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Cytokine induced metalloproteinase expression and activity does not correlate with focal susceptibility of articular cartilage to degeneration[1]

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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).
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Key words: Aggrecanase, Matrix metalloproteinases, Cartilage catabolism.

Abbreviations: MMPmatrix metalloproteinase., IL-1interleukin-1., OSMoncostatin M., GAGglycosaminoglycan., IGDinterglobular domain of the aggrecan core protein., DMMBdimethylmethylene 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

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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 arthritis[2−4]. Nevertheless, synovium from OA joints does secrete significantly more TNF and IL-1

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[7–9].

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.[10]). 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[8,14,15]. 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[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 $\pm 10^{-6}$ 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 dimethylmethylene 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 4–12% gradient (Novex, Frankfurt, Germany) or 10% Tris/glycine SDS-PAGE 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