Topographical variation in glycosaminoglycan content in human articular cartilage

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The weight-bearing status of articular cartilage has been shown to affect its biochemical composition. We have investigated the topographical variation of sulphated glycosaminoglycan (GAG) relative to the DNA content of the chondrocyte in human distal femoral articular cartilage.

Paired specimens of distal femoral articular cartilage, from weight-bearing and non-weight-bearing regions, were obtained from 13 patients undergoing above-knee amputation. After papain enzyme digestion, spectrophotometric GAG and fluorometric DNA assays assessed the biochemical composition of the samples. The results were analysed using a paired *t*-test.

Although there were no significant differences in cell density between the regions, the weight-bearing areas showed a significantly higher concentration of GAG relative to DNA when compared with non-weight-bearing areas ($p = 0.02$).

We conclude that chondrocytes are sensitive to their mechanical environment, and that local loading conditions influence the metabolism of the cells and hence the biochemical structure of the tissue.

Articular cartilage is a biphasic, viscoelastic, porous, permeable material with unique biomechanical properties.¹ An abundant and highly complex extracellular matrix (ECM) surrounds a sparse population of chondrocytes derived from differentiated mesenchymal cells during embryonic development.

Chondrocytes comprise only 10% of the volume of articular cartilage. Their principal function is to produce and maintain the ECM.² The latter consists of water (65% to 80%), type-II collagen (10% to 20%) and aggrecan (complex proteoglycan molecules) and substantially dictates the inherent biomechanical properties of the cartilage.

Proteoglycans contain a protein core to which glycosaminoglycans (GAGs) are covalently bound as side-chains (Fig. 1). Each GAG molecule consists of repeating disaccharide units (chondroitin sulphate, heparan sulphate and dermatan sulphate) and carries a strong polyanionic (positive) charge which draws water into cartilage. This gives it its ability to withstand compressive forces. Its tensile properties are determined more by its collagen structure.³ Aggrecan represents more than 80% of the proteoglycans within articular cartilage (Fig. 1). It is a large aggregating molecule, with up to 100 chondroitin sulphate and

50 keratan sulphate GAG chains covalently bound to the protein core.¹

Since cartilage is aneural and avascular,⁴ chondrocytes lack the ability to respond to neural or immune stimuli, although various environmental factors and substances may influence their metabolism including interleukins, soluble drugs and growth factors, matrix molecules, hydrostatic pressure and mechanical load.⁵⁻⁹ The proteoglycan content of articular cartilage determines its resilience to compression and its viscoelastic properties.

Our study has considered the effect of mechanical load on the metabolism of chondrocytes. Regional variation in the absolute GAG content of cartilage taken from human femoral heads has been described.¹⁰ Furthermore, this variation, and consequently the amount of water held by the tissue, have been shown to influence the stiffness of cartilage.³ The concentration of chondrocytes can be quantified by a DNA assay.¹¹ To our knowledge, there have been no previous studies of animal or human tissue which have assessed the concentration of GAGs in relation to the density of chondrocytes (DNA).

Our null hypothesis was that there was no significant difference in the concentration of

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Fig. 1

Diagram of the aggrecan molecule and its binding to hyaluronate (reproduced with permission from Wright TN, et al. *Proteoglycans: structure and function*. In: Hay ED, ed. *Cell biology of the extracellular matrix*. Second ed. New York: Plenum Press, 1991:45-78).

GAGs per chondrocyte between regions of weight-bearing and non-weight-bearing human articular cartilage.

Materials and Methods

Articular cartilage was obtained from the resected distal femur of 13 patients undergoing surgery for bone or soft-tissue malignancy of the lower limb at The Royal National Orthopaedic Hospital, Stanmore, between October 2004 and February 2005. There were seven male and six female patients, all of whom had undergone adjuvant chemotherapy. Their mean age was 49.1 years (14 to 75). The nature of the study was explained to them before surgery and written consent obtained. The study did not influence or alter their clinical management. We excluded patients with a tumour invading the knee, marked degenerative changes, evidence of large osteochondral defects, joint infection and those with previous joint surgery such as an arthroplasty or autologous chondrocyte implantation.

Full-thickness samples of articular cartilage, measuring approximately 10 mm x 10 mm, were obtained immediately post-operatively using a sterile scalpel and were stored dry at -70°C before biochemical assay. The medial and lateral femoral condylar summits were deemed to be weight-bearing and the patellar groove and margins of the medial femoral condyle and lateral femoral condyle non-weight-bearing. Each sample was identified by number to ensure that the assays were performed blind to the cartilage load-bearing status.

Digestion of cartilage. Each sample was immersed in a papain enzyme solution to dissolve the ECM. This enzyme was made by dissolving 35 mg of L-cysteine (Sigma Chemical Co, St Louis, Missouri) in 20 ml of phosphate-buffered EDTA (PBE) (7.1 g of Na_2HPO_4 , 1.86 g of Na_2EDTA and 500 ml of H_2O) at pH 6. After syringe-filtering (0.2 μm), 0.1 ml of stock sterile papain at an activity of 35 U/mg

(Worthington Biochemical Corp, Lakewood, New Jersey) was added; 1 ml of this solution was then added to the cartilage sample in a 1.5 ml tube, sealed with paraffin film, and placed in a water bath at 60°C for 16 to 18 hours for digestion. For each triplicate of cartilage analysed, a further 1.5 ml tube contained only papain enzyme solution to act as a background control. The samples were stored at -70°C until the DNA, total collagen and GAG assays were carried out. **GAG assay of cartilage.** A dimethylmethylene blue spectrophotometric assay¹² was used to quantify the level of sulphated GAG in the papain-digested cartilage specimens. Chondroitin sulphate standards were made by diluting the working chondroitin-sulphate solution (1 mg/ml) with a PBE-cysteine solution to yield standards of 100, 75, 50, 25, 12.5 and 6.25 $\mu\text{g/ml}$.

Spectrophotometric measurement of the chondroitin sulphate standards and the papain-digested cartilage samples were carried out in triplicate. Spectrophotometric measurements were taken immediately at 540 nm and a standard curve was obtained (coefficient of determination (R^2) > 0.95). Next, the explants were measured in the same way.

The true sample readings were calculated by subtracting the reading for the standard papain solution from those of the cartilage samples. The GAG concentration in the cartilage samples was then obtained by comparison with the standard curve.

DNA assay in cartilage. The total DNA of the samples was quantified by fluorometric assay using the DNA-specific bisbenzimidazole dye, Hoechst 33258 (Sigma)¹¹ by dilution of the working DNA solution (1 mg/ml), DNA standards for 6.25 $\mu\text{g/ml}$ to 50.0 $\mu\text{g/ml}$ were produced. Fluorometric measurement of the DNA standards and the cartilage samples was carried out in triplicate. The standards and samples were then excited at 365 nm with a spectrofluorometer and their emission measured over a range of 445 nm to 460 nm.

Table I. Glycosaminoglycans (GAG) concentrations ($\mu\text{g}/\text{ml}$) in samples of weight-bearing and non-weight-bearing cartilage

Sample	GAG concentration	GAG (μg) per g/wet weight	p-value
Weight-bearing	418.17	21.82	0.03
Non-weight-bearing	352.12	18.92	

Table II. Chondrocyte cell density ($\mu\text{g}/\text{ml}$) in weight-bearing and non-weight-bearing samples

Sample	DNA concentration	Chondrocytes per g	p-value
Weight-bearing	5.889	30 412 301	0.89
Non-weight-bearing	6.089	30 529 623	

The DNA concentration was calculated by comparing the emission of the cartilage samples with that of the standard curve.

Statistical analysis. Comparative statistical analysis between pairs of cartilage explants, that is, articular cartilage taken from the weight-bearing and non-weight-bearing regions of the same patient, was performed using a paired two-tailed *t*-test (SPSS version 11.0; SPSS Inc., Chicago, Illinois). A *p*-value of < 0.05 was considered to be significant.

Results

GAG assay. The GAG concentrations ($\mu\text{g ml}^{-1}$) from the paired weight-bearing and non-weight-bearing cartilage samples are shown in Table I. This shows the pooled mean from the entire series and has been calculated for both GAG concentration and total GAG (μg) per wet weight of sample (g). The results show a significantly greater ($p = 0.03$) absolute GAG concentration in weight-bearing cartilage.

DNA assay. There was no statistical difference in the chondrocyte cell density assessed using the DNA assay between weight-bearing and non-weight-bearing articular cartilage ($p > 0.05$; Table II). The quantity of chondrocytes per gram of wet weight of articular cartilage was calculated by knowing that the cellular content of DNA was 7.7 picogram (pg) per chondrocyte, a figure also derived by fluorometric assay.¹¹

Combined assay. The weight of GAG per chondrocyte was calculated by dividing the weight of GAG per μg by the cellular density determined by the DNA assay. When compared with the pooled mean of the entire series there was a significantly higher GAG per DNA ($p = 0.02$) in the weight-bearing cartilage (Fig. 2).

The amount of GAG present per chondrocyte was calculated using the figure of 7.7 pg of DNA per chondrocyte.¹¹ Figure 3 details paired explants taken from individual limbs, and except for one pair, there is a significantly higher ($p = 0.02$) quantity of GAG per chondrocyte in the weight-bearing cartilage.

The mean ratio of GAG (μg) per μg of DNA in weight-bearing regions compared with non-weight-bearing regions was 1.78 (0.9 to 6.2) indicating that chondrocytes from weight-bearing regions contained, on average, 1.78 times

Fig. 2

Bar chart showing the total glycosaminoglycan (GAG) (μg) per DNA (μg) in weight-bearing and non-weight-bearing cartilage ($p = 0.02$, $N = 13$).

Fig. 3

The amount of glycosaminoglycan (GAG) present per chondrocyte in patient-paired explants ($p = 0.02$, $N = 13$).

more GAG in their ECM than those from non-weight-bearing regions.

Discussion

A number of studies have demonstrated the importance of physiological loading on the biology of chondrocytes^{5,13-15} and the metabolic response of cartilage to loading.^{5-9,13,15-20} Variations in the proteoglycan content of cartilage in different sites have been shown in both animal and human studies.^{18,21-23} These considered the absolute concentration of proteoglycan in cartilage without relating it to chondrocyte density. By contrast, our study has compared the quantity of sulphated GAG present per chondrocyte from different regions of the femoral articular surface of the human knee.

The results obtained from the GAG and DNA assays in our study compare well with those of previous human and animal studies^{2,11,12,23} despite the fact that the cartilage samples came from patients who had undergone chemotherapy. While our study demonstrated no significant difference in chondrocyte density between weight-bearing and non-weight-bearing cartilage, there was on average 1.78 times more GAG in load-bearing articular cartilage ($p = 0.0004$). On average there was 1.78 times more GAG per chondrocyte in weight-bearing locations ($p = 0.02$). These

results refute the null hypothesis and suggest that significant differences in GAG concentration must relate to differences in the metabolism of chondrocytes rather than in their density.

Animal studies have indicated that decreased weight-bearing affects both the thickness^{21,24,25} and GAG concentration of cartilage.^{20,26} The variation in thickness of articular cartilage from the human knee has been mapped using cadaver tissue²⁷ and by arthrography.²⁸ The high loads transmitted through the patellofemoral joint and the resulting thick retropatellar articular cartilage support the theory that cartilage is modelled in response to mechanical load.^{29,30} Our findings confirm those of previous human tissue studies which have quantified the absolute proteoglycan levels from different sites in the knee.²³

Our study has added to the evidence linking the physical forces exerted upon chondrocytes, through the ECM to their metabolic activity by a mechanism termed mechanotransduction.^{5,6} This involves cellular, molecular and genetic pathways leading to some form of altered gene expression.^{15,16,18,19,22} The integrin family of dimeric transmembrane proteins are considered to be the principal vehicle of the cell/matrix interaction.³¹ This triggers a plethora of intracellular pathways which alter gene expression, thus modifying both the biosynthesis and biodegradation of the ECM. These gene products include the following: 1) ECM proteins (collagens and aggrecan); 2) growth proteins regulating one cell cycle (cyclins and Cdks); 3) cytokines (interleukin-1, -4 and -5); 4) growth factors (transforming growth factor beta, fibroblast growth factor); 5) matrix metalloproteinases and tissue inhibitors of metalloproteinase; and 6) angiogenic and anti-angiogenic factors (trophonin and chondromodulin).

Autologous chondrocyte implantation is a new and evolving technique which is used to treat symptomatic defects of articular cartilage especially in young adults.³²⁻³⁴ Non-weight-bearing cartilage is cultured after enzymatic degradation, and implanted into the chondral defect in a load-bearing region. The biochemical response of previously non-load-bearing chondrocytes, cultured and transposed into load-bearing regions, has yet to be clarified or correlated with the clinical response and is of interest given the results of our study. Studies are required to determine whether implanted chondrocytes respond to their new mechanical environment by altering gene expression and consequently the surrounding ECM.

In summary, our findings have shown that there is no significant difference in chondrocyte density between weight-bearing and non-weight-bearing regions of human articular cartilage and that human articular cartilage in weight-bearing regions contains significantly more sulphated GAG than that in non-weight-bearing regions, in both absolute terms and in the individual cell. We conclude that human chondrocytes are sensitive to their mechanical environment, and that local loading influences the metabolism of the cells, and hence the biochemical structure of the tissue.

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