Spectrophotometric measurement of proteoglycans in osteoarthritic synovial fluid

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SUMMARY Cartilage glycosaminoglycans (GAGs) were measured by a spectrophotometric assay in synovial fluid obtained from 30 normal bovine hock joints and 15 osteoarthritic human knee joints. Results were compared with those obtained by radioimmunoassay (RIA). The spectrophotometric method (dimethylmethylene blue (DMB) assay) was found to be simple, safe, and sufficiently reproducible to be of potential value for serial measurement of sulphated GAGs in arthritic joint fluids. The nature of the proteoglycans present in normal bovine and osteoarthritic human synovial fluid was examined by gel chromatography. Whereas normal bovine synovial fluid contained only small molecular weight proteoglycans, osteoarthritic human synovial fluid contained aggregated proteoglycans and predominantly high molecular weight proteoglycan subunits.

Key words: glycosaminoglycans, radioimmunoassay, normal bovine synovial fluid, gel filtration chromatography.

The state of the articular cartilage is of major interest to the clinician evaluating patients with arthritis. Physical examination is often inadequate in this respect and general laboratory investigations are not helpful. Joint radiographs can be informative, but have limitations. These shortcomings have stimulated investigators to develop methods for measuring cartilage proteoglycans in synovial fluid. Gysen and Franchimont have developed a radioimmunoassay (RIA) for this purpose,¹ and Roughley *et al* have developed a similar technique.² Recently, Saxne *et al* reported the use of an enzyme linked immunosorbent assay (ELISA) to measure proteoglycans in synovial fluids from patients with various inflammatory joint diseases.³ The same authors have subsequently used an ELISA to evaluate the effects of corticosteroids on cartilage metabolism.⁴

Glycosaminoglycan assays have been used extensively in the past to measure proteoglycans *in vitro*, but with the exception of urine they have proved unsatisfactory for measurement of proteoglycans in biological fluids, mainly because they are subject to interference by other polyanions.^{5,6} In particular, such assays have been unsuitable for the determina-

tion of proteoglycan concentrations in synovial fluid where there is a large molar excess of hyaluronic acid. By modifying the pH and NaCl concentration of the DMB dye reagent, Farndale *et al* have improved the selectivity of the DMB spectrophotometric microassay for sulphated glycosaminoglycans.^{7,8} In this study the modified DMB assay has been used to determine the concentration of sulphated GAGs in synovial fluid, and the results have been compared with proteoglycan concentrations determined by RIA.

Materials and methods

MATERIALS

Na¹²⁵I (specific activity 625 MBq/μg of iodine) was purchased from Amersham International, Amersham, UK. Chondroitin ABC lyase and Staphylococcus protein A were obtained from Miles Laboratories, Slough, UK. Sepharose CL-2B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. *N*-Acetylcysteine, papain, iodoacetic acid, 1,10-phenanthroline, and phenylmethylsulphonyl fluoride were all obtained from Sigma Chemical Co, London, UK. Chondroitin sulphate was from Koch-Light Laboratories Ltd, Haverhill, UK. 1,9-Dimethylmethylene blue was purchased from Serva Feinbiochemica, Heidelberg, FRG. All other chemicals were AR grade.

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Bovine synovial fluid was obtained from the hock joints of freshly slaughtered cows. Human synovial fluid was obtained from patients with osteoarthritis who at the time of outpatient review were deemed to warrant joint aspiration. Fluids were collected into plastic vials, centrifuged at 1000 g for 10 min to remove cells and debris, and stored in aliquots at -70°C until assayed.

Radioimmunoassays were performed directly on diluted specimens of synovial fluid using the method described by Christner *et al.*⁹ Rabbit antiserum (R-110) directed against bovine proteoglycan A1D1 was a generous gift from A R Poole.

DIMETHYLMETHYLENE BLUE ASSAY FOR GAG

The DMB assay was adapted from the method of Farndale *et al* and is described in detail elsewhere.^{7,8} Samples of synovial fluid (500 μl) were dispensed into plastic vials with a 1 ml Gilson pipette and diluted to 5 ml with 50 mM phosphate buffered saline, pH 7.0. Special care was taken to wash out the pipette tip with the diluent in order to minimise drainage error. Aliquots (100 μl) were mixed by repeated inversion and transferred in duplicate to polystyrene tubes. A further 100 μl of the same buffer containing *N*-acetylcysteine (final concentration 2 mmol/l) and papain 0.13 units was added, and

the tubes were mixed and capped. The samples were then incubated at 65°C for two hours. After digestion the papain was inactivated with iodoacetic acid (final concentration 4 mmol/l) and the NaCl concentration of the digest was adjusted to approximately 0.5 mol/l in a final volume of 250 μl .

Dimethylmethylen blue dye reagent (2.5 ml) was then added and the absorbance at 525 nm was read after 15 s in a Cecil CE 292 digital ultraviolet spectrophotometer. The DMB reagent was prepared by adding 3.04 g of glycine, 2.37 g of NaCl and 16 mg of 1,9-dimethylmethylen blue to 1 litre of deionized water. The pH was adjusted to 3.0 with hydrochloric acid and the reagent was stored in a brown bottle.

The DMB assay was calibrated with shark chondroitin sulphate and suitable solvent blanks.

GEL CHROMATOGRAPHY

The hydrodynamic size distribution of the proteoglycans in normal bovine and osteoarthritic human synovial fluids was determined by gel chromatography on columns (67 $\times$ 1 cm) of Sepharose CL-2B. Samples consisting of 1 ml of synovial fluid and the proteinase inhibitors, 1,10-phenanthroline (1 mM), iodoacetic acid (1 mM), and phenylmethylsulphonyl fluoride (1 mM) were applied to the column and chromatographed at 22°C and 6 ml/h. Fractions

Fig. 1 (a) Chondroitin sulphate was added to osteoarthritic synovial fluid and the recovery determined by the modified DMB assay. (b) High molecular weight proteoglycan subunit was added to osteoarthritic synovial fluid and the recovery determined by RIA.

(500 μ l) were collected and aliquots were assayed in duplicate by the spectrophotometric and radioimmunoassay procedures described. The quantity of proteoglycans recovered after gel chromatography (89–97%, $n=3$) was consistent with that estimated in the starting material by the DMB assay and RIA.

Results

ASSAY PERFORMANCE AND PROTEOGLYCAN RECOVERY

Both assays were sufficiently sensitive to determine proteoglycan or GAG concentrations, or both, in synovial fluid and chromatographic fractions thereof. The sensitivity in synovial fluid was 10 μ g/ml for the RIA and 20 μ g/ml for the DMB assay. In synovial fluid the within assay coefficient of variation was 5.3% ($n=10$) for the DMB assay and 13.8% ($n=5$) for the RIA. The between assay coefficient of variation was 11.2% ($n=3$) for the DMB assay and 29.2% ($n=3$) for the RIA. Thus the DMB assay was more reproducible and the RIA twice as sensitive.

Increasing quantities of proteoglycan were added to undiluted synovial fluid, and the recovery determined by each assay procedure is shown in Fig. 1. Similar recovery was obtained with both methods.

BOVINE SYNOVIAL FLUID PROTEOGLYCAN ASSAYS

With the DMB assay the mean concentration of sulphated GAGs was 155 μ g/ml of synovial fluid (standard error (SE) 11 μ g/ml, $n=30$). With the RIA the mean concentration of proteoglycan was 183 μ g/ml of synovial fluid (SE 20 μ g/ml, $n=30$). The correlation coefficient was 0.60 ($p<0.001$, Student's t test), which indicates satisfactory agreement between the two methods.

HUMAN SYNOVIAL FLUID PROTEOGLYCAN ASSAYS

Aliquots of osteoarthritic synovial fluid were assayed by both methods on three occasions. No drift was evident with storage. Mean values were determined from the three assays and compared. With the DMB assay the mean concentration of sulphated GAGs was 142 μ g/ml of synovial fluid (SE 13 μ g/ml, $n=15$). With the RIA the mean concentration of proteoglycans was 122 μ g/ml of synovial fluid (SE 10 μ g/ml, $n=15$). The scatter of the results obtained with each method is shown in Fig. 2. The correlation coefficient was 0.51 ($0.10>p>0.05$, Student's t test).

SIZE DISTRIBUTION OF THE

PROTEOGLYCANS IN SYNOVIAL FLUID

Normal bovine synovial fluids were found to contain

mainly small proteoglycan molecules (Fig. 3a). In contrast, osteoarthritic human synovial fluids contained a significant proportion of aggregated proteoglycan in addition to monomeric proteoglycans of diverse molecular weight (Fig. 3b). The difference in the position of the peaks indicates that the proteoglycans of varying hydrodynamic size differ with respect to their sulphated GAG content. In normal bovine synovial fluid the proteoglycans of higher molecular weight (corresponding to effluent volumes 38–48 ml) are richer in sulphated GAG than those of lower molecular weight (corresponding to effluent volumes 49–58 ml). In human synovial fluid from patients with osteoarthritis it can be seen from the data in Fig. 3b that the aggregated proteoglycans (corresponding to effluent volumes 20–25 ml) have a lower sulphated GAG content than the large and small proteoglycan monomers. The presence of proteoglycan aggregates in the human synovial fluid indicates that some of the proteoglycan molecules are bound to hyaluronic

Fig. 2 The distribution of GAG concentrations in 15 osteoarthritic knee joint synovial fluids.

Fig. 3 Size distribution of the cartilage degradation products in (a) normal bovine synovial fluid and (b) osteoarthritic human knee joint synovial fluid.

acid and suggests that these molecules still possess a functionally intact binding domain.

Discussion

The DMB assay and RIA were both found to be

sufficiently sensitive for measurement of cartilage degradation products in synovial fluid. Samples of synovial fluid were assayed directly with the RIA, whereas previous digestion with papain was necessary for the DMB assay. The reproducibility of the assays was found to be satisfactory for the DMB

assay, but not the RIA. The results obtained with the RIA, however, are consistent with those reported by Saxne *et al* and also Gysen *et al*.^{3 4 10}

The DMB assay has several advantages over RIAs. It does not require radioisotopes and is therefore safer. The required reagents are readily available and inexpensive. It also has a theoretical advantage since it measures the glycosaminoglycan moiety of proteoglycans, and the polysaccharides are less likely to be degraded than the core protein component. Moreover, since it is a chemical method highly comparable results should be obtained when homogeneous groups of patients are studied in different centres.

The reason for the poor correlation between the DMB assay and the RIA is not entirely clear. The poor reproducibility of the RIA may be relevant. Two other possibilities need to be considered. Proteoglycans from articular cartilage contain approximately equal proportions of chondroitin sulphate and keratan sulphate and have 30–50% protein.¹¹ Use of chondroitin sulphate as standard for the DMB assay is likely to underestimate the total GAG content since chondroitin sulphate and keratan sulphate differ in their reactivity with DMB.⁸ It is also possible that the antiserum used in the RIA does not recognise all of the proteoglycan present in synovial fluid either by virtue of its nature or because some of the proteoglycan molecules in synovial fluid have lost their recognition sites.

Gel chromatography of normal bovine synovial fluid showed the presence of predominantly small proteoglycan molecules. No proteoglycan aggregates were detected. These findings are unlikely to be due to proteolysis during storage or chromatography since consistent results were obtained when fresh specimens were chromatographed immediately in the presence of various proteinase inhibitors. The remote possibility of rapid postmortem catabolism, however, cannot be excluded. Whether the size distribution of the proteoglycans in normal human synovial fluid is similar is not yet known.

In contrast, a significant proportion of the proteoglycans present in osteoarthritic synovial fluid were found to be aggregated and most of the monomers present were of higher molecular weight. This distribution is in agreement with that found by Gysen *et al* and closely resembles the size distribution of the proteoglycans which can be extracted from human articular cartilage with high concentrations of salt.¹⁰ Although the mechanism governing the release of proteoglycans from osteoarthritic cartilage is still a matter of conjecture, the presence in the synovial fluid of proteoglycans with a functionally intact hyaluronic acid binding region suggests that at least a subset of proteoglycans may be

released from cartilage without enzymatic cleavage of this region.

In summary, the modified DMB assay has been shown to be suitable for measuring glycosaminoglycans in synovial fluid. In addition to being sufficiently sensitive, specific, and reproducible the method is simple, safe, and inexpensive. The DMB assay expands the range of methods now available for measuring cartilage degradation products in synovial fluid. These techniques are likely to prove increasingly useful for monitoring disease progression and the effects of various therapeutic strategies. In combination with other lines of inquiry they are also likely to provide further insight into the mechanisms responsible for cartilage resorption in arthritic diseases.

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