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158 AKT1 CONTRIBUTES TO CHONDROCYTE CALCIFICATION DURING ENDOCHONDRAL OSSIFICATION

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Purpose: Endochondral ossification plays crucial roles in skeletal disorders like osteoarthritis as well as in skeletal growth. Since the phosphoinositide-dependent serine-threonine protein kinase Akt has been proposed to be a pivotal signaling molecule for several factors regulating cartilage metabolism, this study examined the possible involvement of Akt in the processes of endochondral ossification.
Methods: Expressions of the three isoforms of Akt (Akt1, 2 & 3) were examined by real-time RT-PCR in chondrocytes derived from ribs of neonatal mice. To know the physiological function of Akt1, we compared the skeletal phenotypes of homozygous Akt1-deficient (Akt1−/−) mice with those of the wild-type littermates by plain radiograph, 3D-μCT, and histological analyses including toluidine blue, von Kossa stainings, type X collagen (COL10) immunostaining, and BrdU labeling. For gain- and loss-of-function analyses, we established stable lines of mouse chondrogenic ATDC5 cells with retroviral overexpression of constitutively active Akt1 (CA-Akt1) and small interfering RNA (siRNA) of Akt1, respectively, and compared the functions with respective empty-vector controls. Cell proliferation was assessed by CCK-8 assay. The differentiation was determined by Alcian blue and alkaline phosphatase (ALP) stainings under the stimulation of insulin. The hypertrophic differentiation was determined by luciferase assays using ATDC5 cells transfected with a luciferase-reporter gene construct containing a 4.5-kb fragment of the COL10 promoter. Calcification was assessed by Alizarin red staining under the stimulation of insulin and inorganic phosphate ion (Pi). Expressions of inorganic pyrophosphate (PPi)-related factors were determined by semi-quantitative RT-PCR.
Results: Since Akt1 was most highly expressed in chondrocytes among the three isoforms, we generated Akt1−/− mice and examined the skeletal phenotype. The Akt1−/− mice exhibited dwarfism with about 20% shorter limbs and trunks than the wild-type littermates during observation periods from embryos to 12 weeks postnatal. The entire width and columnar structure of the growth plate cartilage were normal, and BrdU-positive proliferative zone and COL10-positive hypertrophic zones were also unaffected by the Akt1 deficiency. However, the width of the calcified layer and the number of calcified chondrocytes determined by the von Kossa staining were significantly decreased at the bottom of the Akt1−/− growth plate. In the ATDC5 cell culture, neither the CA-Akt1 nor the Akt1 siRNA overexpression altered cell proliferation, differentiation, or the COL10 promoter activity. Contrarily, calcification of ATDC5 cells cultured in the presence of insulin/Pi was significantly enhanced by the CA-Akt1 and suppressed by the Akt1 si-RNA. These in vivo and in vitro findings demonstrate that Akt1 is essential for chondrocyte calcification without affecting the prior processes of endochondral ossification. As PPi has been known to be a crucial regulator of chondrocyte calcification, we finally examined expressions of principal modulators of PPi: ANK, NPP1 and Pit1. Although all increased during chondrocyte calcification under the insulin/Pi stimulation, none of them was affected by the CA-Akt1 or the Akt1 si-RNA.
Conclusions: Akt1 controls skeletal growth by maintaining chondrocyte calcification during endochondral ossification, without affecting the proliferation or differentiation of chondrocytes. Further understanding of the molecular network related to Akt1, probably independent of the putative PPi pathway, will quite probably lead to a breakthrough for the treatment of skeletal disorders like osteoarthritis.

159 ECM REMODELLING PLAYS A CRUCIAL ROLE IN THE LOSS OF MATURATIONAL ARREST OF OA CHONDROCYTES

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Purpose: IKKα, one of the two NF-κB activating kinases, has as yet unknown roles in cartilage pathophysiology. Our previous work revealed an unexpected role of IKKα in ECM remodelling and progression to hypertrophy and terminal differentiation of primary osteoarthritic chondrocytes, with a “chondrogenic” and abundant ECM being a key feature of cells lacking IKKα. Here we have begun to mechanistically define how IKKα affects the terminal chondrogenic differentiation program.
Methods: Primary chondrocytes were derived from 14 Osteoarthritis (OA) patients undergoing joint arthroplasty. Stable and efficient expression of IKKα or IKKβ or MMP-13 shRNAs was achieved by transduction with a pSUPER retroviral vector and populations of shRNA expressing cells were selected for puromycin resistance. Knock-down (KD) efficiencies were determined as follows: by Western blotting for each IKK in comparison to cells transduced by a firefly luciferase shRNA retrovector; and by ELISA for MMP-13 released by Il-1β stimulation of monolayer or micromasses established from MMP-13 KD or control chondrocytes.
High density monolayer and micromass cultures (1, 2 and 3 weeks) in mineralizing conditions were selected to investigate the effects of either IKK or MMP-13 KD on key regulators of chondrogenesis (including SOX-9, Runx-2 and β-catenin), and of angiogenesis (VEGF) at the protein (immunohistochemistry or western blot) and RNA (real time PCR) levels. The viability and subcellular features of micromass chondrocytes were also evaluated to assess their progression to terminal differentiation. ECM turnover was assessed by comparison of the C1,2C and TIMP staining of micromasses evaluated by immunohistochemistry.
Results: MMP-13 KD was found to mimick the effects of IKKα KD in the control of the chondrogenic program, as recapitulated in micromass cultures. Thus loss of either IKKα or MMP-13 suppressed the accumulations of SOX-9, Runx-2 and VEGF (at the protein and RNA levels) contributing to the maturational arrest of articular chondrocytes. On the other hand, in control micromasses, the chondrogenic program progressed up to the calcium deposition stage in conjunction with reduced cell viability, scant ECM and a paucity of specialized cell-cell and cell-ECM junctions. IKKα, IKKβ and MMP-13 KD cells also displayed a lower C1,2C staining at 1 week, compared to control micromasses. Noteworthy IKKα KD cells were markedly and reproducibly characterized by a high level of TIMP-3 staining in 1 week micromasses, while TIMP-1 and TIMP-4 levels were elevated in some but not in all IKKα samples derived from different OA patients.
Conclusions: Since micromasses established with IKKα and MMP-13 (the key collagenolitic enzyme in OA) KD chondrocytes, presented comparable phenotypes, our data reveal that ECM remodelling has a crucial role in the capacity of OA chondrocytes to progress towards terminal differentiation. Moreover, our observations also suggest that IKKα functions at least in part as a positive effector of ECM remodelling by suppressing TIMP-3 (given the unique attributes of TIMP-3 to control MMPs, as well as ADAMTS-4, 5 and 17), thereby allowing for progression to hypertrophy in Osteoarthritis and possibly also in normal bone development.

160 PTH ACTS AS AN ANABOLIC EFFECTOR ON OSTEOARTHRITIC ARTICULAR CARTILAGE: COULD PTH BE A NEW TREATMENT OPPORTUNITY FOR OSTEOARTHRITIS?

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Purpose: In view of the fact that pathogenesis of osteoarthritis (OA) involves both the bone and cartilage compartments of the affected joints, an increasing amount of attention is directed to potential treatments of OA with positive effect on calcium metabolism. One of the factors that are known to have an anabolic effect on bone is parathyroid hormone (PTH). However, *in vivo* effects of PTH on cartilage has until now been an open question. In bone, PTH acts on osteoblasts, initiating the G-coupled receptor pathway through cAMP. Interestingly, cAMP has recently been shown to be of major importance for the chondrocyte phenotype. Since chondrocytes and osteoblasts originate from the same mesenchymal lineage, we hypothesize that chondrocytes and osteoblasts, respond anabolically similar to PTH treatment. Thus, PTH could potentially represent a novel treatment opportunity for OA. To test this hypothesis, we used validated human OA cartilage *in vitro* models and a pre-clinical model of accelerated cartilage loss *in vivo* for our investigations.
Methods: *In vitro:* Human chondrocytes were isolated and maintained serum-free for 1 hour in the presence of 100 μM IBMX (PDE inhibitor), and subsequently stimulated with PTH 1 nM-100 nM + 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 10 nM PTH treatment, and 5 μCi ^{35}sulphate was added for the last 24 hours. Soluble proteoglycans were released by 4M GuHCl and incorporated sulphate was measured. Neo-epitopes of pro-peptides of collagen type II (PIINP) were quantified as a measure of formation in the conditioned medium. *In vivo*: Thirty 5-month old female rats were subjected to either sham

or ovariectomy (OVX), and administered either vehicle or 30 μg/kg hPTH (1−34aa) by s.c. injection for 5 weeks, n = 10. Serum levels of osteocalcin and CTX-II were measured by specific ELISAs.
Results: 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 vehicle control. In the explants 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% ($p = 0.002$). The serum level of the bone turnover marker osteocalcin was significantly ($p < 0.001$) elevated in OVX animals that were PTH treated compared to sham and vehicle treated, while the level of serum cartilage degradation marker CTX-II decreased by 30% ($p < 0.01$).
Conclusions: The current data strongly suggest that PTH, in addition to osteoblasts and bone turnover, also has direct anabolic effects on chondrocytes and cartilage. 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.

161 NMDA RECEPTOR FUNCTION IN OSTEOARTHRITIC CHONDROCYTES IS DEPENDENT ON β1 INTEGRINS

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Purpose: Osteoarthritis (OA) is associated with abnormal loading of articular joints. In normal joints physiological loading maintains structure and function of cartilage and chondrocytes within. This is mediated by a process known as mechanotransduction whereby the mechanical forces are recognized by the resident chondrocytes and transduced into biochemical and molecular responses. In chondrocytes $\alpha5\beta1$ integrin is a major mechanoreceptor and activation triggers signaling events and autocrine/paracrine signaling via IL4 which lead to up-regulation of anabolic genes and down-regulation of catabolic activity. In OA however this mechanism is disrupted and mechanically stimulate chondrocytes express pro-inflammatory and catabolic IL1β. The reasons for this are unclear but recent research from our group has raised the possibility that differential involvement of ionotropic glutamate receptors, NMDARs in the mechanotransduction pathway may be important. NMDARs are associated with neurons, although non-neuronal tissues have been shown to express these receptors. In neurons, NMDAR interact with intracellular signaling proteins through the scaffold protein PSD-95. As potential roles for integrins in modifying NMDAR activity have also been shown we have now investigated the relationship between NMDAR signaling in chondrocytes and molecules that are involved in chondrocyte mechanotransduction.
Methods: Articular cartilage was removed from OA and chondrocytes released using sequential enzymatic digestion. Chondrocytes were maintained as non-confluent primary monolayer cultures at a concentration of 5×10^5 cells/ml. To measure the function of NMDAR cell membrane potentials of OA cells were measured by electrophysiology in resting cells, after treatment (integrin modifying or CD47 antibody (1 μg/ml)) and following stimulation with NMDA (50 μM). Chondrocytes seeded onto cell culture dishes coated with substrates (poly-L-lysine or fibronectin; 10 μg/ml) were used to assess cell membrane potential before and after NMDA treatment. Co-immunoprecipitation of PSD-95 with β1 integrin and CD47 using specific antibodies was analysed by Western blot.
Results: Incubation of OA chondroctytes with anti-β1 integrin and anti-CD47 (integrin associated protein) function blocking antibodies inhibited the electrophysiologcal response to NMDA; antibodies to β3, β5 and anti-α integrin antibodies had no effect. Integrin dependency of NMDAR signaling with NMDA treatment was analysed; Chondrocytes adherent to poly-L-lysine (integrin independent attachment) showed no electrophysiological response to NMDA while cells adherent to fibronectin and type II collagen (β1 integrin dependent attachment) showed a membrane depolarization. Co-immunopreciptiation experiments of PSD-95 with β1 integrin and CD47 identified that these molecules are physically linked and therefore may be involved in regulating NMDAR function.
Conclusions: We identify that NMDAR function in chondrocytes is linked to β1 integrin and CD47, and that the electrophysiological response to NMDA requires integrin dependent cell-matrix interactions. Linkage of β1 integrins and CD47 to PSD-95, possibly as part of a complex that may contain NMDAR, appears to be important in NMDAR activity in chondrocytes. In neurons studies have shown integrins can be involved in regulating the expression and function of NMDAR, although the mechanisms by which this occurs have yet to be defined. The role of NMDAR in OA chondrocyte/cartilage function is yet to be fully understood, but parallel studies indicate that these receptors may participate in the signal cascade that results in anabolic/catabolic responses to applied mechanical forces.

162 CHONDROCYTES FROM OSTEOARTHRITIS PATIENTS REVERT TO THEIR ORIGIN PHENOTYPE ONCE GROWN ONTO A HYALURONAN-BASED SCAFFOLD

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Purpose: To evaluate the expression of some specific extracellular matrix molecules in human chondrocytes from healthy and osteoarthritis cartilages freshly isolated and after their growth onto a hyaluronan-based scaffold used in autologous transplantation procedure.
Methods: Chondrocytes were isolated from human articular cartilage obtained from the knees of patients with osteoarthritis and from multiorgan donors. First, the cells were expanded in monolayers and then they were seeded on a hyaluronic-acid derivative scaffold. Constructs were analyzed at 0, 3, 7, 14, 21 and 28 days after seeding. Immunohistochemical analysis for collagen type I, II, proteoglycans, Sox-9, MMP-1, MMP-13, TIMP-1, cathepsin-B was carried out on freshly isolated cells, on cells grown in monolayer culture and after they were grown onto the scaffold. A Real-Time RT-PCR analysis was performed on the constructs to evaluate the expression of the specific genes at the different experimental times evaluated.
Results: Chondrocytes freshly isolated from control and OA patient cartilages expressed the same extracellular matrix molecules even if at different amount. These differences, which were appreciable both at protein and molecular levels, were not evident once the cells were grown onto Hyaff®-11 scaffold. In this experimental culture condition the cells derived from control and OA patients showed a significative increase of collagen type II, Sox-9 and aggrecan and a decrease of collagen type I compared to chondrocytes grown in monolayer. On the other hand, MMPs were downregulated in both the cell types evaluated by the specific action of TIMP-1 which was highly expressed at molecular and protein levels in the two groups.
Conclusions: The growth of chondrocytes onto Hyaff®-11 membrane seems to erase the differences between the cells derived from normal and OA cartilages. The hyaluronan-based scaffold is able to recapitulate some embryonic events which allow to the expression of the extracellular matrix specific genes and to the production of the appropriate proteins. This is of particular relevance hypothesizing the use of tissue engineering therapeutical approach also in osteoarthritis patients.

163 RELATIONSHIP BETWEEN INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR EXPRESSION AND MICROSCOPIC SCORE IN NORMAL EQUINE JOINTS

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Purpose: Osteoarthritis (OA) is a chronic degenerative disease of the articular cartilage which appears to reflect a failure in the attempted repair of the cartilage. Comprehensive data regarding the biological nature of both normal and early OA tissue are lacking. Until such data are available, accurate comparison of variables between cartilage from different joints and potential therapeutic strategies may be compromised. Insulin-like growth factor-1 (IGF-1) is a potential therapeutic agent in OA due to its potent anabolic effect in normal cartilage. In an OA joint, chondrocytes are phenotypically disturbed, and demonstrate altered receptor expression. Disruption in levels of IGF-1 and expression of its receptor (IGF-1R) causes enhanced catabolism of proteoglycans. Therefore the aim of the current study was to investigate IGF-1R expression in macroscopically normal cartilage from grossly normal joints and correlate this with microscopic score, in an attempt to further understand the normal condition of cartilage.
Methods: Macroscopically normal cartilage from grossly normal joints was sampled from six sites on the mediodistal metacarpal condyle at distances from the transverse ridge (dashed line in figure 1) of