understand the role of norepinephrine in human osteoarthritic chondrocytes with regard to inflammation and its impact on metabolic activity.
Methods: Human chondrocytes were isolated from post-surgery discarded human osteoarthritic articular cartilage. Expression of β2-adrenergic receptor on articular cartilage was tested with standard immunohistochemical analysis. Employing 3D cell cultures in fibringel, effects of norepinephrine on interleukin-1β induced gene expression of pro-inflammatory cytokines and matrix metalloproteinases (MMP) were analyzed with quantitative real-time PCR. The impact of norepinephrine on cell proliferation was determined in monolayer culture with BrdU and XCelligence analyses.
Results: ß2-adrenergic receptors are abundantly expressed in human osteoarthritic cartilage. Stimulation with norepinephrine has significantly reduced interleukin-1β induced gene expression of interleukin 8 and MMP-13 in human osteoarthritic chondrocytes cultured in 3D fibringel. Notably, we were unable to detect an impact on interleukin-1β induced gene expression of interleukin-6, MMP-2 and MMP-3. Furthermore, norepinephrine inhibits BrdU incorporation compared to the controls. Additionally, we measured a lower cell spreading, assessed through electrical impedance using the XCelligence System (Roche), starting 6 h after norepinephrine stimulation until analysis was stopped (50 h after stimulation). All effects were observed exclusively with 10-6 M and not with 10-8 M norepinephrine indicating for a signaling via β-adrenergic receptors.
Conclusions: Neurotransmitters of the sympathetic nervous system like norepinephrine presumably mediate an anti-inflammatory / chondroprotective effect in human osteoarthritic chondrocytes via reducing interleukin-8 and MMP-13. Furthermore, norepinephrine is able to modulate the metabolic activity by inhibiting cell proliferation of human osteoarthritic chondrocytes. These findings indicate, together with β2-adrenergic receptor expression in human osteoarthritic articular cartilage, a yet unknown function of catecholaminergic neurotransmitters in adult human cartilage and might have an impact on osteoarthritis pathology.

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EGR-1 MEDIATES THE SUPPRESSIVE EFFECT OF IL-1 ON PPARG EXPRESSION IN HUMAN OA CHONDROCYTES

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Purpose: PPARg has been shown to down-regulate several inflammatory and catabolic responses in articular cartilage and chondrocytes and to be protective in animal models of OA. We have previously shown that IL-1 down-regulated PPARg expression in OA chondrocytes. In the present study we will investigate the mechanisms underlying this effect of IL-1.
Methods: Chondrocytes were stimulated with IL-1, and the level of PPARg and Egr-1 protein and mRNA were evaluated using Western blotting and real-time reverse-transcription polymerase chain reaction, respectively. The PPARg promoter activity was analyzed in transient transfection experiments. Egr-1 recruitment to the PPARg promoter was evaluated using chromatin immunoprecipitation (ChIP) assays. Small interfering RNA (siRNA) approaches were used to silence Egr-1 expression.
Results: We demonstrated that the suppressive effect of IL-1 on PPARg expression requires de novo protein synthesis and was concomitant with the induction of the transcription factor Egr-1. ChIP analyses revealed that IL-1 induced Egr-1 recruitment at the PPARg promoter. IL-1 inhibited the activity of PPARg promoter and overexpression of Egr-1 potentiated the inhibitory effect of IL-1, suggesting that Egr-1 may mediate the suppressive effect of IL-1. Finally, Egr-1 silencing with small interfering RNA blocked IL-1-mediated down-regulation of PPARg expression.
Conclusion: These results indicate that Egr-1 contributes to IL-1-mediated down-regulation of PPARg expression in OA chondrocytes and suggest that this pathway could be a potential target for pharmacologic intervention in the treatment of OA and possibly other arthritic diseases.

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THE FUNCTIONAL EFFECTS OF DIO2 IN AN IN-VITRO MODEL FOR CHONDROGENESIS

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Purpose: Previously, we identified the deiodinase iodothyronine type-2 gene (DIO2) as a relevant susceptibility gene for osteoarthritis (OA). The type 2 deiodinase (D2) is a selenoprotein responsible for catalyzing the conversion of intracellular inactive thyroid (T4) to active thyroid (T3), a process that occurs in specific tissues including the growth plate. In the growth plate, T3 specifically signals terminal maturation of the chondrocytes. Here, we investigate the role of DIO2 during chondrogenesis by interfering with D2 protein function in an in-vitro chondrogenesis model.
Methods: The 'RAAK' study contains human bone marrow derived mesenchymal stem cells (BM-MSCs) which were isolated from OA affected hips joints of subjects undergoing total hip arthroplasty. These BM-MSCs were cultured as micromasses and differentiated into cartilage particles. First, we assessed expression of DIO2 at consecutive weeks of chondrogenesis by RT-qPCR on samples previously isolated by the Rotterdam departments. Next, we tested the RAAK samples for the effect of T3 and Iodopanoic Acid (IOP), a DIO2 inhibitor, on chondrogenesis by adding them to the chondrogenic culture medium of the micromass cultures. These micromass cultures were than maintained up to 49 days. To measure the effects of interfering with D2 protein function we initially assessed pellet sizes at different time points and histochemistry with Alcian blue to determine glycosaminoglycan and Alizarin red to determine calcifications.
Results: DIO2 gene expression significantly increased in untreated BM-MSC micromasses during in-vitro chondrogenesis from 3 weeks onwards of differentiation. Upon inhibition of DIO2 protein function with IOP, the BM-MSC micromasses showed no significant changes in size after 7 weeks of differentiation when compared to untreated controls. In contrast, excess of T3 resulted in significantly smaller micromasses after 7 weeks of differentiation suggesting decreased extracellular matrix deposition. This observation was supported by histological analysis, which showed decreased Alcian blue staining already after 5 weeks of differentiation in T3 treated micromasses compared to untreated controls. In addition, only upon treatment with T3, calcium deposition could be detected by Alizarin Red staining from 5 weeks of differentiation onwards.
Conclusions: In addition to the association between DIO2 and osteoarthritis and the body of literature on the role of DIO2 in endochondral ossification, we are investigating the functional effect of DIO2 on chondrogenesis. Our first results show a significant up-regulation of DIO2 gene expression during chondrogenic differentiation. Furthermore, we show that excess T3 in this in-vitro chondrogenesis model, appears to interfere with the chondrogenesis process reflected by significant decreased pellet sizes and decreased Alcian blue staining accompanied by increased calcium deposition. Currently, we are using both lentiviral-induced DIO2 over expression and siRNA knock-down to explore directly the effect of DIO2 interference during in-vitro chondrogenesis.

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EXPRESSION OF NUCLEOSTEMIN IN SYNOVIAL TISSUES AND IN NORMAL AND OSTEOARTHRITIC CHONDROCYTES

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Purpose: Osteoarthritis is a common disease with a strong genetic component. Despite this, previous attempts to identify genetic variants that predispose to osteoarthritis have met with limited success. In the past few months, the results of a large genome wide association study for osteoarthritis have been reported and identified a novel susceptibility locus for the disease on chromosome 3 (Panoutsopoulou et al OARSI 2011). The strongest association within this region was found with a SNP located within the coding region of *GNL3* gene which encodes nucleostemin ($p=7.24x10^{-11}$). Nucleostemin is a protein that is found within the nucleolus of stem cells and tumour cells. It is thought to play a role in regulating cell cycle progression by modulating the action of p53, but its role in the joint is unknown. Here we wanted to determine if nucleostemin was expressed in articular chondrocytes and other joint tissues to determine if

there was any dysregulation of expression in cartilage from patients with osteoarthritis.

Methods and Results: We looked for expression of nucleostemin mRNA in cartilage, bone and synovial tissue by RT-PCR and this confirmed expression of full length transcripts from all three tissues. Further studies using western blotting showed that nucleostemin was expressed in cultured articular chondrocytes and that expression levels (corrected for GAPDH) were slightly higher in chondrocytes cultured from OA cartilage (n=5) versus those cultured from normal cartilage (n=4) (0.90±0.12 *vs.* 0.59±0.11) although the differences were not significant (p=0.12). Nucleostemin protein was also detected by western blotting in fresh extracts of articular cartilage. Analysis of fresh and cultured articular chondrocytes by immunohistochemistry showed evidence of nucleostemin expression localised to the nucleoli.

Conclusions: We conclude that nucleostemin is expressed within several articular tissues including chondrocytes where it is localised within the nucleoli. Although we found that levels of protein expression were slightly raised in osteoarthritic chondrocytes as compared with normal chondrocytes, the number of samples studied was small and this will have to be confirmed by further research.

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DELPHINIDIN BLOCKS IL-1β-INDUCED ACTIVATION OF NF-κB BY MODULATING THE IKKβ GENE EXPRESSION AND THE ACTIVATION OF NIK IN HUMAN CHONDROCYTES

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Purpose: Osteoarthritis (OA) is a highly prevalent disease that affects the quality of life of its victims. Interleukin-1 β (IL-1β) plays a major role in the pathogenesis of OA and is present in the arthritic joints at high levels. IL-1β has been shown to cause increased production of catabolic enzymes such as MMPs, and inflammatory mediators such as prostaglandins and nitric oxide through the activation of several signaling pathways including NF-κB. Novel approaches using plant derived polyphenols to inhibit signal transduction pathways have shown promise and could overcome some of the toxicity issues related to current therapies. One key signaling mechanism implicated in OA that could be a target of orally bioavailable inhibitors is the NF-κB pathway. Delphinidin (2-(3,4,5-trihydroxyphenyl) chromenylium-3,5,7-triol) is an anthocyanidin that is widely distributed in pigmented fruits and flowers and has been shown to possess anti-inflammatory activity and block the activation of NF-κB in cancer cells. No such study with human chondrocytes has been reported. In the present study using primary human chondrocytes we determined (a) the chondroprotective effect of Delphinidin; and (b) mechanism of NF-κB inhibition in IL-1β-stimulated human chondrocytes.

Methods: Chondrocytes were derived by enzymatic digestion of human cartilage (OA chondrocytes) from patients undergoing total knee arthroplasty. OA chondrocytes were pretreated with Delphinidin for different time points and were then stimulated with IL-1β (10ng/ml) *in vitro* and total RNA or cell lysate was prepared following our standard protocols. To determine the gene expression, single stranded cDNA was synthesized and the expression of target mRNAs (COX-2, MMP-13, iNOS) was quantified using TaqMan Assays. HeLa cells were transfected with promoter reporter vectors and negative control vectors using the Fugene-6 reagent and the effect on promoter activity was verified by Luciferase assay. Activation of NF-κB p65 was determined by specific ELISA-based DNA binding assay. Total protein levels were determined by detergent compatible Biorad Protein Assay. Nitric oxide production was determined by Griess reaction. Protein expression and the phosphorylated forms of different kinases were determined by Western immunoblotting. Data were analyzed using Origin 6.1 software package and $p<0.05$ was considered significant.

Results: Pretreatment of chondrocytes with Delphinidin (50 μg/ml) for 2 hrs significantly inhibited the IL-1β induced expression of COX-2, iNOS and MMP-13 and the production of nitric oxide. Delphinidin also inhibited the phosphorylation of IKKβ and was also a potent suppressor of IKKβ gene and protein expression in OA chondrocytes. IKK promoter had a strong activity in HeLa cells and this activity and production of the Luciferase reporter enzyme was blocked by pre-treatment with Delphinidin. Functional pathway analyses showed that IL-1β-induced phosphorylation of NF-κB-inducing Kinase (NIK), which interacts with and activates IKKα and IKKβ which then phosphorylate IκB, was inhibited by pretreatment of OA chondrocytes with Delphinidin. In agreement with these findings, pretreatment with Delphinidin inhibited the phosphorylation and degradation of IκB, as determined by Western immunoblotting, and the activation and the DNA binding activity of NF-κB p65 determined by a highly specific ELISA based assay.

Conclusions: Taken together, the data presented here identifies a novel mechanism of NF-κB inhibition by Delphinidin by inhibiting NIK, a kinase upstream of IKK complex in the NF-κB activation pathway. Given the important role played by IL-1β and NF-κB in OA, these results may provide important clues to develop useful pharmacological inhibitors of NF-κB for the prevention and/or treatment of OA.

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REDIFFERENTIATION OF HUMAN ARTICULAR CHONDROCYTES IN 2D VERSUS 3D CULTURE

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Purpose: For cartilage regenerative techniques human articular chondrocytes (HACs) are cultured *in vitro*. Unfortunately, long expansion time and multiple passaging lead to 'dedifferentiation'. To overcome dedifferentiation, chondrocytes are 'redifferentiated' in 3D-culturemodels (pellets, alginate cultures etc.). However, 2D (monolayer) culture methods are less laborious and have better reproducibility. We therefore set out to compare redifferentiation characteristics of HACs in 2D and 3D systems

Methods: HACs were expanded in monolayer from 15 independent donors. Redifferenitiation in monolayer was performed at subconfluency. Identical medium conditions were used to redifferentiate cells in pellets or alginate beads. After 7 days chondrogenic marker-expression was analyzed.

Results: Monolayer redifferentiation did not support expression of Col2a1 and Sox9 mRNAs, whereas chondrocyte hypertrophy markers Col10A1, Runx2 and ALP were significantly increased. Col2a1 and Sox9 mRNA expression in HACs redifferentiated in pellets or alginate beads increased significantly, as did Col10a1 and RunX2. Suggesting that redifferentiation in monolayer does not fully support a chondrogenic phenotype. However, when corresponding protein samples were analyzed we detected a profound upregulation of Col2A1 and Sox9 protein expression in monolayer samples which was quantitatively equal to samples from 3D cultures. Upregulation of RunX2 and Col10A1 protein expression was also found in both culture systems, with the monolayer cultures inducing these markers to higher levels than 3D cultures.

Conclusions: For the first time redifferentiation capacity of HACs was compared between 2D and 3D culture techniques by gene expression as well as by protein expression. Although gene expression analyses indicate that monolayer does not support chondrogenic redifferentiation, protein expression analysis proved the opposite. Besides a fundamentally interesting contradictory observation, our data show that redifferentiation of HACs is possible in monolayer but behaves differently on the gene expression as compared to 3D culture systems. Monolayer provides a reproducible and low cell amount-demanding culturesystem better suited for molecular and genetic interference studies into chondrocyte biology.

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IMPAIRMENT OF MITOCHONDRIAL 8-OXOGUANINE DNA GLYCOSYLASE (OGG1) AGAINST ACCUMULATION OF 8-OXOGUANINE IN OSTEOARTIRITIC CHONDROCYTES.

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Purpose: It is well known that chondrocytes produce excess amounts of reactive oxygen species (ROS) as well as proinflammatory cytokines and chemokines in response to mechanical and chemical stresses. An oxidized form of guanine, 8-oxo-7,8-dihydroxyguanine (8-oxoguanine) is a major causative lesion for mutagenesis by ROS, since it can form a stable base pair with adenine as well as with cytosine during DNA replication. 8-