Aggrecanase activity was assessed by measuring in the glycosaminoglycan (GAG) release and ARGSVIL neoepitope formation. Explants were further examined by histology after safranin O proteoglycans (PG) staining/light green counterstaining, and by immunohistochemistry for aggrecan NITEGE neoepitope. PG and neoepitope content was then quantified applying a scoring system to individual images.
In addition, cytokine-induced matrix degradation was assessed in a new rat chondrosarcoma cell model ('RCS assay') in which the amount of sulphated proteoglycan was quantified using Alcian blue staining and image processing.
Results: TNFα+OSM or IL1β+OSM strongly induced ADAMTS4 and ADAMTS5 in human cartilage explants. OSM is strictly needed for induction of aggrecanases but not for MMP13.
A specific ADAMTS5 inhibitor (cpd A, IC50 80% inhibition of GAG release, >80% inhibition of neoepitope release at 3μM). In contrast, a selective ADAMTS4 inhibitor (cpd B, IC50 <100 nM) did not inhibited cartilage degradation by more than 50%. Cpd C, a non-selective 3-OH-3-methylpipecolic hydroxamate type metalloproteinase inhibitor completely inhibited GAG and Neoepitope release.
Histological evaluation confirmed that cpd A prevented PG loss and inhibited neoepitope formation in cartilage matrix but not in the pericellular matrix (PM), while cpd B only inhibited neoepitope formation in PM. Cpd C prevented PG loss and inhibited neoepitope formation in both cartilage matrix and PM.
In addition, the compound ranking obtained in the explant model was reproduced with the simple 'RCS assay'.
Conclusions: This study represents the first pharmacological evidence for the importance of ADAMTS5 over ADAMTS4 in human cartilage degradation. Strong induction of ADAMTS5 by cytokines and significant prevention of cartilage degradation by its pharmacological inhibition using a selective inhibitor are demonstrated. This observation underscores the important role of ADAMTS5 in the degradation of human cartilage. Furthermore, these ADAMTS5 effects were recapitulated in a new RCS-based assay, which provides a good substitute for the complex cartilage explant model.
Finally, histological analysis delivered the unexpected observation that matrix aggrecan is subject to different proteolytic events depending on its localization, providing mechanistic information on the cartilage degradation process.

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THE MELANOCORTIN SYSTEM IN HUMAN CHONDROCYTES

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Purpose: Melanocortins (MCs) are structurally related peptides that elicit their actions via binding to melanocortin receptors (MCRs). Originally defined as neurohormones these peptides exhibit an astonishing spectrum of effects on many peripheral cell types and tissues. However, our present knowledge on the function of the MC system in cartilage is scarce. We could show that MC-1R is expressed in human articular cartilage and chondrocytes. α-Melanocyte-stimulating hormone (α-MSH), a prototype of MCs, is capable of regulating a number of important genes involved in extracellular matrix (ECM) composition and inflammation in chondrocytes. The major goal of this study is to define the role of MCs and the MC-1R in the context of osteoarthritis (OA).
Methods: Cartilage slices were aseptically dissected from femoral condyles of OA patients who underwent total knee arthroplasty. Articular chondrocytes isolated from OA-cartilage were proliferated in monolayer until passage one and either cultured in 3D-micromass pellets for 7 days or kept in monolayer. After stimulation for 48h with 10^{-6} M α-MSH only (micromass pellet culture) or in presence of 1 ng/ml IL-1β (monolayer culture); the cDNA copy number of Sox9, collagens, MMPs; TGF-β1,TNF-α and interleukins was assessed with quantitative RT-PCR. POMC, converting enzymes and MC-receptor gene expression was determined by end point RT-PCR and, MC-R1 protein expression *in situ* on cryosections of OA-cartilage by immunohistochemistry. Signal transduction of α-MSH was evaluated by cAMP ELISA and Ca^{2+}-assays using Fura2-AM. Protein concentration of MMP-2, MMP-13, IL-6, TNF-α, TGF-β1 and collagen I and II in cell culture supernatants and cell lysates was determined with ELISA.
Results: Articular OA-chondrocytes express receptors MC-1R, MC-2R and MC-5R plus POMC and converting enzymes PC1, PACE4 and furin convertase. MC-1R protein was preferentially found on chondrocytes from the middle and deep zones of articular cartilage. The MC-1R detected in human chondrocytes appears to be functional as stimulation with 10^{-6} M α-MSH resulted in a moderate but significant increase in intracellular cAMP levels but not in changes of the intracellular Ca^{2+} level. *In vitro* application of 10^{-6} M α-MSH to articular chondrocytes, kept in micromass pellet culture induced gene expression of COL1A1, COL2A1 and COL10A1. Of note, gene expression of MMP-2, MMP-7, MMP-9 and MMP-13 was induced, however, MMP-13 protein concentrations in culture supernatant revealed profound interpatient fluctuations. MMP-2 protein concentration was consistent within the sample collective and remained unaltered in the presence of α-MSH. IL-1β and TNF-α gene expression was reduced, IL-6 remained mostly unchanged and Sox9 and TGF-β1 were induced upon stimulation with α-MSH. IL-6 and TNF-α protein concentration were upregulated. α-MSH attenuates IL-1β-induced expression of IL-4 and IL 8, MMP-2, -9 and -13 in articular OA-chondrocytes.
Conclusions: Human articular chondrocytes are target cells for α-MSH. The effects of α-MSH on cytokine- and MMP expression suggest a chondroprotective role of this neuropeptide in inflammatory and degenerative processes in cartilage. It is conceivable that inflammatory reactions can be mitigated by induction of endogenous melanocortins or administration of α-MSH to the affected joints. The induction pattern of regulatory and structural ECM components as collagens, Sox9, anabolic and catabolic cytokines points towards a function of α-MSH as a trophic factor in skeletal development during endochondral ossification or as anti-inflammatory agent in degenerative joint pathologies.

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THE FUNCTION OF p53 ON HUMAN CHONDROCYTE APOPTOSIS IN RESPONSE TO SHEAR STRESS

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Purpose: Chondrocyte apoptosis plays an important role in cartilage degeneration in osteoarthritis (OA), and mechanical injury to cartilage induces chondrocyte apoptosis. p53 has been identified as a 53 kDa cellular protein and 'ultimate tumor suppressor gene'. p53 is recognized as a pivotal regulatory protein that responds to a variety of signals and recruits an array of biochemical activities to trigger diverse biological responses, most notably cell cycle arrest and apoptosis. In response to DNA damage, p53 expression is upregulated, increases transcriptional activity, and initiates apoptosis signals. p53-regulated apoptosis-inducing protein 1 (p53AIP1) is one of the p53-regulated genes, and is activated in response to severe DNA damage with the phosphorylation of p53 at Ser-46. In this study, we speculated that p53 and p53AIP1 might play an important role in DNA damage-induced apoptosis of articular carti-

lage, and analyzed the function of p53 and p53AIP1in chondrocyte apoptosis by shear stress.
Methods: Shear stress was introduced to NHAC-kn cells (human normal chondrocytes) with Flexcell system. DNA damage was detected and quantified by comet assay, DNA fragmentation, and phosphorylation of ataxia-telangiectasia mutated kinase (ATM). To explore the p53 function, NHAC-kn cells were pretreated with pifithrin- alpha, which is specific inhibitor of p53, or p53 siRNAs before induction of shear stress. Chondrocyte apoptosis was detected by expression of cleaved caspase-9 with Western blotting and TUNEL staining. Expressions of p53 and p53AIP1, and phosphorylation of p53 at Ser-15 and -46 were analyzed by Western blotting.
Results: DNA damage was actually induced by shear stress, with the results of comet assay, DNA fragmentation, and phosphorylation of ATM. After loading shear stress, TUNEL positive cells and expression levels of p53, p53AIP1 and cleaved caspase-9 were increased in a force and time dependent manner. Phosphorylation of p53 at Ser-15 and Ser-46 increased, compared with non-loaded samples. Chondrocyte apoptosis was reduced when pretreatment with pifithrin-alpha or p53 siRNAs. Western blotting revealed that expressions of p53 and p53AIP1 were still detected when chondrocytes were pre-treated with pifithrin-alpha. Pifithrin-alpha inhibited the phosphorylation of p53 at Ser-15 compared with non-treatment of pifithrin-alpha. However, pifithrin-alpha did not inhibit the phosphorylation of p53 at Ser-46 compared with non-treatment of pifithrin-alpha. When p53 siRNA was transfected, p53, phosphorylation of p53 at Ser-15 and Ser-46, and p53AIP1 expressions decreased.
Conclusions: We demonstrated that expression of p53 and p53AIP1 increased with the activation of apoptotic processes in human normal chondrocytes following shear stress. However, pifithrin-alpha reduced chondrocytes apoptosis after loading shear stress. Furthermore, down regulation of endogenous p53 by transfection of p53 siRNA inhibited chondrocytes apoptosis after loading shear stress. Our results suggest that shear stress induces apoptosis via p53, mainly phosphorylation at Ser-46, and the mitochondrial pathway. In conclusion, we demonstrated that p53 and p53AIP1 act as an important molecule for chondrocyte apoptosis induced by elongation shear stress, and down regulation of exogenous or endogenous p53 expression inhibited chondrocyte apoptosis.

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A FUNCTIONAL LINK BETWEEN ECM HOMEOSTASIS AND SOX-9 MEDIATED CONTROL OF β-CATENIN ACTIVITY

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Purpose: Our recent findings have unexpectedly linked IKKα to MMP-13 activity dependent ECM remodeling (Olivotto et. al A&R 2008) in differentiating chondrocytes, revealing a linkage between ECM stabilization in the absence of IKKα with a block in chondrocyte differentiation towards a hypertrophic-like phenotype. Here we present evidence that knocking down MMP-13 or IKKα, either of which blocked ECM remodeling, resulted in pronounced alterations in the pattern of Sox-9 nuclear localization that can be linked to changes in β-Catenin levels and activation status.
Methods: Accumulation of β-catenin protein was evaluated by IHC in maturing (1, 2 and 3 week) control, IKKα KD or MMP-13 KD micromasses and gene expression was assessed by Real Time PCR at 1 week maturation and also in high density monolayers. Sox-9 subcellular distribution was analyzed in one week micromasses by confocal microscopy, wherein permeabilized cells were stained with anti-Sox9 Ab linked to Cy5 in conjunction with Sybr green labeling of DNA. Activation of the wnt/β-catenin signaling pathway was investigated by IHC and WB analysis of active β-catenin in three week micromasses. Confocal microscopy analysis of Sox-9 subcellular localization was also carried out on full thichness slices of cartilage derived from either normal cartilage/early OA or late OA cartilage, which were also assessed for ECM integrity by Safranin-O staining.
Results: Both IKKα KD and MMP-13 KD prevented the accumulation of total β-catenin protein in maturing micromasses and high density monolayers, which was also associated with significantly lower (n=5, p=0.043) β-catenin RNA expression. Loss of either MMP-13 or IKKα expression also blunted the accumulation of activated β-catenin. Moreover, confocal microscopy revealed strong intranuclear Sox-9 staining in the absence of MMP-13 or IKKα, while the nuclei of control differentiating chondrocytes presented a halo-like, peri-nuclear Sox-9 staining pattern. Noteworthy, in cartilage tissue with strong safranin-O staining, Sox-9 was mainly localized in chondrocyte nuclei while the Sox-9 in chondrocytes within areas of degraded OA cartilage presented a peri-nuclear like staining pattern.
Conclusions: IKKα and MMP-13 are both required for ECM remodeling in differentiating chondrocyte micromass cultures expression. Loss of either MMP-13 or IKKα stabilized the ECM in conjunction with the subcellular localization of Sox-9 to cell nuclei, an event which was associated with a reduction in β-catenin expression and activity. A similar pattern of Sox-9 subcellular localization was also associated with the ECM integrity of cartilage tissue. On the basis of these observations, we suggest that when chondrocytes abandon their natural maturation arrested state in cartilage, IKKα dependent, MMP-13 initiated ECM remodeling can act as a focal point for functional cross-talk between early (Sox-9) and late (β-Catenin) positive effectors of chondrocyte differentiation, thereby pushing chondrocytes towards a more hypertrophic-like state.

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CROSSTALK BETWEEN RESIDENT CELLS IN THE JOINT: IMPACT ON CHONDROCYTE METABOLISM

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Purpose: Osteoarthritis (OA) is a multi-factorial disorder that results in a net loss of cartilage from synovial joints, often leading to pain. Cartilage lesions are a product of blunted extracellular matrix (ECM) synthesis by chondrocytes, and enhanced enzyme-mediated ECM degradation. Although often characterized by the single pathological feature of cartilage degeneration, OA is increasingly recognized as a disease of the whole joint in which interplay between the different cells within the joint are important. Here we explore the impact of various joint resident cells on ECM metabolism of chondrocytes in an *in vitro* co-culture system.
Methods: Primary human chondrocytes were isolated by enzymatic digestion of adult articular cartilage and encapsulated in 3D alginate beads. Primary human osteoblasts, purchased from PromoCell, were isolated by enzymatic digestion of bone from patients undergoing knee endoprothesis due to arthrosis of the knee joint. Human macrophages were differentiated from $CD14^+$ peripheral blood monocytes (Allcells) by treatment with macrophage colony