Cytokine induced metalloproteinase expression and activity does not correlate with focal susceptibility of articular cartilage to degeneration

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Summary

Objective: To determine whether the focal susceptibility to cartilage degeneration in joints is related to a differential response to cytokine stimulation.

Methods: Compare aggrecan and collagen catabolism in in-vitro models of cartilage degradation induced by retinoic acid (RA), interleukin-1 (IL-1), tumor necrosis factor alpha (TNF) and IL-1 plus oncostatin M (OSM). Glycosaminoglycan (GAG) and hydroxyproline (HyPro) quantification and Western immunoblot analyses of aggrecan and collagen degradation products were undertaken in explant cultures of normal cartilage from regions of equine joints with a known high and low susceptibility to degeneration in disease. RNA isolation and semi quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis were performed to determine the expression of aggrecanases, matrix metalloproteinases (MMPs) and their inhibitors.

Results: Although the rate of basal cartilage aggrecan turnover was dependent on joint region there was no difference in the response of different cartilages to cytokines. Individual animals did show a significant difference in the response of certain cartilages to cytokines, with both decreased and increased aggrecan loss in cartilage with a low susceptibility to degeneration. Aggrecan release in both short- and long-term cultures from all cartilages was associated with increased cleavage by aggrecanases rather than MMPs. There was a poor correlation between expression of aggrecanases, MMPs or their inhibitors and cytokine induced aggrecan catabolism. IL-1 alone was able to stimulate collagen breakdown in equine articular cartilage and surprisingly, significantly more collagen loss was induced in cartilage from regions less susceptible to degeneration.

Conclusions: Collectively, these studies suggest that a regional difference in response to catabolic cytokines is unlikely to be a factor in the initiation of focal cartilage degeneration in osteoarthritis (OA).

Key words: Aggrecanase, Matrix metalloproteinases, Cartilage catabolism.

Abbreviations: MMPmatrix metalloproteinase., IL-1interleukin-1., OSMoncostatin M., GAGglycosaminoglycan., IGDinterglobular domain of the aggrecan core protein., DMMBdimethylmethylen blue., MAbmonoclonal antibody., ADAMa family of proteinases containing A Disintegrin region And a Metalloproteinase domain., ADAMTSADAM proteinases containing one or more thrombospondin type-1 repeats.

Introduction

Articular cartilage degeneration and erosion are hallmarks of both degenerative and inflammatory joint diseases. In the inflammatory arthritides, it is well accepted that cytokines such as interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF) are key mediators of the disease pathophysiology. There is extensive evidence from in vitro studies, that both IL-1 and TNF can stimulate the synthesis and activation of proteolytic enzymes by chondrocytes, resulting in the catabolism of the principal extracellular matrix components of cartilage, aggrecan and type II collagen (reviewed in Ref.¹). Although similar proteolytic events occur in the cartilage of both the degenerative arthropathies such as osteoarthritis (OA) and the inflammatory diseases such as rheumatoid arthritis, the role of inflammatory cytokines in OA is less clear. IL-1 and TNF have been detected in OA synovial fluid but the levels are lower than those in rheumatoid arthritis²–⁴. Nevertheless, synovium from OA joints does secrete significantly more TNF and IL-1...
**Osteoarthritis and Cartilage Vol. 13, No. 2**

in vitro when compared with normal joints\(^5\) and more chondrocytes in the superficial zones of OA cartilage are immuno-positive for these two cytokines\(^6\). Finally, chondrocytes from OA cartilage express more p55 TNF receptor and type I IL-1 receptor than cells from normal cartilage and as a consequence are more responsive to these catabolic cytokines\(^7,8\).

Despite the evidence implicating catabolic cytokines in the pathogenesis of OA, it is difficult to reconcile with the distinct topographical distribution of cartilage degeneration. The role of biomechanics in the initiation and development of cartilage damage in OA is still the subject of investigation and both overloading and underloading have been implicated (reviewed in Ref.\(^15\)). Nevertheless, the joint regions with the most advanced cartilage lesions in late stage OA are generally those exposed to the greatest load bearing, such as the superolateral femoral head and the medial femorotibial compartment\(^11,12\), implicating mechanical rather than humoral factors in disease pathogenesis. However, the chondrocytes from distinct joint regions have metabolic differences that are maintained in culture suggesting that they are phenotypically distinct cell populations\(^13\). It has been reported that a differential response to TNF by chondrocytes from topographically defined areas could contribute to the focal nature of cartilage degeneration in OA\(^6,14,15\). In these studies pathological cartilage from human knees was used and it is therefore unclear whether the different response was a cause or an effect of focal disease. The purpose of the present investigation was to examine what role differential cytokine response might play in the initiation of focal cartilage damage in joint disease. To this end, we compared normal equine cartilage from defined joint regions with a known low or high susceptibility to degeneration in disease\(^6,16–18\). The expression and activity of the matrix degrading metalloproteinase enzymes and their natural inhibitors was evaluated using in-vitro models of cartilage degradation. Collectively, these studies have demonstrated that the regional susceptibility of cartilage to degeneration in arthritic diseases was not associated with a differential response to cytokines in vitro.

**Materials and methods**

**IN-VITRO MODELS OF CARTILAGE DEGENERATION**

Full-depth articular cartilage was harvested from three areas of horses (2–12 years of age): high degeneration susceptibility regions of the distal metacarpus (MCP) and the dorsal rim of the third carpal bone (DR), and the low susceptibility palmar condyle of the third carpal bone (PC). The variable incidence of degenerative change, biomechanical loading and biochemistry of the DR and PC along with the location of these regions within the joint have been described previously\(^16–21\). The MCP was chosen to represent another cartilage region with a high incidence of degeneration (load-bearing region of the medial condyle) in a joint separate from the carpus to see if similarities existed between susceptible cartilages in different joints\(^22,23\). All joints were assessed visually to ensure that there was no gross evidence of joint disease such as osteophytes or cartilage fibrillation. Histological evaluation of selected cartilage specimens was also undertaken to ensure that there was no evidence of proteoglycan loss, chondrocyte cloning or surface fibrillation indicative of disease. Cartilage was pre-cultured for 3 days in Dulbecco’s Modified Eagles Medium (DMEM; Gibco BRL) + 10% foetal bovine serum, washed (3 × 5 min) and cultured in serum free DMEM.

Cartilage degradation models characterised by aggrecan loss but little or no collagen catabolism, were established using 4 day serum free cultures + 10–10\(^{-6}\) M retinoic acid (RA), 10 ng/ml IL-1 beta or 100 ng/ml TNF alpha\(^24\). To try and model cartilage degeneration characterised by type II collagen proteolysis, long-term (up to 28 days) serum free cultures + 10 ng/ml IL-1 beta or 1 ng/ml IL-1 beta plus 50 ng/ml OSM with media changed every 7 days were established\(^25\). Quadruplicate samples from each joint region of individual horses were cultured and the cartilage and media were harvested after 4 days (short-term culture) or 7, 14, 21 and 28 days (long-term culture). At the termination of culture, medium was frozen at −20°C until analysed and cartilage was blotted dry, weighed and extracted for 48 h at 4°C using 10 volumes of 4 M guanidinium hydrochloride (GuHCl), 0.05 M sodium acetate pH 6.8 containing the proteinase inhibitors 0.01 M EDTA, 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine HCl and 0.01 M N-ethylmaleimide. Extracts were dialysed for 16 h against 400 volumes of ultra-pure deionised water at 4°C and stored at −20°C until analysed further. The cartilage remaining after extraction was digested with papain\(^26\).

**QUANTITATION OF PROTEOGLYCAN AND COLLAGEN CATABOLISM**

The proteoglycan content in the medium, dialysed cartilage extracts and papain digests was measured as sulphated glycosaminoglycan (GAG) by colorimetric assay using dimethylmethylen blue (DMMB) and chondroitin sulphate-C from shark cartilage (Sigma) as a standard\(^27\). Collagen in the medium, extracts and papain digests was measured as hydroxyproline (HyPro) content\(^28\). Data were normalised by log transformation and differences in GAG or HyPro release in association with the catabolic agent used and joint region were studied by analysis of variance (ANOVA) and Bonferroni Dunn post hoc analysis. Data were analysed using the Stat View 4.02 package for Macintosh (Abacus Concepts Inc., Berkley, CA) with P < 0.05 being considered significant.

**SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOTTING**

Proteoglycan and collagen metabolites in the conditioned medium or cartilage extracts were electrophoretically separated as described previously\(^24\). Briefly, chondroitin sulphate and keratan sulphate were removed by 2–4 h digestion at 37°C with proteinase free chondroitinase ABC (Sigma), keratanase (Seikagaku) and keratanase II (Seikagaku), dialysed against ultra-pure deionised water, lyophilised, separated under reducing conditions on 4% sodium dodecyl sulphate-polyacrylamide gels and transferred to nitrocellulose membranes. Loading of samples onto the gels was standardised either by loading an equal amount of GAG for analysis of most proteoglycan epitopes\(^24\) or an equal wet weight of tissue for aggrecan G1 domain and type II collagen metabolites. Immunoblotting of membranes was performed using monoclonal antibodies (Mabs) BC-3 (1:2000\(^29\)) recognising the aggrecanase-generated N-terminal interglobular domain of the aggrecan core protein (IGD) neoepitope ARG...; BC-14 (1:1000\(^29,30\)) recognising the matrix metalloproteinase (MMP)-generated N-terminal IGD neoepitope FFA... (equine sequence\(^31\); BC-13 (1:500\(^24\)) recognising the aggrecanase-generated C-terminal neoepitope ...EGE; BC-4 (1:1000\(^29\)) recognising the
MMP-generated C-terminal IGD neoepitope …PEN; and 9A4 (1:100025) recognising the collagenase generated C-terminal type II collagen neoepitope …AEGPPGPQG. Incubations with primary and secondary (alkaline phosphatase conjugated) antibodies were performed for 1 h at room temperature and the immunoblots were incubated with substrate for 5–15 min at room temperature to achieve optimum colour development.

RNA EXTRACTION AND REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSES

Replicate explants to those used for biochemical analysis (short-term culture experiments only) were used for analysis of metalloproteinase and inhibitor mRNA expression. Total RNA was extracted from explants and isolated using RNeasy mini-columns and reagents (Qiagen Ltd.) according to the manufacturers protocol and eluted in sterile water33. The RNA obtained from explants derived from three individuals was pooled and RT-PCR was performed using the RNA PCR kit (Perkin–Elmer) as previously described33. First strand cDNA was synthesised by RT (2 μl RNA solution/20 μl reaction volume) using MuLV reverse transcriptase and “hot-start” PCR amplification was performed (1 μl RT-mRNA solution/50 μl reaction volume) using oligonucleotide primers corresponding to cDNA sequences for A Disintegrin And a Metalloproteinase with thrombospondin repeat (ADAMTS)-4 (ACCACCTTTGAC-CAACACGCACTTC and ACCCCCAAGGTCGAGGCA; GenBank™ accession number: AF368321), ADAMTS-5 (TGCTGCTGATTGAAGCAT and GACCTGAG-AAGGTCGAGG; GenBank™ accession number: AF368322), MMP-3 (TTTCTGGGMAAATCCTCAG and AAARRACCAATATCCTCAG; GenBank™ accession number: J03209), MMP-13 (TKCTGGCWCAYGCTTTT-CCT and GGTGCGGGCTCCTCCTG; GenBank™ accession number: NM002427), TIMP-1 (CCACCTTATAC-CAGGCTTAT and GCCAGCAGCCAACAGTTAGG; GenBank™ accession number: S68252), TIMP-2 (GTGG-ACCTTGGAAAYGACAT and TCTTCTCTGGTGTGTCGTA; GenBank™ accession number: S48568), TIMP-3 (GGGAAAGAACTGTGAAADAGG and GCCGATGCG-ACCATTGTGTT; GenBank™ accession number: U02571) and GAPDH (TGCTATCGGAAAGAGCT and GTGGTCTGTGGTAAAGCTC; GenBank™ accession number: X01677), where M = A or C, Y = C or T, R = A or G, K = G or T and W = A or T. The PCR products were visualised on 3% agarose gels stained with ethidium bromide and their nucleotide sequences verified using an ABI 310 Genetic Analyser.

Results

SHORT-TERM CARTILAGE CULTURES

There was no detectable release of HyPro in any of the joint regions over 4 days of culture under any condition (data not shown). Data pooled from all horses (n = 5) on the release of GAG into the medium (expressed as a percentage of total) are shown in Fig. 1(A). In control cultures more GAG was released from the high weight-bearing carpal cartilage (DR) compared with the other regions (P < 0.006 for both analyses). There was a significant effect of RA, IL-1 and TNF on GAG release (P < 0.0001). RA, which was used as a positive control to determine maximal GAG release from the different cartilages, induced a significantly greater release than either IL-1 or TNF (P < 0.0006). There was no difference between the three regions in their response to any of the catabolic stimuli (expressed either as % of total or as a percentage of the mean control value). When the data from individual horses were analysed separately, one animal showed significantly less response to catabolic stimulation in the PC cartilage [Fig. 1(B), Horse 1; P < 0.0001], while in a second animal cartilage from the PC was significantly more responsive [Fig. 1(B), Horse 2; P < 0.0001] to all three catabolic stimuli compared with the other regions. No correlation between the age of the horses and the response to catabolic stimulation was evident.

The proteases responsible for aggrecan release were evaluated by Western blot analyses with Mabs recognising aggrecanase- and MMP-generated neoepitope sequences (Fig. 2). Medium from control cultures of all horses (n = 5) regardless of age or joint region contained aggrecanase-generated (BC-3 positive) aggrecan metabolites of approximatively 120 kDa molecular mass [Fig. 2(A)]. Cartilage from all three regions had an increased release of BC-3 positive aggrecan fragments. No MMP-generated aggrecan metabolites (BC-14 positive) were detected in culture medium from any region or treatment (not shown). When an equivalent amount of equine aggrecan (20 μg GAG) was subjected to MMP-3 digestion and Western blot analysis, numerous BC-3 positive bands were observed [Fig. 2(A)], one described previously with porcine and bovine aggrecan24,34. BC-14 was able to detect equine MMP-generated aggrecan metabolites when 3% or more of the loaded sample initiated with the FFA…sequence (data not shown). Both aggrecanase- and MMP-generated G1-bearing metabolites were detected in control cartilage [Fig. 2(C)]. Extracts of cartilage at the time of harvest from the joint demonstrated that these metabolites were present prior to the initiation of culture and no difference was found between joint regions (data not shown). The increase in BC-3 [Fig. 2(A)] correlated with increased BC-13 reactive metabolites in the cartilage extracts from all regions [Fig. 2(C)]. In contrast, the levels of BC-4 remained largely unchanged, consistent with the lack of BC-14 [Fig. 2(B)] and results in other species24.

Analysis of the expression of mRNA for aggrecanases, MMPs and TIMPs using RT-PCR was performed on pooled RNA to determine whether any differences existed between cartilages commonly (MCP and DR) or rarely (PC) affected by arthritic degeneration (Fig. 3). The mRNA for ADAMTS-4 was not expressed in any cartilage prior to culture or in any control or RA stimulated cartilages. Expression of ADAMTS-4 was induced by IL-1 (MCP and DR) and TNF (DR and PC). The mRNA for ADAMTS-5 was present in all cartilages prior to culture but this expression was markedly decreased or abolished in control cultures. ADAMTS-5 mRNA was induced by RA (MCP and DR), IL-1 (MCP) and TNF (DR and PC). mRNA for MMP-3 and -13 was detected in MCP but not other cartilages prior to culture. When compared with control cultures, IL-1 and TNF induced expression of MMP-3 and -13 in all cartilages. Message for TIMPs-1, -2 and -3 was present prior to culture in cartilage from all regions. TIMP-1 message was unaffected by any culture condition. In contrast, TIMP-2 mRNA was generally

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C. B. Little et al.: Cytokines in focal cartilage breakdown

[References and data not shown for brevity.]

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(Scientific abstract, not a complete research paper. Table and figures are not included.)
diminished in all cultured cartilages, particularly by TNF. TIMP-3 mRNA expression was still detected in most cultured cartilage although it was generally down regulated by TNF treatment in all three regions.

LONG-TERM CARTILAGE CULTURES

IL-1 and IL-1/OSM significantly increased GAG release from cartilage when compared with controls [Fig. 4(A); P < 0.0001], with 60–80% of total cartilage GAG being released during the first 7 days of culture. There was a significantly greater release of GAG induced by IL-1/OSM compared with IL-1 alone during days 0–7 (P = 0.03). There was no difference between joint regions in GAG release on any day when pooled or when individual animal data (results not shown) were analysed. Treatment with IL-1 but not IL-1/OSM significantly increased HyPro release from both DR and PC cartilage [Fig. 4(B); P < 0.0001]. Approximately 30% of the total cartilage HyPro was released from both cartilages over the 4 weeks of culture in the presence of IL-1. The IL-1/OSM increase in HyPro release from both PC and DR cartilage during days 22–28 [Fig. 4(B)], failed to reach statistical significance. There was a difference between PC and DR cartilage HyPro release during days 8–14 of culture (P = 0.001), with PC cartilage releasing significantly more HyPro in response to IL-1 stimulation at this time.

In order to determine whether the release of GAG and HyPro from the DR and PC cartilages was due to similar proteolytic events, and whether this differed between catabolic stimuli or time in culture, Western blot analysis of the aggrecan and type II collagen metabolites released into the culture media was performed. No differences in Western blot analysis between the DR and PC cartilages were detected at any time point, so for simplicity blots representative of both cartilages are shown in Fig. 5(A,B). Throughout the 4 weeks, both IL-1 and IL-1/OSM increased aggrecanase-mediated cleavage of aggrecan compared with controls, as shown by the BC-3 Western blot analysis [Fig. 5(A)]. There was a decrease in the molecular mass of the BC-3 positive metabolites with time. Most notably, a novel band of BC-3 positive metabolites between 75 and 100 kDa molecular mass became apparent in IL-1 treated cultures during days 15–21 and 22–28 and also in IL-1/OSM treated cultures in days 22–28 [Fig. 5(A)]. No BC-14 positive metabolites were detected in media from any cultures at any time (data not shown). Collagen catabolism...
was associated with an increase in detection of collagenase-generated neoepitopes in both the GuHCl extracts (not shown) and culture medium [Fig. 5(B)]. The 9A4 positive metabolites were of multiple molecular weights consistent with secondary N-terminal catabolism of the collagenase-generated 3/4 fragment.

Discussion

In joint diseases such as OA, cartilage damage is not uniform across the joint surface, rather it occurs in well-defined regions. Recent evidence has implicated variability in responsiveness of chondrocytes to catabolic cytokines, in the focal distribution of cartilage degeneration in human OA.\(^{8,14,15}\) In the present investigation we could not demonstrate a significant difference in RA, IL-1, TNF or IL-1/OSM stimulated proteoglycan catabolism in equine cartilage from joint regions with a low vs high incidence of susceptibility to arthritic degeneration. In the previous work, much lower doses of catabolic cytokines were used.\(^{8,14,15}\)

With the high cytokine doses used in the present study we did nevertheless demonstrate significant differences between cartilage regions in certain individuals [Fig. 1(B)]. However, even in these two animals, the differences were not consistent with one showing increased and one decreased catabolism in the low susceptibility region (PC). While species differences could account for the divergent results of the present and previous reports, it seems more likely that the disease state of the joints from which the cartilage was obtained is responsible for the disparity. In the present study all joints were free from disease while the previous investigations used cartilage from late stage OA joints at the time of replacement surgery. Taken together these results suggest that differential response to cytokine stimulation is a result rather than a cause of focal cartilage degeneration.

Consistent with other species (reviewed in Ref.\(^{1}\)), aggrecan loss from equine articular cartilage was associated with increased cleavage by aggrecanases and not MMPs. There was no evidence for a difference in the proteolytic mechanisms involved in cartilage aggrecan release either from different joint regions or in response to different catabolic stimuli. In contrast to normal cartilage from other species, aggrecanase-generated metabolites were readily detected in medium from control cultures of all equine cartilages regardless of the age of the animal at levels similar to those previously demonstrated in OA human cartilage.\(^{24,35,36}\) This suggests that articular cartilage in normal equine joints has a higher basal aggrecanase activity, which could render it more susceptible to degeneration. ADAMTS-5 but not -4 was expressed in all cartilage, it was still detected in the high weight-bearing DR region, which also had a higher basal release of GAG [Fig. 1(A)]. This finding is consistent with data suggesting that ADAMTS-5 is constitutively expressed and as such may be the aggrecanase responsible for basal aggrecan turnover in cartilage.\(^{24,35,36}\) While expression of ADAMTS-5 was markedly decreased in control cultures when compared with ex vivo cartilage, it was still detected in the high weight-bearing DR cartilage [Fig. 3], which also had a higher basal release of GAG [Fig. 1(A)]. This finding is again consistent with the suggested role of ADAMTS-5 in normal/basal aggrecan turnover. Expression of ADAMTS-5 was increased, albeit inconsistently, by culturing with RA, IL-1 or TNF, indicating that similar regulation of this gene could occur in vivo. Unchanged, decreased and increased ADAMTS-5 expression have all been reported in human OA compared with normal cartilage.\(^{26,38,39}\)
ADAMTS-4 message was not detected prior to culture in these normal equine cartilages but was stimulated by IL-1 and TNF but not RA. The pattern of ADAMTS-4 and -5 mRNA regulation by catabolic agents was not consistent and did not appear to correlate with cartilage regions with high vs low incidence of disease. There are a number of possible explanations for cartilage cultures where, despite evidence of increased aggrecanase activity, no mRNA for either ADAMTS-4 or -5 could be detected (e.g., TNF stimulated MCP or RA stimulated PC). Up-regulation of ADAMTS-4 and -5 expression may only occur during the initial 24 h after stimulation and then decline. Aggrecanases other than ADAMTS-4 or -5 may be responsible for aggrecan catabolism in these culture systems or the regulation of activity may not be primarily at the level of transcription. Recent evidence indicates that ADAMTS-4 activity may be controlled through C-terminal proteolysis of the secreted enzyme. Finally, the level of natural inhibitors of ADAMTS, such as TIMP-3, may modulate enzyme activity. The expression of both MMP-3 and -13 in MCP but not other cartilages prior to culture did not appear to be related with increased basal matrix turnover in this joint region. The metacarpophalangeal joint has a high incidence of disease, however there was no evidence grossly or histologically (not shown) for pathological change. Although this does not rule out the possibility that the MCP cartilage used in this study had early degenerative change, the fact that the basal GAG release was not elevated and there was no difference in cytokine response argues against this. Further investigation of the significance and consequences of MMP-3 and -13 expression in MCP cartilage in vivo is required. While both MMPs were expressed in control cultures of DR cartilage, the higher basal aggrecan turnover in this region was not associated with detectable MMP cleavage. Increased expression of both MMPs in response to IL-1 and TNF stimulation did not differ between regions.
and again was not associated with detectable proteolysis of aggrecan or collagen in these short-term cultures.

It is well recognised that MMP activity is tightly controlled post-transcriptionally and that activation of latent enzyme is responsible for regulating collagenolysis in explant cultures. Normal equine articular cartilage differed from other species in that IL-1 alone rather than IL-1 plus OSM, induced significant collagenolysis despite the fact that OSM did augment aggrecan release from cartilage as expected. The earlier increase in collagen catabolism in PC cartilage conflicts with the lower incidence of cartilage pathology in this joint region. This finding argues strongly against regional cytokine responsiveness being responsible for the focal nature of cartilage degeneration observed in OA. The collagen release in equine cartilage was due to MMP-cleavage and increased generation of 9A4 neo-epitope as expected. This increase in MMP activity, while not resulting in aggrecan IGD cleavage, was correlated with a change in the distribution of aggrecanase-generated aggrecan catabolites initiating with the neoepitope sequence ARG... released into the medium were detected with the neoepitope specific monoclonal antibody 9A4. The migration position of pre-stained globular protein standards is shown on the left.
In conclusion, these studies suggest that a regional difference in response to catabolic cytokines is unlikely to be a factor in the initiation of focal cartilage degeneration in OA. Nevertheless, local differences such as the elevated basal aggrecan turnover in high weight-bearing cartilage in normal joints may well play a role in localised degeneration. These regional differences in basal chondrocyte metabolism may be related to the mechanical loading history of the cartilage and represent a distinct topographical cell phenotype. Increased mechanical loading above a physiological range induces changes in chondrocyte metabolism and initiates cartilage degeneration. The changes induced by early degeneration may include altered sensitivity and response to catabolic cytokines that could contribute to the progression of cartilage breakdown.

References


