Chondroitin and keratan sulphate epitopes, glycosaminoglycans, and hyaluronan in progressive versus non-progressive osteoarthritis

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Abstract

***Objective*—To determine if a single time point estimation of chondroitin sulphate (CS) or keratan sulphate (KS) epitopes, hyaluronan (HA), or total glycosaminoglycans (GAG) in knee synovial fluid at time of hospital referral can predict subsequent radiographic progression of knee osteoarthritis.**

***Methods*—Two groups of hospital referred patients with knee osteoarthritis were compared: (1) a "progressive" group (n = 45), showing further reduction in radiographic joint space of at least one grade (0-3) in at least one compartment; and (2) a "non-progressive" group (n = 25) in whom radiographs showed no change during the mean follow up period of 2.3 years (median 2, range 1 to 5 years). Knee synovial fluid obtained at the first visit was examined by ELISA for: CS epitopes, using monoclonal antibodies 3B3 and 7D4; KS epitope, using monoclonal antibody 5D4; and HA, using biotinylated HA binding region of cartilage proteoglycan. Total sulphated GAG were measured by dye binding with 1:9 dimethylmethylene blue.**

***Results*—In patients with bilateral synovial fluid data right and left knee values were closely correlated for all variables. There were no significant differences between CS and KS epitopes, HA, total sulphated GAG, or ratios of individual CS or KS epitopes to total GAG, between progressive and non-progressive groups.**

***Conclusions*—Single time point estimation of CS, KS, HA, or total GAG in synovial fluid does not distinguish radiographically progressive and non-progressive knee osteoarthritis patients followed for two years.**

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There is considerable interest in "markers" of the osteoarthritic process that could prove useful for diagnosis, assessment of disease activity, or prognosis.

Estimation of keratan sulphate (KS) and chondroitin sulphate (CS) epitopes may be valuable in this respect. The monoclonal antibody 5-D-4 which recognises "oversulphated" domains in a heptasaccharide of KS[1] has been used to determine KS in body fluids, principally serum.[2–4] High levels of serum KS are reported in patients with hypertrophic osteoarthritis compared to normal subjects,[5] though similar increases have not been found in patients with polyarticular osteoarthritis.[4] Certain neo-epitopes expressed on CS chains may also reflect aspects of the osteoarthritic process. For example, native carbohydrate epitopes on CS chains recognised by the monoclonal antibodies 3-B-3 and 7-D-4 are absent or only weakly expressed in normal adult canine cartilage but expressed to a much higher degree in experimental osteoarthritic cartilage.[6] Increased levels of these epitopes also occur in human articular cartilage from osteoarthritic knees[7] and in synovial fluid from knees with traumatic cruciate ligament or meniscal damage.[8] It is postulated that increased expression of these epitopes may reflect the response of articular cartilage to insult, requiring reinitiation of a high level of matrix synthesis comparable to that seen during development.[8]

To date, these markers of matrix metabolism have not been investigated in longitudinal synovial fluid studies of patients with well characterised knee osteoarthritis. We recently reported clinical and radiographic features of a cohort of hospital referred patients with knee osteoarthritis followed prospectively for more than one year.[9] The aim of the present study was to determine whether synovial fluid concentrations, at time of referral, of CS, KS, or hyaluronan (HA) might distinguish those patients who developed subsequent radiographic progression from those who showed no further *x* ray change.

Methods

Local research ethics committee approval was obtained for this study.

PATIENT CHARACTERISATION

Patients with knee osteoarthritis all had radiographic joint space narrowing and osteophyte in one or more compartments (medial or lateral tibiofemoral, patellofemoral). Other joint pathology was excluded on the basis of clinical assessment, radiographic features, synovial fluid examination, and serological and biochemical tests.[9] In each patient standardised radiographs were taken: standing, knee extended, anteroposterior views (tibiofemoral joints), plus lateral 30° flexion views (patellofemoral joint). Radiographic severity of osteoarthritis in each knee compartment was

graded according to the Kellgren and Lawrence system[10] and for individual features, including cartilage loss (0-3), in each compartment, by two observers. Scores for each compartment were summated to give a single total score for each variable for each knee. Low inter- and intraobserver variability was demonstrated.[9] Knee aspiration was attempted on all patients at their first attendance. From this prospective cohort of 188 consecutive patients two groups were chosen on the basis of (1) their subsequent radiographic progression, and (2) availability of initial synovial fluid. The "progressive" group showed further radiographic deterioration of at least 1 grade of narrowing (0-3) and 1 Kellgren grade in at least one compartment (n = 49; osteoarthritic knees = 73). The "non-progressive" group showed no change in any *x* ray feature (n = 31; osteoarthritic knees = 38). Mean follow up of the two groups was 2.3 years (median 2, range 1 to 5).

SYNOVIAL FLUID SAMPLE COLLECTION AND ESTIMATIONS

Synovial fluid was collected onto ice, centrifuged at 2500 *g* for 15 minutes at 4°C, and the supernatant stored at –80°C. The fluid was treated with 0.5 units hyaluronidase (*Streptomyces hyalurolyticus*) per 100 ml fluid for 30 minutes at room temperature. Samples for HA and GAG measurement were digested with papain.[8]

Monoclonal antibodies to 3-B-3 and 7-D-4 were a generous donation from Professor B Caterson, University of Wales, Cardiff.[8 11] For the 3-B-3 enzyme linked immunosorbent assay (ELISA), wells were coated with 25 ng ml^{-1} chondroitinase ABC digested pig laryngeal cartilage proteoglycan (PLCP) in 20 mM sodium carbonate buffer (pH 9.6); standards were prepared from PLCP in the range 3.9-500 ng ml^{-1}. A maximum binding well was prepared containing no competitor. 3-B-3 antibody was used at 1:10 000. The ratio of absorbances to the absorbance of the maximum binding well (read at 405 nm) was calculated for standards and samples; a standard curve of ratio against standard concentration was used to determine sample concentrations. The 7-D-4 ELISA was carried out similarly, with wells coated with 3 mg dye binding ml^{-1} bovine tracheal cartilage proteoglycan (BTCP) freshly prepared in 20 mM sodium carbonate (pH 9.6); standards were prepared from BTCP in the range 0.0234-6 mg ml^{-1}. 7-D-4 antibody was used at 1:25 000. The 5-D-4 ELISA was modified from the procedure described by Thonar *et al.*[2 8] Wells were coated with 50 ng ml^{-1} PLCP in 20 mM sodium carbonate buffer (pH 9.6); standards were prepared from PLCP in the range 0.78-200 ng ml^{-1}. 5-D-4 antibody was used at 1:70 000. Absorbances were read at 405 nm and results calculated as before.

The HA ELISA was a modification of the procedure described by Fosang *et al.*[12] Wells were coated with 25 mg ml^{-1} hyaluronan in 20 mM sodium carbonate buffer (pH 9.6); standards were prepared from hyaluronan in the range 0.0195-5 ng ml^{-1}. Absorbances were read at 405 nm and results calculated as before. Total sulphated GAG were measured by a modification of the procedure described by Farndale *et al.*[13] A working solution of dye was prepared by mixing 20 mg 1,9-dimethylmethylene blue, 5 ml ethanol, and 1 litre formate buffer (0.1 M, pH 3.5). Standards were prepared from chondroitin sulphate in the range 5-90 mg ml^{-1}. Forty millilitres of standard or sample were added to 250 ml dye in a microtitre plate. Absorbances were read at 570 nm after two minutes.

Because of low synovial fluid volumes for some knees, different sample numbers were analysed for some of these variables.

STATISTICAL ANALYSIS

Results of KS and CS were expressed as both concentrations and as ratios to GAG concentrations. Correlation between samples taken from the right and left knees of the same individual was calculated using Spearman's rank correlation. Comparison between the two groups for each measurement was made using the Mann-Whitney U test with a Bonferroni correction.

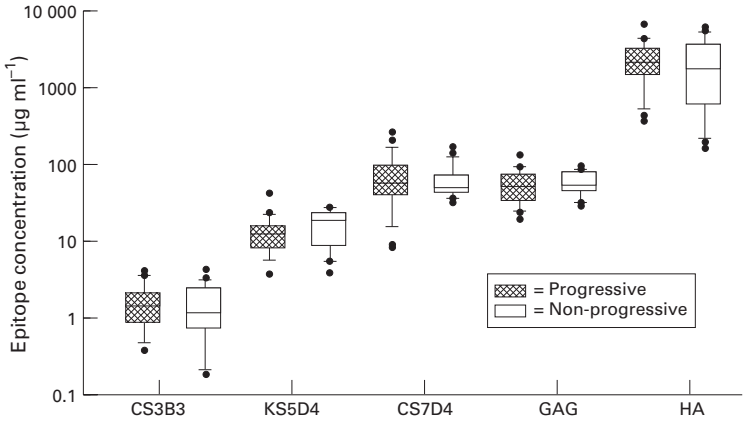

Figure 1 Comparison between progressive and non-progressive groups for all epitopes measured. The central solid line represents the median, with the box representing the middle 50% of the data. The error bar cap lines mark the 10th and 90th centiles. Dots represent individual data points outside the 10th and 90th centiles. CS3B3, chondroitin sulphate epitope 3B3; KS5D4, keratan sulphate epitope 5D4; CS7D4, chondroitin sulphate epitope 7D4; GAG, total sulphated glycosaminoglycans; HA, hyaluronan.

Results

There was no significant difference between progressive and non-progressive groups with respect to age (mean 75, range 62-88, and 71, 48-89 years, respectively) or gender mix (male:female, 1:2 in each group). At entry no knee had complete obliteration of joint space in all three compartments. The median Kellgren score for the progressive group at entry was 4 (mean 3.96, range 1 to 7), and for the non-progressive group it was 3 (mean 2.5, range 1 to 5).

For subjects with synovial fluid data on right and left knees (n = 13) there was strong correlation between knees in a single individual for all assays (5-D-4, $r = 0.8$*; 3-B-3, $r = 0.61$*; 7-D-4, $r = 0.54$; HA, $r = 0.65$*, GAG, $r = 0.49$, *$P<0.05$); thus we could not treat the data as independent. Therefore, for patients with bilateral knee data, one knee was chosen at

random for between group analysis. For patients with one knee progressive and the other non-progressive (n = 7), all data for that patient were deleted.

For between group analyses the sample number for individual assays varied from 19 to 22. Results are shown in the table and in figs 1 and 2. There were no significant differences between the two groups for: individual CS epitopes or KS epitope; GAG; ratios of individual CS and KS epitopes to total GAG; ratios of 3-B-3 and 7-D-4 to 5-D-4, or 3-B-3 to 7-D-4; or HA.

Discussion

This is the first study to examine the predictive value of synovial fluid CS and KS epitopes, HA, and sulphated GAG with regard to radiographic progression of knee osteoarthritis. Previous studies have shown the CS epitopes 3-B-3 and 7-D-4 to be increased in animal models of osteoarthritis[6 11] and human osteoarthritis[7] compared to normal, but no study has investigated whether such increases predict future damage. Serum KS is reported to be increased in patients with knee osteoarthritis, inversely correlating with the degree of radiographic joint space loss,[4] though some studies have not found these associations.[14] Serum markers have the major disadvantage that they may reflect changes in multiple joints. If in a patient with bilateral knee osteoarthritis one knee shows radiographic progression and the other does not, the serum level should legitimately be applied to both knees, presenting difficulties in interpretation. We therefore examined synovial fluid levels, rather than serum levels, of possible "markers" to determine any prognostic value. The potential of these estimations as markers of diagnosis or disease severity, involving examination of normal knee synovial fluid, is to be the subject of a separate report (unpublished).

From a large prospective study of hospital referred patients with symptomatic knee osteoarthritis[9] we derived two groups of patients: one with progressive structural change and cartilage loss, and the other with no structural change over a mean period of 2.3 years. Interestingly there was close correlation for all synovial fluid estimations between right and left knees of patients with bilateral data, suggesting that synovial fluid levels may reflect more an individual (systemic) characteristic than the local extent of joint disease. Such a correlation has also been shown for synovial fluid inorganic pyrophosphate measurements.[15] This is an important consideration for future studies with respect to analysis of two affected knees within the same individual. Our results showed no major difference between the two groups when CS and KS epitopes, HA, and GAG were measured.

Numbers in each group for individual assays

Assay	*Progressive (n=)*	*Non-progressive (n=)*	*Difference, (P=) (Mann-Whitney)*
CS 3B3	20	20	0.636
KS 5D4	20	19	0.100
CS 7D4	22	21	0.913
GAG	21	19	0.607
HA	22	20	0.458
3B3/GAG	20	19	0.392
5D4/GAG	19	17	0.419
7D4/GAG	21	19	0.946

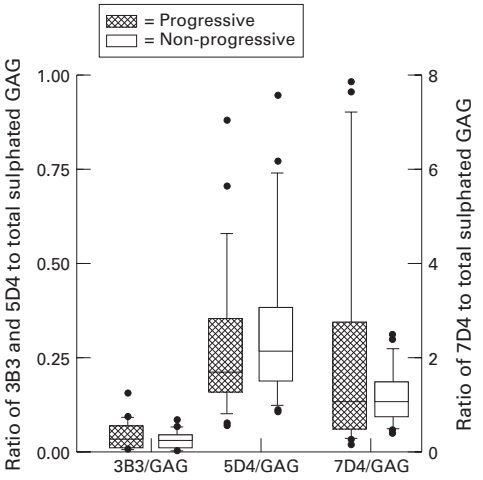

Figure 2 Comparison between progressive and non-progressive groups for ratio of chondroitin sulphate and keratan sulphate epitopes to total sulphated glycosaminoglycans. The central solid line represents the median, with the box representing the middle 50% of the data. The error bar cap lines mark the 10th and 90th centiles. Dots represent individual data points outside the 10th and 90th centiles. The left hand y axis is used for 3B3/GAG and 5D4/GAG. The right hand y axis is used for 7D4/GAG.

This study has several important caveats. There are problems inherent in measuring any synovial fluid concentration, with no information on rate of release, breakdown, or clearance. We studied a hospital based population with established, relatively severe structural change and only took a single time point measurement. We selected patients at the two extremes of radiographic outcome and only included those with available synovial fluid. Serial estimations in patients showing a wider range of osteoarthritis severity, followed for longer, may yield more information. Our data, however, strongly suggest that these synovial fluid measures have no clear prognostic value for symptomatic, hospital referred patients with knee osteoarthritis.

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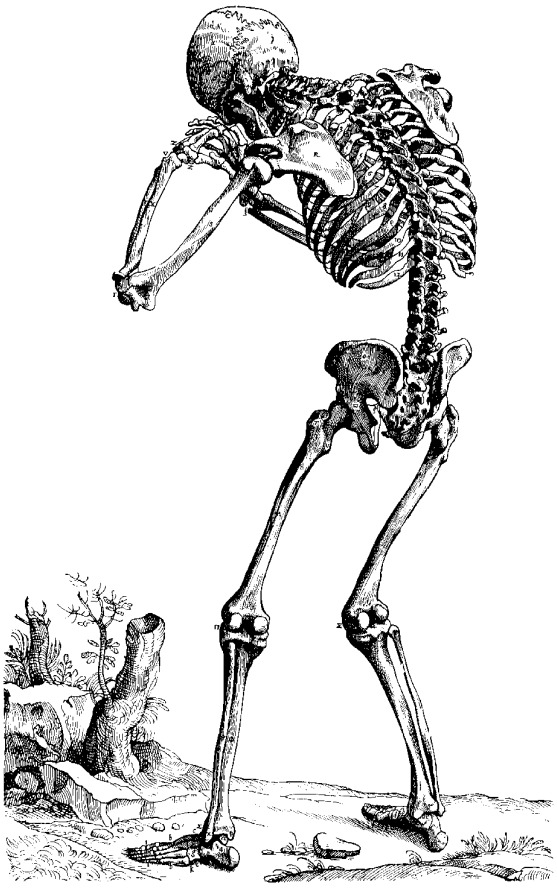

The bones of the human body presented from the posterior aspect. From the anatomical drawings of Andreas Vesalius, 1543.