of up to 112 μm. Histological features of the subchondral bone resorption pits were analysed with H&E staining. Tartrate resistant acid phosphatase (TRAP) positive cells were identified by immunohistochemistry. Normal cartilage and subchondral bone were obtained from femoral heads at post mortem from cadavers with no clinical history of OA (n = 9).

**Results:** Resorption pits were divided into five groups which were classified by morphological phenotype based on the content of cells present within the pit and the location of the pit within the subchondral/calcified cartilage space. All resorption pits examined contained TRAP positive osteoclast-like cells indicating the destructive nature of these lesions. The concurrent presence of osteoblasts and osteoclasts within the resorption pits suggests the involvement of the resorption pits in the process of bone remodelling and possibly sclerosis. Histological analysis of samples suggested that resorption pits may arise from the normal pit structure that is seen within the subchondral plate and may extend through a process of bone resorption and remodelling, to involve a larger area of the subchondral region and encroach on overlying calcified and articular cartilage. 3D reconstruction of subchondral bone resorption pits demonstrated a branching morphology with smaller pits interlinking with a larger resorption pit which linked with bone marrow. This branching morphology suggesting possible development of resorption pits through pre-existing vascular channels or the interaction of bridging areas of bone resorption.

**Conclusions:** Subchondral bone resorption pits can be classified into various types based on their morphology; these separate classifications may represent a development sequence from focal (normal) areas of endochondral ossification or expansion of bone vascular channels. The extensive nature of these lesions may also be a reflection of altered biomechanical stresses on the subchondral plate and may, in turn, contribute to altered bone responses and joint instability in OA.

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**Cartilage/Chondrocyte Biology**

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**ALK1 OPPOSES ALK5/SMA3 SIGNALING AND EXPRESSION OF EXTRACELLULAR MATRIX COMPONENTS IN HUMAN CHONDROCYTES**

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**Purpose:** Transforming growth factor-β (TGF-β) is a multifunctional regulator of chondrocyte proliferation, differentiation and extracellular matrix production. Dysregulation of TGF-β action has been implicated in cartilage diseases such as osteoarthritis. TGF-β signaling is transduced through a pair of transmembrane serine/threonine kinases, known as the type I (ALK5) and type II (TβRII) receptors. Activation of ALK5 leads to phosphorylation of intracellular Smad2 and Smad3, which associate with the common Smad4 and then translocate to the nucleus where they interact with coactivators and/or corepressors to modulate expression of target genes in a cell-specific manner. However, recent studies on endothelial cells have identified another TGF-β receptor system in human chondrocytes, as evidenced by its interaction with ALK5, TβRII, endoglin and betaglycan in the presence or absence of TGF-β ligand. Our results indicate that both ALK1 and ALK5 are required for TGF-β-induced phosphorylation of Smad1/5 whereas only ALK5 is essential for TGF-β-induced phosphorylation of Smad3 in these cells. In addition, our results demonstrate that ALK1 inhibits, while ALK5 potentiates, TGF-β-induced Smad3-driven transcriptional activity and the expression of PAI-1, fibropectin and type II collagen.

**Conclusions:** Our results suggest that, in human chondrocytes, TGF-β signals through two TGF-β type I receptors: ALK1 and ALK5. Activation of the ALK5 pathway leads to activation of the Smad3 pathway and increased expression of extracellular matrix components, PAI-1, fibropectin and type II collagen, whereas activation of the ALK1 pathway opposes these functions. Our study suggests that ALK1 plays an essential role in the regulation of TGF-β signaling and function in the human cartilage.

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**CPII, A C-TERMINAL PEPTIDE FROM PROCOLLAGEN TYPE II, ENHANCES HTRA1 ACTIVITY AND CARTILAGE PROTEOGLYCAN TURNOVER**


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**Purpose:** The serine protease HtrA1 (also referred to as PRSS11) has been shown to be one of the most abundant proteases in cartilage, wherein its levels are upregulated during osteoarthritis (OA). A hexapeptide derived from the C-terminus of procollagen type II (and type III), designated CPII, has previously been shown to stimulate HtrA1 protease activity by tightly interacting with its PDZ domain. The purpose of this study was to investigate HtrA1-mediated cleavage of aggrecan and the agonistic effect of CPII on HtrA1 activity, and to better understand the role of HtrA1 in OA disease progression.

**Methods:** Identification of potential HtrA1 substrates: Wildtype HtrA1 (amino acids 157-480) and an inactive HtrA1 mutant (S328A) were overexpressed in 3-D alginate cultures of primary human chondrocytes. A mass spectrometry-based method was utilized to identify potential HtrA1-generated cleavage products released into the culture medium. In vitro cleavage of aggrecan: Purified human aggrecan and recombinant aggrecan constructs comprising either the G1-interglobular domain (IGD)-G2 or IGD alone were incubated with purified recombinant HtrA1 (aa 157-480) or an inactive HtrA1 mutant (aa 157-480, S328A). A control peptide or CPII was added to the reactions to evaluate potential
agonistic effects. The reaction products were separated by SDS-PAGE, followed by Coomassie Blue or silver staining. Treatment of cartilage explants with HtrA1: Human osteoarthritic cartilage explants were treated with recombinant HtrA1, with or without CPII. Conditioned media were assayed for sGAG release by DMMB. Proteoglycans in the media were precipitated using 1% cetylpyridinium chloride and subjected to SDS-PAGE followed by Coomassie Blue or silver staining.

Results: The cartilage proteoglycan aggrecan was identified as one of the potential substrates of HtrA1 in the mass spectrometry-based “degradomics” analysis. Incubation of recombinant aggrecan G1-IGD-G2 and IGD constructs with wild-type HtrA1, but not mutant HtrA1, resulted in distinct cleavage of these substrates. HtrA1 activity was further enhanced by the peptide agonist CPII, and inhibited by the HtrA inhibitor Ucl-101. In addition, recombinant HtrA1 cleaved native human aggrecan in the presence of the CPII peptide agonist. Treatment of cartilage explants with recombinant HtrA1 significantly increased (p<0.05) the amount of sGAG released compared to control. Further, the addition of CPII significantly increased (p<0.05) the amount of sGAG release compared to treatment with HtrA1 alone.

Conclusions: Our data suggest that the collagen type II C-propeptide may induce proteoglycan catabolism by stimulating the protease activity of HtrA1. Elevated levels of collagen type II C-propeptide have been detected in osteoarthritic human articular cartilage, due to increased collagen synthesis. Excessive HtrA1 protease activity in OA cartilage may represent another contributing factor in OA disease progression.

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CRUCIAL ROLE OF VISFATIN/PBEF IN MATRIX DEGRADATION AND PGE2 SYNTHESIS IN CHONDROCYTES: POSSIBLE INFLUENCE ON OSTEOARTHRITIS

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Purpose: To evaluate the contribution of visfatin, an adipose tissue-derived hormone, to the pathophysiology of osteoarthritis (OA) by examining its role in prostaglandin E2 (PGE2) synthesis and matrix degradation. In an inflammatory context, PGE2 synthesis is catalysed by cyclooxygenase type 2 (COX-2) and microsomal prostaglandin E synthase type 1 (mPGES-1), whereas NAD+-dependent 15-hydroxy prostaglandin dehydrogenase (15-PGDH) degrades PGE2.

Methods: The synthesis of visfatin by human chondrocytes from OA patients, with and without stimulation with interleukin-1β (IL-1β–10 ng/ml), was assessed by real-time RT-PCR and immunoblotting. The effects of visfatin (1 to 10 ng/ml) on mPGES-1, 15-PGDH, PGE2, MMP-3 and MMP-13 expressions by human OA chondrocytes and by primary mouse articular chondrocytes were examined by quantitative RT-PCR, immunoblotting and ELISA. A siRNA strategy was used to assess the influence of visfatin on the IL-1β induced release of PGE2. Finally, the role of IGF-1R in visfatin signalling was studied using primary chondrocytes from IGF-1R knockout mice (IGF-1R−/−).

Results: (1) Visfatin was constitutively expressed by cultured human OA chondrocytes. Its expression increased 6-fold in response to 10 ng/ml IL-1β (p<0.05).
(2) Visfatin at 1 to 5 μg/ml triggered MMP-3 and MMP-13 mRNA expression (up to 6-fold, p<0.01) by primary mouse articular chondrocytes. Stimulation with 5 μg/ml visfatin led to a release of 572 ± 280 ng/ml MMP-3 protein (p<0.05). Visfatin also induced i) PGE2 release (controls 47 ± 8 versus 141 ± 10 pg/ml when treated with 10 μg/ml visfatin, p<0.05), ii increased expression of the mPGES-1 (14-fold increase, p<0.01) and iii) a 90% decrease (p<0.05) of the 15-PGDH. Interestingly, 1 ng/ml IL-1β plus visfatin (1, 2.5 or 5 μg/ml for 24 hours) had additive effects on PGE2 release (19-fold, 31-fold and 35-fold compared to IL-1β, p<0.05; [1 ng/ml IL-1β released 1506 ± 67 pg/ml]). Moreover, IL-1β dramatically decreased 15-PGDH expression by 95% (p<0.001).
(3) Blocking visfatin expression by siRNA inhibited IL-1β-induced PGE2 release: triggered the release of (1430 ± 467 pg/ml in presence of IL-1β (10ng/ml) versus 985 ± 292 pg/ml in presence of IL-1β + siRNA visfatin, -35%, p<0.01) probably due to a 40% inhibition of mPGES-1 expression (p<0.01). (4) Visfatin is known to bind to, and to activate insulin receptor (IR). However, IR is not considered to be usually present on chondrocytes. We therefore tested the implication of IGF-1R, a close homologue to IR, in visfatin signalling. When stimulated with 5 μg/ml visfatin, IGF-1R−/− chondrocytes unexpectedly exhibited higher PGE2 release than IGF1R+/+ controls (228 ± 4 compared to 86 ± 29 pg/ml, p<0.05) which rules out the direct implication of IGF-1R in visfatin action.

Conclusions: Visfatin triggers the synthesis and the release of MMP-3 and MMP-13 and induces PGE2 synthesis resulting from an increase of mPGES-1 and a decrease of 15-PGDH expression in chondrocytes. We therefore consider that visfatin is a novel and a potential critical target for OA. In vivo experiments are now needed to test this hypothesis.

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AN EXPERIMENTAL MODEL TO STUDY THE MECHANISMS OF EPIGENETIC DNA DE-METHYLATION OBSERVED IN HUMAN OSTEOARTHRITIS

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Purpose: Previous studies (Arthritis Rheum 52:3110-24) showed that DNA de-methylation at specific CpG sites in the promoters was associated with the abnormal synthesis of matrix-degrading enzymes in human osteoarthritis. However, it is not known whether DNA de-methylation actually causes the abnormal expression of the proteases. To demonstrate possible cause-effect relationships and to study the mechanisms involved in the loss of DNA methylation requires an in vitro system in which experimentally induced gene induction is correlated with de-methylation at specific CpG sites.

Methods: Since monolayer cultures of articular chondrocytes are an established model to study the induction of the typical OA proteases by inflammatory cytokines, we used this system. Healthy chondrocytes were harvested from human femoral head cartilage after hemi-arthroplasty following a fracture of the neck of femur. The chondrocytes from each patient were divided into five groups: non-culture; control culture; culture with the de-methylating agent 5-aza-deoxycytidine (5-aza-DC) or the inflammatory cytokines IL-1β or TNF-α/oncostatin M. At confluency (4-5 weeks), total RNA and genomic DNA were extracted simultaneously. Relative mRNA expression was quantified by SybrGreen-based real-time PCR and a method for quantifying the percent of cells with DNA methylation at one specific CpG site was developed (Epigenetics 2: 86-95). ELISA was used to analyze IL-1β in the culture.

Results: Initial non-quantitative experiments confirmed IL-1β - induced expression of MMP-3 and MMP-13 and also demonstrated induction of IL-1β by itself, which correlated with loss of