inability of the specific IGFR inhibitor, PPP, to decrease insulin-induced 2-DG uptake and Akt phosphorylation, a signaling event common to IR and IGFR. Insulin responses mediated by its specific receptor may overcome the previously reported defective IGFR signaling in OA chondrocytes.

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DIFFERENT OF AREAS OF THE HUMAN OSTEOARTHRITIC KNEE CARTILAGE RESPOND DIFFERENTLY TO CATABOLIC AND ANABOLIC STIMULATION WHEN CULTURED
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Purpose: There is a vast need for developing models for investigation of the pathobiology of cartilage turnover. A model system that have proven worthy in investigation of cartilage turnover is the bovine explant model; it show distinct patterns in response to defined catabolic and anabolic stimuli. It is desirable to extent this model to human articular cartilage, however, obtainable human cartilage will most likely be affected by OA. We therefore investigated how different areas of human knee OA cartilage would respond to catabolic and anabolic stimuli, when cultured for 3 weeks.

Methods: Human articular cartilage was isolated from patients undergoing knee replacement. Explants were taken from either the center (slightly macroscopically damaged) or the rim of the tibia plateau (macroscopically undamaged), or the femur condyle (no macroscopic damage). Explants were cultured at optimized conditions with and without cytokines and growth factors: 1) without stimulation (w/o), 2) TNFα and oncostatin M (O+T), 3) IGF-1, and 4) Parathyroid hormone (PTH). The explants were cultured for 21 days and medium was renewed every 2-3 days. At day 21, the explants were formalin-fixed and paraffin embedded. Explants were analyzed by safranin O/fast green. Supernatants were analyzed by formation and degradation biomarkers (ELISA assays): PIINP, CTX-II, 342-G2, 374-G2 and CIIM (new Col II marker).

Results: All stimulated samples were compared with w/o. Aggrecanase fragments (374-G2) were continuously released from all explants throughout the culturing period, and there was only little effect of the different stimuli. MMP fragments of aggrecan (342-G2) and type II collagen (CTX-II and CIIM) were significantly released by explants from both damaged and undamaged tibial cartilage when treated with O+T, but not by femur explants (all time points). Neither IGF-1 nor PTH could significantly alter the release of aggrecanase- or MMP-derived fragments. Type II collagen formation (PIINP) was suppressed when treated with O+T in explants from the femur and the undamaged tibial cartilage, but only slightly in the late stage of the damaged cartilage explants. Both IGF-1 and PTH could induce collagen type II formation, but again the effect was only significant in the femur and the tibial undamaged explants. These data was supported by histological assessments.

Conclusion: We found that different areas of the OA cartilage display different biological potential. We found that the aggreganase activity could not be induced further by either anabolic or catabolic stimulation. In contrast, MMP activity could be induced by cytokines in both slightly damaged and undamaged cartilage explants. Furthermore, anabolic stimulation had greater effect on femur condyle and undamaged tibial cartilage, than on slightly damaged tibial cartilage. This means that different areas of the cartilage display different biological profile and this information should be incorporated when designing OA models (e.g. cartilage explants). Moreover this could with advantage be used for investigation of particular OA situation.

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AN IKKα ASSOCIATED GENE EXPRESSION PROFILE IN DIFFERENTIATING CHONDROCYTES
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Purpose: We previously reported that IKKα, one of the two NF-κB activating kinases, has an unexpected role in ECM remodelling and progression to hypertrophy and terminal differentiation of primary osteoarthritic chondrocytes grown in 3D mineralizing cultures. As an unbiased approach to deciphering IKKα’s mechanisms of action in this context we have begun to evaluate the effects of IKKα ablation on the gene expression profile of primary OA chondrocytes. In addition we have also begun to investigate if the expression of genes affected by IKKα in this in vitro context reflects similar or different alterations in gene expression associated with OA disease.

Methods: IKKα expression was evaluated by immunohistochemistry in sections of full thickness explants of cartilage and subchondral bone derived from OA patients, and graded by Safranin-O staining. To investigate IKKα dependent gene expression, primary OA chondrocytes, derived from several independent patients undergoing joint arthroplasty, were seeded into differentiating micromass cultures. Endogenous IKKα expression was ablated just prior to micromass seeding by stable retrotransduction with IKKα shRNAs (Olivotto et al A&R 2008) with a firefly luciferase shRNA retrovector as a control. After ~1 week of micromass differentiation, Affymetrix arrays were employed to reveal the gene expression profile linked to IKKα in this context and the latter gene expression profile was subsequently evaluated in the context of normal and OA cartilage.

Results: IHC analysis showed that the degree of IKKα protein expression correlated with OA severity, and was more upregulated in the vicinity of the middle to deep zones of late stage OA cartilage compared to normal cartilage. The expression profile of ninety-two genes in differentiating micromass cultures were linked to IKKα. Pathway analysis (Kegg map) indicated that the pathways significantly affected by IKKα specify gene products associated with ECM-receptor interaction, focal adhesion, cell communication, T cell receptor signaling pathway and the cell cycle. Noteworthy, most of the genes encoding ECM proteins were up-