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