

EXTENDED REPORT

Tenascin and aggrecan expression by articular chondrocytes is influenced by interleukin 1 β : a possible explanation for the changes in matrix synthesis during osteoarthritis

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Objective: To analyse the distribution patterns of tenascin and proteoglycans in normal and osteoarthritic cartilage, and to determine the effect of interleukin 1 β (IL1 β) on aggrecan and tenascin expression by human articular chondrocytes in vitro.

Methods: Normal and osteoarthritic cartilage and bone samples were obtained during total knee replacements or necropsies. After fixation and decalcification, paraffin embedded specimens were sectioned perpendicular to the surface. Specimens were graded according to Mankin and subdivided into those with normal, and mild, moderate, and severe osteoarthritic lesions. Serial sections were immunostained for tenascin. Tenascin expression by healthy and osteoarthritic chondrocytes was quantified by real time polymerase chain reaction (PCR). Furthermore, in cell culture experiments, human articular chondrocytes were treated with 0.1 or 10 ng/ml IL1 β . Real time PCR analyses of aggrecan and tenascin transcripts (normalised 18S rRNA) were conducted to determine the effect of IL1 β on later mRNA levels.

Results: Tenascin was immunodetected in normal and osteoarthritic cartilage. In osteoarthritic cartilage increased tenascin staining was found. Tenascin was found specifically in upper OA cartilage showing a strong reduction of proteoglycans. Greatly increased tenascin transcript levels were detected in osteoarthritic cartilage compared with healthy articular cartilage. IL1 β treatment of articular chondrocytes in vitro significantly increased tenascin transcripts (~200% of control) and strongly reduced aggrecan mRNA levels (~42% of control).

Conclusions: During progression of osteoarthritis the switch in matrix synthesis occurs mainly in upper osteoarthritic cartilage. Furthermore, changes in synthesis patterns of osteoarthritic chondrocytes may be significantly influenced by IL1 β , probably diffusing from the joint cavity within the upper osteoarthritic cartilage.

Osteoarthritis (OA) a degenerative disease of articular cartilage is accompanied by typical changes of the periarticular bone (osteophytes, sclerosis) and inflammatory reactions of the synovial membrane.¹ Specifically, fibrillations and fissuring of the surface, progressive loss of proteoglycans, formation of chondrocyte clusters, and in late stage OA decreased cell density are signs of the progressive damage of hyaline cartilage. It has been repeatedly shown that disease progression depends on the severity of the cartilage damage. Therefore, matrix synthesis by articular chondrocytes is a critical factor in the maintenance of cartilage integrity in healthy joints. Many efforts have been devoted to characterising the different matrix compositions under normal and pathological conditions such as OA.^{2–5} Evidence has shown that articular chondrocytes change their synthesis pattern, resulting in an insufficient matrix unable to absorb and respond to mechanical load.⁶

In our previous studies we showed an increased deposition of tenascin, collagen type I, and fibronectin in upper cartilage during experimental OA in rabbits.⁷ Tenascin is a large glycoprotein composed of six subunits. Each tenascin subunit consists of an epidermal growth factor-like repeat, a fibronectin (Fn) type III repeat, and a fibrinogen sequence.⁸ On the other hand, it has been shown that distinct matrix proteins—for example thrombospondin and aggrecan, are decreased in OA, and it is reasonable to assume that the

unique biomechanical properties of OA cartilage are weakened.^{9–10} Furthermore, it has been shown that proinflammatory cytokines such as interleukin 1 β (IL1 β), tumour necrosis factor α and others are secreted by synoviocytes into the joint cavity.^{11–12} In healthy joints IL1 β is counteracted by IL1 receptor antagonist, whereas in OA joints this balance is disturbed and IL1 β initiates different catabolic pathways by activating the transcription factor NF κ B and several signal cascades.^{13–15}

Beside its effects on apoptosis, inducible nitric oxide synthase expression, and matrix metalloproteinases expression, IL1 β has significant effects on matrix molecule expression by articular chondrocytes (for review see Martel-Pelletier *et al*¹⁵). IL1 β decreases type II collagen and aggrecan expression.^{16–17} As mentioned above osteoarthritic chondrocytes are considered to change their synthesis pattern, thereby down regulating aggrecan expression and enhancing the expression of other matrix molecules.^{2–6 18 19} This phenomenon could be explained by intrinsic mechanisms such as differentiation or de-differentiation processes of chondro-

Abbreviations: HBSS, Hanks's buffered salt solution; IL, interleukin; OA, osteoarthritis; PBS, phosphate buffered saline; PCR, polymerase chain reaction

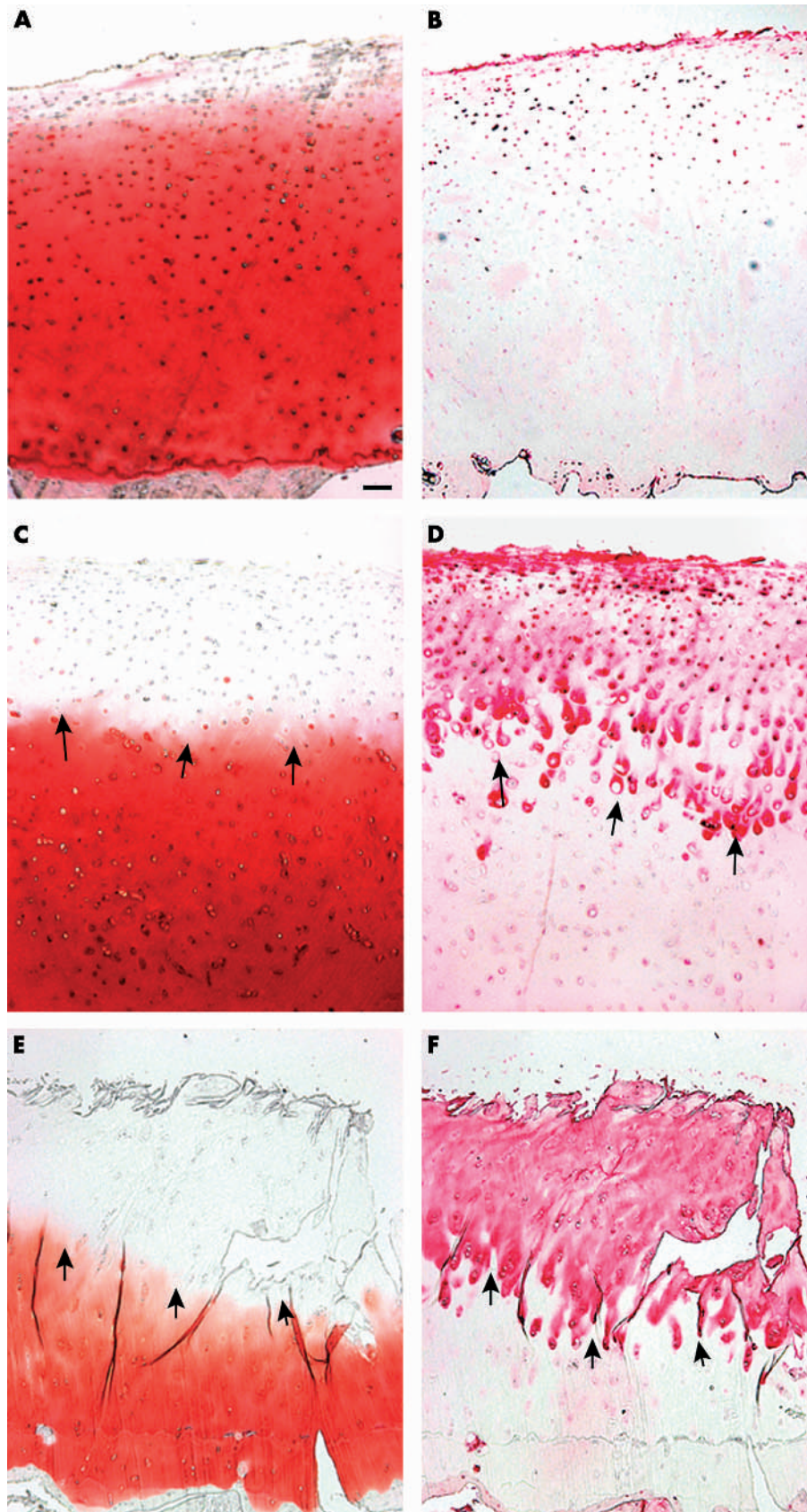


Figure 1 Serial sections of normal and OA cartilage. The tenascin deposition in healthy cartilage varied interindividually. Normal cartilage stained for proteoglycans (A) and immunostained for tenascin (B) shows pericellular/territorial tenascin deposition around the superficial and upper middle zone chondrocytes (weak immunostaining). (C) Mild OA lesion with decreased proteoglycan staining of the upper cartilage (safranin O staining). (D) Immunohistochemical analysis of tenascin in the same section as shown in C (note the territorial staining around the middle zone chondrocytes indicated by arrows). (E) Severe OA cartilage lesion showing a strong reduction of proteoglycans, deep clefs within the deep zone, and chondrocyte clusters in all zones (safranin O staining). (F) Immunostaining of tenascin in the same section as shown in E (note the strong tenascin staining in cartilage areas showing the absence of proteoglycans indicated by arrows). The bar in A represents 100 μ m in A-F.

Table 1 Sequences of primers and probes

	Forward	Reverse	Probe
Tenascin	ATCCGGACCAAAACCATCAG	AATGTCGGAATGGTTAGGTTTC	CGACAGAGGCCCTGCCCTTC
Aggrecan	ACTTCCGCTGGTCAGATGGA	TCTCGTCCAGATCATCACC	CCATGCAATTTGAGAACTGGCGCC

cytes, as well as by extrinsic factors, for example, proinflammatory cytokines or mechanical load.²⁰

Two previous studies have shown that tenascin is present in human normal and OA cartilage.^{21, 22} We have additionally demonstrated that beside tenascin other matrix components such as fibronectin and type I collagen are deposited within the upper OA cartilage of rabbits. In view of these observations, we suggested that a common mediator controlled pathway may induce these changes in matrix synthesis by OA chondrocytes.⁷ However, it is still unclear whether IL1 β is responsible for the changes in matrix gene expression which probably weaken the unique biomechanical properties of articular cartilage. To test this hypothesis we determined the presence and distribution of tenascin compared with proteoglycans in normal and OA cartilage. Furthermore, cell culture experiments with human articular chondrocytes, cultured in the absence and presence of IL1 β , were conducted. The effect of IL1 β on aggrecan and tenascin mRNA expression levels was determined by real time polymerase chain reaction (PCR).

MATERIALS AND METHODS

Patients and samples

Cartilage and bone samples were obtained from patients who had been admitted to hospital in the Division of Orthopaedic Rheumatology, University Erlangen-Nuremberg, Germany. The following clinical data had been obtained: age, sex, patient history, physical examination, haematological analysis (complete blood count, rheumatoid factors, erythrocyte sedimentation rate, serum electrophoresis, C reactive protein, and other serum diagnostic tests), and x ray examinations of the respective knee joints carried out. Data were carefully reviewed to exclude any secondary forms of OA and rheumatoid arthritis. Thirty one human cartilage samples were obtained from 29 patients undergoing total knee replacement. Nine human normal cartilage samples were obtained from five donors during necropsies and from one patient undergoing a reconstruction of the anterior cruciate ligament. The localisation of the excised samples was carefully documented by videoprints. The specimens were immediately fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), decalcified in diethyl-pyrocabonate treated 0.2 M EDTA, embedded in paraffin wax, and then cut into 6 μ m thick sections perpendicular to the surface. Serial sections were stained with safranin O and used for immunohistochemistry.

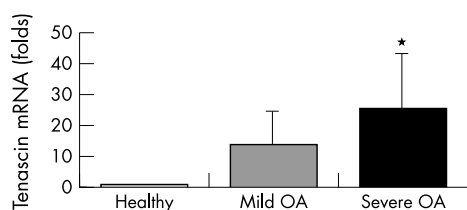


Figure 2 Bars represent the several-fold changes of tenascin mRNA levels (normalised to 18S rRNA) in mild and severe OA lesions compared with healthy cartilage samples (one fold expression). Bars are means (SD). * $p < 0.05$ versus healthy samples.

Histological-histochemical grading

Sections of normal and OA cartilage specimens routinely stained with safranin O were graded according to Mankin *et al* by two different observers.²³ The samples were classified as normal as well as mild, moderate, and severe OA lesions as described previously⁹: Mankin score 0–1, normal cartilage (9 samples); 2–5, mild OA lesion (8 samples); 6–9, moderate OA lesion (12 samples); ≥ 10 , severe OA lesion (11 samples).

Immunohistochemistry

After deparaffinisation, the sections were incubated with 2 mg/ml hyaluronidase (Merck, Darmstadt, Germany) in PBS (pH 5.5) for 15 minutes, followed by digestion with 1 mg/ml pronase in PBS (pH 7.5) for 30 minutes (Boehringer, Mannheim, Germany). Non-specific binding of the antibodies was blocked with 5% bovine serum albumin in PBS. Each step was followed by extensive washing in PBS. Sections were then incubated overnight at 4°C, with a human tenascin-specific monoclonal antibody (Sigma, Munich, Germany) diluted 1:1000. Negative control cartilage sections were incubated with mouse control isotypes. After incubation with the first antibody each step was followed by extensive washing in Tris buffered saline. This was then followed by incubation with a biotinylated antimouse IgG from rabbits, diluted 1:500. Then, a complex of streptavidin and biotin labelled with alkaline phosphatase was added according to the manufacturer's protocol (Dako, Hamburg, Germany). Finally the sections were coloured with Fast Red (Sigma, Munich, Germany).

Sample acquisition and RNA preparation

Eight samples of OA cartilage were obtained from eight patients who had undergone total knee replacement. Additionally, three normal samples were obtained at necropsy from patients who had had no known history of joint disease. The cartilage areas for RNA isolation were macroscopically graded as four mild (softening, fibrillations) and four severe (softening, yellowish colour, fissuring down to the bone or calcified cartilage) OA lesions and documented by videoprints. Cartilage samples were rapidly dissected and stored until preparation at -70°C . The frozen tissue was shredded under liquid nitrogen and total RNA was isolated from cartilage with the Nucleo-Spin-RNA-II-Kit (Clontech

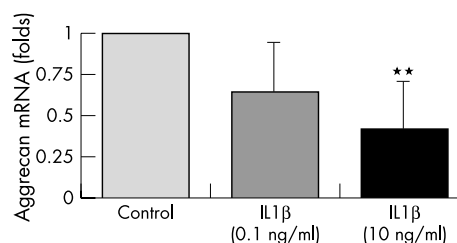


Figure 3 Bars represent the several-fold changes of aggrecan mRNA levels (normalised to 18S rRNA) of IL1 β treated chondrocytes compared with untreated control chondrocytes (one fold expression). Articular chondrocytes were cultured under serum free conditions for 18 hours in the absence or presence of 0.1 and 10 ng/ml IL1 β , respectively. Results are from four independent cell culture experiments. Bars represent means (SD). ** $p < 0.01$ versus control.

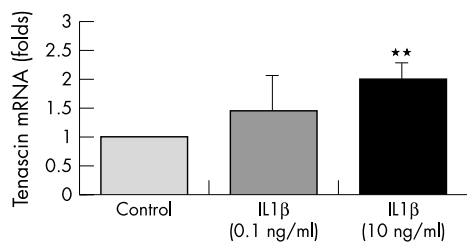


Figure 4 Bars represent the several-fold changes of tenascin mRNA levels (normalised to 18S rRNA) of IL1 β treated chondrocytes compared with untreated control chondrocytes (one fold expression). Articular chondrocytes were cultured under serum free conditions for 18 hours in the absence or presence of 0.1 and 10 ng/ml IL1 β , respectively. Results are from four independent experiments. Bars represent means (SD). ** $p < 0.01$ versus control.

Laboratories, Palo Alto, USA) as recommended by the manufacturer. Total RNA yields were calculated by measurement of the extinction at 260 nm (UltraspecIII, LKB).

Reverse transcription and real time PCR

After isolation, RNA was transcribed into single complementary DNA using AMV reverse transcriptase (Boehringer, Mannheim, Germany) and random hexamer primers according to the manufacturer's instructions.

For real time PCR analyses cDNAs from different cartilage samples or four independent cell culture experiments were diluted to a final concentration of 10 ng/ μ l. For PCR reactions TaqMan-Universal Mastermix (Applied Biosystems) was used. 50 ng cDNA was used as template to determine the relative amount of mRNA by real time PCR (ABI Prism 7700 sequence detection system) using specific primers and probes (table 1) (Qiagen Operon, Cologne, Germany). The reaction was conducted as follows: 95°C for 4 minutes, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. 18S rRNA was amplified as an internal control. Cycle threshold values were measured and calculated by the sequence detector software. Relative amounts of mRNA were normalised to 18S rRNA (Applied Biosystems) and calculated with the software program Microsoft Excel. Relative mRNA contents were calculated as $x = 2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = \Delta E - \Delta C$ and $\Delta E = Ct_{\text{sample}} - Ct_{18S}$ and $\Delta C = Ct_{\text{control/normal}} - Ct_{18S}$. For statistical analyses the Mann-Whitney U test was used.

Chondrocyte culture

Human articular chondrocytes were isolated from cartilage obtained during knee replacements with two digestion steps. Firstly, articular cartilage was digested in Hanks's buffered salt solution (HBSS) containing pronase 2 mg/ml (30 minutes at 37°C) followed by a collagenase 180 U/ml in HBSS (6 hours at 37°C). After quantification of isolated chondrocytes with a haemocytometer, two million chondrocytes were plated onto 5 cm culture dishes in Dulbecco's modified Eagle's medium containing 10% bovine fetal serum, 2 mM glutamine, and 50 U/ml streptomycin/penicillin. Chondrocytes were cultured until they reached confluency (day 7 after plating). Cells were then treated with either 0.1 or 10 ng/ml IL1 β under serum free conditions. After 18 hours, total RNA was harvested with the Nucleo-Spin-RNA-II-Kit (Clontech Laboratories, Palo Alto, USA) as recommended by the manufacturer.

RESULTS

In the first set of our experiments we analysed the tenascin distribution in human normal and OA cartilage samples. In normal cartilage showing a smooth surface and homogeneous proteoglycan staining, tenascin was present in the

pericellular territorial matrix of the superficial and middle zone chondrocytes and on the surface lining. In a few specimens a slight interterritorial matrix staining in the deep zone was even seen. Further, osteocytes and osteoblasts of the subchondral bone showed tenascin expression. The intensity of the tenascin staining in healthy cartilage varied between specimens from different donors (figs 1A and B and figs W1A and B (available at <http://www.annrheumdis.com/supplemental>)). Control slides incubated with control isotypes showed no staining (not shown).

In mild and moderate OA lesions decreased proteoglycan staining next to the cartilage surface was accompanied by increased territorial and interterritorial tenascin deposition. In addition, we detected the highest tenascin depositions specifically in those cartilage areas showing the greatest reduction of safranin O staining (figs 1C and D and figs W1E and F (<http://www.annrheumdis.com/supplemental>)). In the middle and upper deep zones some specimens showed a territorial tenascin deposition (figs 1C and D). Pannus-like tissue covering OA cartilage showed a very strong immunostaining for tenascin (figs W1C and D (<http://www.annrheumdis.com/supplemental>)). Even in the surrounding matrix of chondrocyte clusters a strong tenascin staining was determined.

In OA specimens with high Mankin's grade (severe OA lesion) strongly increased immunostaining of tenascin was found (figs 1E and F). As described previously, the highest tenascin depositions were immunodetected specifically in upper OA cartilage characterised by great reduction of proteoglycans (figs W1G and H (<http://www.annrheumdis.com/supplemental>)). Significantly higher transcript levels of tenascin were detected in severe OA cartilage lesions than in healthy articular cartilage by real time PCR (normalised to 18S rRNA) (fig 2).

In a previous study we detected increased protein levels of tenascin, fibronectin, and type I collagen next to the surface of rabbit OA cartilage.⁷ Thus we suggested that a common mediator controlled pathway is responsible for the changes in chondrocytic synthesis pattern next to the joint space. In addition, Aigner *et al* showed that OA chondrocytes next to the cartilage surface reduce the expression of aggrecan.¹⁰ On the other hand, we and other groups have demonstrated that tenascin and other molecules are up regulated in OA. To test the hypothesis that IL1 β one of the most prominent mediators in OA, diffusing into upper OA cartilage, is responsible for at least some of the changes in expression pattern, cell culture experiments were conducted. Therefore OA chondrocytes were isolated and cultured in monolayer. After the cells reached confluency, chondrocytes were treated with two different concentrations of IL1 β under serum free conditions. After 18 hours total RNA was harvested and aggrecan and tenascin transcripts were quantified by real time PCR (normalised to 18S rRNA). Figures 3 and 4 show the results of four culture series. As shown, we determined a strong stimulation of tenascin mRNA expression (~200% of control) by IL1 β , whereas the aggrecan transcript levels (~42% of control) were significantly reduced, further supporting the idea that IL1 β is responsible for changes in matrix synthesis during OA.

DISCUSSION

In this study we have shown that changes of matrix gene expression in OA cartilage are strongly influenced by IL1 β . Firstly, we analysed the protein expression pattern of tenascin in healthy and OA cartilage. In healthy articular cartilage different staining intensities of tenascin were seen in the superficial zone and in the upper middle zone, mainly in the territorial matrix of the articular chondrocytes, confirming the results of Salter.²¹ In pannus-like tissue

covering the surface of OA cartilage, strong tenascin staining was detected. In moderate and severe OA lesions increased territorial and interterritorial immunostaining was determined, mainly in cartilage areas showing a great reduction of safranin O staining. Our previous observations on rabbit OA cartilage and these extended findings further strengthen the idea of a common mediator controlled pathway responsible for the observed changes in cartilage matrix composition. Several groups have provided evidence that IL1 β is one of the most dominant cytokines in OA, stimulating different catabolic pathways by activating the transcription factor NF κ B and several other signal cascades as well.¹⁵ Interestingly, it has been repeatedly shown that IL1 β inhibits aggrecan expression and stimulates expression of matrix metalloproteinases and aggrecanase, thereby contributing to the pathogenesis of OA.¹⁵ Furthermore, IL1 β has been shown to accumulate in upper OA cartilage, thus eventually influencing the synthesis pattern of articular chondrocytes.¹³ A previous study on stromal cells has demonstrated that IL1 stimulates the expression of tenascin on RNA, and the protein level.²⁴ Taken together, these findings suggest that IL1 β could be a key mediator in the regulation of chondrocytic synthesis pattern. To prove the hypothesis that chondrocytic matrix molecule expression is modulated by IL1 β , cell culture experiments were conducted. Consistent with this notion, real time PCR analyses disclosed that the action of IL1 β significantly reduced aggrecan mRNA levels. Beside its inhibiting effects on aggrecan expression, tenascin message was significantly stimulated by addition of IL1 β to the chondrocyte cultures. Our findings are in accordance with a previous study, which demonstrated that IL1 β enhances the immunohistological staining of tenascin in cartilage explants.²⁵ In addition, we and other groups found significantly increased tenascin transcripts in OA cartilage, further indicating the significance of changes in chondrocytic expression patterns in vivo.²⁶ However, we cannot rule out the possibility that chondrocytic expression patterns are influenced by other important mechanisms, such as mechanical load, oxygenation levels, growth factors, and differentiation processes.^{27, 28} None the less, our findings and those of others suggest a crucial role for IL1 β in the observed switch of matrix gene expression during disease progression, thereby contributing to the cartilage changes seen in OA.

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