Increased concentrations of proteoglycan components in the synovial fluids of patients with acute but not chronic joint disease

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SUMMARY Synovial fluid samples (139) from 121 patients with rheumatoid arthritis, osteoarthritis, pseudogout, chronic pyrophosphate arthritis, gout, and reactive arthritis were analysed for cartilage proteoglycan components. Keratan sulphate (KS) epitope was determined by a competitive radioimmunoassay, and total sulphated glycosaminoglycans (S-GAG) were determined after papain digestion by a specific dye binding assay. Increased concentration of both KS epitope and S-GAG were found in synovial fluid from joints with acute inflammatory arthropathy (gout, pseudogout, and reactive arthritis). Analysis of consecutive samples from the same joint at different stages showed that the concentration of KS epitope or total S-GAG varied with acute inflammatory activity. In samples from patients with chronic conditions during active and inactive inflammatory phases concentrations were much lower and not distinguishable among these disease groups. The detection of raised concentration of proteoglycan components may reflect the rapid depletion or greatly increased turnover of proteoglycan in the articular cartilage during acute inflammation in the joint. This did not appear to be sustained in most patients with chronic joint diseases.

Key words: articular cartilage, glycosaminoglycans, keratan sulphate.

The diseases that affect joints vary greatly in their symptoms and clinical presentation. They are often phasic in character and may involve a range of tissue processes such as synovitis, fibrosis, cartilage and bone damage, and remodelling. Current classification is based on clinical symptoms and presumed pathogenic or aetiological mechanisms.¹ Cartilage damage is an important feature in any joint disease¹ and may result in cartilage loss from the articular surface. This can be detected by x ray changes only at a late stage of disease, however, and there is an urgent need for better methods of detection.^{2,3}

Normal articular cartilage is composed of an extracellular matrix, which consists mainly of collagens (types II, VI, IX, and XI) and proteoglycans, principally of the large aggregating type, and a

sparse population of chondrocytes.⁴⁻⁸ The proteoglycans are complex macromolecules (Mr 1-4×10⁶) and consist of an extended protein core, to which are attached many glycosaminoglycan chains (chondroitin sulphate and keratan sulphate).^{9,10} In normal cartilage proteoglycans are slowly but continuously turned over, the degraded molecules are released from the cartilage and are replaced by newly synthesised components.^{5,11} It is the coordinate control of synthesis and degradation of the matrix components by the chondrocytes that maintains normal cartilage. In experimental models of joint disease, however, there is evidence of changes in the rates of biosynthesis and turnover of proteoglycans, which may contribute to cartilage degeneration.¹²⁻¹⁷ The degradation products of proteoglycan turnover are released from the cartilage matrix and appear in the synovial fluid and serum. It is therefore possible that concentrations of proteoglycan components in body fluids, particularly in synovial fluid, may reflect metabolic changes occurring within the cartilage, and their determination

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may enable cartilage degeneration to be detected and monitored and thereby help more effective treatments to be developed.

In this study we determined the concentrations of proteoglycan in synovial fluids from patients with well characterised joint diseases (rheumatoid arthritis, osteoarthritis, acute and chronic pyrophosphate arthropathy, gout, and reactive arthritis). In addition, the results were correlated with the clinical assessment of joint inflammation (inactive or active) at the time of aspiration to determine whether there is a correlation between increased proteoglycan release from cartilage and clinically assessed disease activity.

Patients and methods

One hundred and thirty five synovial fluid samples from 117 patients attending the rheumatology unit of the City Hospital, Nottingham, with clinically evident disease affecting at least one knee, and samples from four patients with reactive arthritis attending the rheumatology department of Charing Cross Hospital, London were included in this study. Minimum investigation in each case included full blood count, erythrocyte sedimentation rate, serum rheumatoid factor, plain radiographs of hands, feet, and knees, and examination of fresh knee synovial fluid for birefringent crystals (compensated polarised light microscopy); further investigation was determined by the individual patient characteristics. The principal diagnostic categories were rheumatoid arthritis (RA), osteoarthritis (OA), and crystal associated arthropathy (acute and chronic pyrophosphate arthropathy and gout). All patients with RA had erosive disease and fulfilled the criteria of the American Rheumatism Association for classic or definite disease¹⁸; patients designated as seropositive had raised titres of serum IgG rheumatoid factor recorded on at least one occasion. Patients with OA had symptomatic seronegative gonarthrosis with knee radiographs showing cartilage loss plus subchondral sclerosis or osteophyte, or both; none had radiographic chondrocalcinosis, synovial fluid

calcium pyrophosphate dihydrate (CPPD) crystals, or evidence of other primary joint disease. Patients with acute pyrophosphate arthropathy (pseudogout) had typical self limiting acute episodes of knee synovitis with synovial fluid CPPD crystals and no evidence of coexistent joint disease (e.g., sepsis). Chronic pyrophosphate arthropathy (CPA) was defined as persistent (>3 months) symptomatic gonarthrosis with synovial fluid CPPD crystals and x ray features of OA (often with predominant patellofemoral involvement, bi- or tricompartmental disease, and/or chondrocalcinosis). Acute and chronic gout was confirmed by demonstration of monosodium urate crystals in fresh knee synovial fluid. The diagnosis of other conditions was similarly based on clinical presentation/distribution of arthropathy together with the appropriate serological, radiographic, and synovial fluid characteristics; no patient with indeterminate or 'probable' disease was included. Reactive arthritis was diagnosed as described by previously published criteria.¹⁹

At each aspiration knees were examined and assessed by a single observer for six clinical parameters of inflammation (Table 1), and the joint was assessed as 'active' or 'inactive'. To make a clear distinction between active and inactive disease those knees scoring three active parameters were excluded. Synovial fluid samples were taken immediately into 10 mM edetic acid; samples were then centrifuged for five minutes and the synovial fluid stored in vapour phase liquid nitrogen (-186°C) within three hours of collection.

DETERMINATION OF KERATAN SULPHATE (KS) EPITOPE BY RADIOIMMUNOASSAY
The radioimmunoassay procedure^{20 21} used to determine KS epitope was that described previously²² using the mouse monoclonal antibody MZ15 to keratan sulphate.^{22 23} The antibody MZ15 binds to pentasulphated hexasaccharides and larger related oligosaccharides of KS,²³ and this suggests that it binds to highly sulphated sequences. The KS epitope was determined by competition with ¹²⁵I labelled purified proteoglycan monomer from pig

Table 1 Clinical assessment of knees

	'Inactive'	'Active'
Individual parameters		
Increased warmth	Absent	Present
Effusion	Absent-mild/not tense	Moderate-marked/tense
Synovial thickening	Absent	Present
Joint line tenderness (0-3)	0-1	2-3
Early morning stiffness (h)	<1	≥1
Inactivity stiffness (min)	<15	15
Global assessment	<3 parameters inactive	4-6 parameters active

laryngeal cartilage, and concentration of KS epitope was therefore expressed in molar units of proteoglycan (PG) standard (PG units/ml).^{16 22}

DETERMINATION OF SULPHATED GLYCOSAMINOGLYCANS (S-GAG)

Before assay the synovial fluid samples were digested by incubation with papain overnight at 60°C. The reaction was stopped by heating the samples at 100°C for 15 minutes. The concentration of S-GAG in the synovial fluids was determined with 1,9-dimethylmethylene blue in a newly developed automated procedure.²⁴ Briefly, 40 µl of shark chondroitin sulphate standard (5–40 µg/ml) or sample diluted in 0.05 M sodium acetate pH 6.8 was added to wells of a 96 well microtitre plate, and to each well was added 250 µl of the dye in a formate buffer pH 3.5.²⁴ The absorbance (at wavelength 600 nm) of the wells was determined immediately. The change in absorbance (a negative change at 600 nm) was calculated from the mean absorbance of four blank wells in which only sample buffer and dye were added. The concentration of S-GAG in the samples was determined from the linear portion of the chondroitin sulphate standard curve (5–40 µg/ml). The microtitre plate reader was interfaced with a Norsk minicomputer to facilitate calculation of the results. With this system both chondroitin sulphate and keratan sulphate can be detected without interference from hyaluronate (high molecular weight or oligosaccharide).

ANALYSIS OF DATA

Statistical analysis was performed using a 'Minitab' data analysis system operated on a Norsk-Data ND570/CX minicomputer. Comparison of the data was by one way analysis of variance.

Results

PROTEOGLYCAN CONTENT OF SYNOVIAL FLUIDS

KS epitope determinations

Keratan sulphate is abundant in cartilage but not in other tissues of the joint.²⁵ It is part of the proteoglycan structure and its determination with specific monoclonal antibody forms a sensitive assay for proteoglycan, though the KS epitope detected may not be distributed evenly among the proteoglycans and the fragments derived from them that appear in synovial fluid.²⁶ During preliminary investigation of synovial fluids from selected patients with different joint diseases it was observed that raised KS epitope was detected in those with reactive arthritis. The study was then enlarged to include samples from 117 patients who were assessed for their disease activity at the time of sampling on the basis of a six parameter score (Fig. 1). The concentrations of KS epitope in the synovial fluids of patients with chronic crystal related arthropathies (CPA and gout), RA, and OA, assessed as either active or inactive in terms of clinical inflammation were low (<50 PG units/ml) and not significantly

Fig. 1 Determination of keratan sulphate (KS) epitope by radioimmunoassay in synovial fluids of patients with characterised joint disease. Each point represents the average of triplicate determination of separate synovial fluid samples. Disease activity was based on the six clinical assessment parameters as described in 'Patients and methods'. CPA=chronic pyrophosphate arthropathy.

different from each other ($p>0.1$). No difference was found between patients who were seronegative or seropositive (results not shown).

The concentration of KS epitope in the synovial fluids of patients with acute gout, acute pseudogout, and reactive arthritis (all clinically assessed as active) was significantly raised ($p<0.001$) (Fig. 1). In the synovial fluids of patients with acute pseudogout 10 out of 14 contained KS epitope at concentrations above 50 PG units/ml, the mean value was 156 PG units/ml, and the maximum concentration detected was 480 PG units/ml. The synovial fluid of one patient with acute gout also had a high concentration of KS epitope. In the synovial fluids of patients with reactive arthritis three out of four patients had concentrations above 50 PG units/ml (mean value 107.7 PG units/ml, maximum concentration 192 PG units/ml). The synovial fluids of four patients with RA showing active inflammation also contained KS epitope at concentrations above 50 PG units/ml (range 97–147).

Total glycosaminoglycans

The concentration of proteoglycans was also determined in the same synovial fluids (Fig. 2) using a dye binding assay that detects all the S-GAG (chondroitin sulphate and keratan sulphate) which form a major part of the proteoglycan structure.²⁷ In synovial fluids from patients with clinically inactive

or active chronic CPA, inactive RA, and active and inactive OA the concentration of S-GAG was less than 100 $\mu\text{g/ml}$, with mean values of 59, 40, 49, 62, and 49 $\mu\text{g/ml}$ respectively. In the synovial fluids of patients with active RA most also had concentrations of S-GAG below 100 $\mu\text{g/ml}$ (mean 62 $\mu\text{g/ml}$), but six samples had concentrations above this (106–218 $\mu\text{g/ml}$) and these included the four samples that had significantly raised concentrations of KS epitope.

The concentrations of total S-GAG in the synovial fluids of patients with pseudogout (mean 118 $\mu\text{g/ml}$), acute gout (mean 314 $\mu\text{g/ml}$), and reactive arthritis (mean 99 $\mu\text{g/ml}$) were significantly higher than those of the other patient groups ($p<0.01$). There was no significant difference between the acute crystal arthropathy and reactive arthritis ($p>0.5$).

A comparison of the concentrations in the synovial fluids of KS epitope and total S-GAG showed considerable variation between individuals in their content of the keratan sulphate structure responsible for the epitope. Those synovial fluids containing raised concentrations of KS epitope also had raised levels of S-GAG. Overall, the KS epitope determinations appeared to show more discrimination between the high concentrations found in acute inflammatory conditions and the lower values of other samples.

Fig. 2 Determination of total sulphated glycosaminoglycans (S-GAG) in the same series of synovial fluids as in Fig. 1.

CONSECUTIVE SYNOVIAL FLUID SAMPLES

Five patients with CPA and superimposed pseudogout attacks underwent serial sampling of the same knee during different phases of clinical activity. Determinations of KS epitope and total S-GAG were made on these samples (Fig. 3). When joints were assessed as showing active clinical inflammation many showed raised concentrations of KS epitope. Of these, the highest values were seen during acute pseudogout attacks where all samples had raised concentrations. Fig. 4 shows the results of an analysis of four samples obtained from one knee joint of a patient with CPA during clinically inactive and active phases and during an acute episode of pseudogout. The general pattern of results from this

limited number of consecutive samples showed that KS epitope concentrations in synovial fluid changed during different phases of inflammatory activity and were consistently raised only during the acute pseudogout attack.

Discussion

The purpose of measuring proteoglycans in synovial fluid is to detect and monitor underlying processes of degradation that may occur in the cartilage of different joint diseases.^{2,3} Initial studies are exploratory as the uneven rates of progress of many joint diseases suggest that the processes involved are episodic, and it is not known how processes such

Fig. 3 Determination of KS epitope in synovial fluids obtained from five patients (●■▲▽○) assessed at different stages of disease activity. Each point represents the average of triplicate determinations of separate synovial fluid samples. Assignment of the patients to inactive, active, and pseudogout (acute) was based on the six clinical assessment parameters as described in 'Patients and methods'.

Fig. 4 Determination of KS epitope in four consecutive synovial fluids from a patient with chronic pyrophosphate arthropathy, obtained at different stages (inactive, active, and acute) and assessed as described in 'Patients and methods'. Each value represents the average of triplicate determinations of separate synovial fluid samples.

cartilage depletion of proteoglycan may coincide with clinical symptoms and other parameters of disease activity (pain, swelling, etc). Previous studies showed that the concentration of KS in serum was on average slightly higher in osteoarthritic patients than in normals (357 (SD 73) ng/ml compared with 251 (78) ng/ml),²⁵ and Saxne *et al* showed that for patients with acute arthritides the concentration of proteoglycans in synovial fluid fell on remission resulting from glucocorticoid treatment of the disease.²⁸ Lohmander *et al* also noted increased concentration of proteoglycans in synovial fluid from hip joints of children with transient synovitis.²⁹ Concentrations were also shown to be high in the synovial fluid of patients with reactive arthritis,³⁰ whereas those with rheumatoid arthritis, psoriatic arthritis, and HLA-B27 associated arthritides were lower.³⁰⁻³¹ These results showed that high synovial fluid concentrations of proteoglycans were detected in acute inflammatory conditions, but that a broad range of lower concentrations was found in chronic conditions. As no account was taken of the different stages of the chronic conditions, however, it is apparent that within the broad range there may be different subgroups.

The present study has confirmed the findings with acute inflammatory conditions, and an attempt was made to discriminate between the scattered values detected in each diagnosed condition by further classifying patients according to their clinically assessed disease activity. This was successful with acute arthropathies such as pseudogout where 10 of 14 patients showed higher concentrations of proteoglycan components than those found in less active or inactive phases (Figs 1, 3, and 4). The results suggest that a rapid increase in proteoglycan concentrations in synovial fluid occurs at the acute stage of disease development but is not sustained during non-active stages.

With more chronic joint diseases the concentrations of proteoglycans in synovial fluid were lower than in acute disease and showed little apparent correlation with clinically assessed disease activity. The failure to detect greatly increased concentrations in most patients with chronic disease may result from a number of factors. Firstly, in chronic disease the activity involved in releasing proteoglycans from cartilage may be less than that found in acute disease. Secondly, the disease may be advanced, and some loss of cartilage may already have occurred. Thirdly, if episodes of rapid release occur in chronic disease they may be of short duration or infrequent, or both, or they may precede clinical symptoms, such that they rarely coincide with clinical sampling. Fourthly, if only modest changes occur they may be masked by the broad range of

natural variation in proteoglycan concentration that is evident in the patient population.

These factors require more detailed study to assess their importance. Saxne *et al* showed that the loss of cartilage measured by joint space narrowing in patients with RA correlated with a fall in proteoglycan concentration in synovial fluid.²⁸ Patients with advanced joint disease may thus contain low concentrations irrespective of other parameters of disease activity. Experimental studies would predict that proteoglycan loss is likely to precede cartilage damage.³⁻¹⁶⁻¹⁷ There is therefore a need to study the very early stages of disease, but it may be difficult to identify 'patients' at preclinical stages and would require the identification of at-risk groups. In established clinical groups the value of determination would be improved if the total volume of synovial fluid and the rate of proteoglycan clearance were measured so that a rate of release of proteoglycan could be calculated. This is impractical for all patients, and although Saxne *et al* reported that volume determinations did not significantly alter the basic trend of results,²⁸ a limited study would enable the importance of these parameters to be assessed. Evidence from the sequential sampling of patients suggests that each individual shows little variation in proteoglycan concentration when sampled at different times compared with the large variation evident in all the clinically defined groups. The development of longitudinal studies may thus reveal more from patients with chronic disease than is evident merely from comparison of data from the different disease groups. Some results have shown that for patients with RA whose synovial fluids were sampled 10 years ago the concentration of synovial fluid proteoglycan showed a direct correlation with the subsequent extent of cartilage damage (joint space narrowing).³¹ Longitudinal studies of patients from the earliest stages of disease will help to substantiate these results and establish if there are phases of proteoglycan release that are of predictive value in different chronic joint diseases.

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