Characterisation of human articular cartilage link proteins from normal and osteoarthritic cartilage

JUNNOSUKE RYU, CHRISTINE A. TOWLE, AND BENJAMIN V. TREADWELL

From the Orthopaedic Research Laboratories, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA

SUMMARY Proteoglycan link proteins were isolated from human articular cartilage obtained from normal and osteoarthritic femoral heads and purified to homogeneity employing a method previously described by this laboratory. The link proteins were analysed for amino acid composition, molecular weight on sodium dodecyl sulphate polyacrylamide gels, and ability to stabilise proteoglycan aggregates. The results of these studies were compared with those obtained with bovine link proteins. Two link proteins were identified in the purified fraction from normal and osteoarthritic human cartilage with apparent molecular weights of 54 000 (link 1) and 48 000 (link 2). Functionally the link proteins, isolated from osteoarthritic and normal cartilage, were indistinguishable as measured by their ability to stabilise aggregate. The amino acid compositions of normal and osteoarthritic link proteins were also found to be similar to each other but significantly different from the amino acid composition reported for the bovine link proteins. The quantities of these proteins in extracts from normal and diseased tissue were similar, as was the ratio of link protein 1 to link protein 2.

Articular cartilage contains few cells and an abundance of surrounding extracellular matrix, consisting mainly of collagen and proteoglycans. The elasticity of cartilage is largely related to the content and the structure of the proteoglycans in the matrix. Proteoglycan aggregate, a structural unit of cartilage, is composed of proteoglycans, hyaluronic acid, and 2 link proteins which differ in molecular weight and chemical composition. The relationship between proteoglycan aggregation and cartilage structure and function is not clearly understood.

The link proteins are believed to stabilise the binding of proteoglycan monomer to hyaluronate. Although the interaction between subunit and hyaluronic acid can occur independently, stability of the aggregate to alteration in ionic concentration, pH, centrifugal pressure, and temperature is enhanced by the presence of link proteins.^{1,2} In all hyaline cartilage studied with the exception of rat chondrosarcoma³ and fetal cartilage⁴ 2 link proteins have been reported. The relative amounts of each of these 2 proteins (molecular weight 45–54 000 (link 1), and 40–48 000 (link 2)) are different depending

on the source of cartilage. The importance of this ratio or in fact of link 2 is not known. There is evidence that this lower molecular weight species is derived from link 1.^{5,6}

Osteoarthritis is a progressively destructive disease of the articular surfaces of joints. It is characterised by localised erosion of the cartilage surface and is accompanied by a variety of biochemical and metabolic changes. An example of a biochemical change reported by several laboratories is the decreased capacity of extracted proteoglycan from osteoarthritic cartilage to interact with hyaluronic acid to form aggregate.^{7–9} Palmoski and Brandt¹⁰ examined the ability of proteoglycans from hip cartilage of patients with osteoarthritis to bind to hyaluronic acid. Their results suggested that progressive morphological changes found in osteoarthritic tissue are associated with progressive defects in proteoglycan aggregation, owing at least in part to impaired ability of the proteoglycans to interact with hyaluronic acid. However, more recent work by Brandt *et al.*¹¹ showed that the hyaluronate binding region of proteoglycans isolated from bovine normal and osteoarthritic cartilage to be functionally intact. They suggested that diminished aggregation of proteoglycans in osteoarthritic cartilage may be due to an abnormality in

some other constituent of the aggregate. It was this impairment which stimulated this study to determine if there was any structural, functional, or quantitative change in the link proteins in osteoarthritic cartilage.

Materials and methods

The 2 groups of patients in this series included 5 women (average age 65.2 years, age range from 53 to 74) with osteoarthritis of the hip (as diagnosed by roentgenogram and histological section),¹² and the normal group consisted of 2 women and 1 man (average age 68 years, age range from 53 to 74) who had replacement of a normal femoral head with an end prosthesis after fracture of the neck of the femur. All osteoarthritic femoral heads, obtained at the time of total hip replacement, had partial areas of cartilage loss, denudation down to sclerotic bone, and localised osteophyte formation in marginal areas. Therefore sampling was obtained from less involved areas, and special care was taken to exclude osteophytic newly formed repair tissue or fibrocartilage and to avoid resecting underlying bone. All material was obtained in the fresh state at the time of resection of the femoral head. Resected femoral heads were stored at -40°C until ready to use.

ISOLATION OF LINK

The procedure used was as described by Amadio *et al.*¹³ with some modification. All procedures were carried out at 4°C unless otherwise stated. Normal and osteoarthritic human articular cartilage was resected and minced into 1–2 mm size pieces. Total resected wet weight of each cartilage type was 11.3 g from the normal femoral heads and 12.3 g from the osteoarthritic femoral heads. The cartilage was extracted by continuous stirring in 250 ml of a solution containing 6 M guanidium chloride (GdmCl), 75 mM sodium acetate pH 7.0, 10 mM EDTA, and 5 mM benzamidine for 72 hours. The nonsolubilised cartilage was separated from the solution by passage through 2 layers of cheese cloth. The solution was further clarified by centrifugation at 15 000 g for 20 min.

The clear, viscous supernatant was centrifuged at 100 000 g for 17 hours. The upper 2/3rds of the supernatant was removed and dialysed overnight against buffer A: 1.0 M NaCl, 20 mM TrisCl pH 7.5. The protein in the solution was precipitated by the addition of ammonium sulphate to 80% saturation adjusted to pH 3.8 with concentrated HCl and collected by centrifugation at 15 000 g for 15 min. The precipitate was dissolved in Buffer A and dialysed overnight against the buffer. 1 mg of high molecular weight hyaluronic acid was then added to the dialysed extract for each 25 mg of protein. The solution was

dialysed for 3 hours against buffer B: 4M GdmCl, 20 mM TrisCl, pH 5.8, and finally, the extract was dialysed for 2 hours against 0.5 M sodium acetate, pH 7.0. 8 ml aliquots of this solution were passed over a column of CL-Sepharose 6B equilibrated with 0.5 M sodium acetate, pH 7.0, and eluted with this buffer (2.6 × 35 cm, flow rate 15–20 ml/hour). Protein containing fractions eluting in the void volume were pooled and dialysed against buffer B containing 50% glycerol. The dialysed solution (4–5 ml) was applied to a Sephacryl S-200 column (2.6 × 43 cm, flow rate 6 ml/hour) which had been equilibrated and eluted with buffer B. Protein containing fractions from the included volume just beyond the excluded peak were pooled, dialysed against Buffer A in 50% glycerol, and stored at -20°C until ready to use. Further purification of the link preparation by refiltration on the Sephacryl column was only occasionally necessary. The preparation was usually more than 90% pure (Table 1).

AMINO ACID ANALYSIS OF LINK PROTEIN

Samples for amino acid analysis were hydrolysed in HCl for 6 hours at 110°C in vacuo before application to a Durham Amino Acid Analyzer.

SODIUM DODECYL SULPHATE

POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED LINK

Gel electrophoresis was performed according to the procedure of Weber and Osborn¹⁴ in the presence of sodium dodecyl sulphate, with 7.5% polyacrylamide gels. Samples containing 10 µg of protein were dialysed against a buffer containing 1% 2-mercaptoethanol, 10 mM sodium phosphate pH 7.0, and 50% glycerol for 4 hours at 37°C prior to electrophoresis, which was carried out at room temperature for 4 hours at a constant current of 6 mA/gel. Molecular weight standards were procollagen (140 000), albumin (68 000), ovalbumin (45 000), and myoglobin (17 800).

GEL FILTRATION OF PROTEOGLYCAN AGGREGATES

Bovine proteoglycan subunit (fraction A1D1) was

Table 1 Purification of link proteins from normal and osteoarthritic femoral heads

	Normal (3 heads)	Osteoarthritic (5 heads)
Total resected cartilage (wet wt.)	11.3 g	12.3 g
GdmCl extracted protein	200 mg	400 mg
Protein in upper 2/3rds of centrifuge tube (after dialysis)	119 mg	153 mg
Protein in (NH ₄) ₂ SO ₄ precipitate	60 mg	42 mg
Protein in excluded peak of 6B column	9.2 mg	16 mg
Link protein after S-200 column	0.4 mg	0.4 mg

prepared by the procedure described by Heinegard.¹⁵ Purified AIDl was lyophilised and stored at -20°C until ready to use.

Samples in a final volume of 0.5 ml contained the following components: (1) 500 μg AIDl; (2) 500 μg AIDl with 6 μg of high molecular weight hyaluronic acid; (3) 500 μg AIDl with 6 μg of high molecular weight hyaluronic acid; and 40 μg of either normal or osteoarthritic articular link protein. Each sample was prepared in buffer A and dialysed against buffer B for 6 hours to dissociate the elements of the sample and then reassociated by dialysis against several change of 0.5 M sodium acetate buffer at either pH 7.0 or pH 4.0 and then applied to a column of CL-Sepharose 2B (1 $\times$ 22 cm, flow rate 18 ml/hour) equilibrated and eluted with 0.5 M sodium acetate buffer at either pH 7.0 or pH 4.0. An automated carbazol method for uronic acid determination¹⁶ was used to detect the elution pattern of the proteoglycan under the various pH conditions.

Results and discussion

This work describes the isolation, purification, and characterisation of human articular cartilage link proteins purified from human normal and osteoarthritic femoral heads. The purification procedure yields link preparations from both tissue types consisting of 2 proteins, link 1 and 2. These 2 proteins migrated on 7.5% SDS polyacrylamide gels as species with apparent molecular weight of 54 000 (link 1) and 47 500 (link 2) (Fig. 1). The purified link proteins from normal and diseased tissue had similar ratios of link 1 to link 2 (1:1), as is apparent from Fig. 1. This is in contrast to the 2:1 ratio obtained with link preparations isolated from calf articular cartilage and bovine nasal septum.¹⁷ The apparent molecular weight of human and bovine link proteins, however, are identical as is evident from the figure showing the 2 comigrating on SDS gels.

The amino acid compositions of these proteins is essentially the same for osteoarthritic and normal human articular preparations (Table 2), though very different from those reported for the bovine preparations.¹⁷ One striking difference is the presence of 6 methionine residues in human as compared to only 2 in the bovine links. It will be interesting to determine the number of cleavage products produced by cyanogen bromide treatment of the human link proteins.

A method to measure link protein's ability to stabilise aggregate has been reported^{1 2} and was used here to assay for this activity in preparations of normal and osteoarthritic link proteins. The results of this assay (Fig. 2) demonstrate the ability of either

Fig. 1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of link proteins. Link proteins (8–10 μg) were subjected to electrophoresis in 7.5% polyacrylamide gels in sodium dodecyl sulphate and stained with Coomassie blue. Link fractions run on individual gels were as follows: (1) 10 μg of calf articular cartilage link; (2) 8 μg of normal human articular cartilage link; (3) 8 μg of osteoarthritic human articular cartilage link; (4) 3 μg each of normal and osteoarthritic human articular cartilage link and bovine link.

preparation to stabilise the proteoglycan hyaluronic acid interaction at pH 4.0. Although not shown, this assay was performed with several lower concentrations of link proteins, and the results again showed no significant difference in aggregate stabilising activity between link preparations.

The link purification scheme given in Table 1 shows that the quantity of link purified from either source was nearly identical. As is also evident from the table, there is nearly twice as much protein

Table 2 Amino acid analysis of purified link fractions

Amino acid	Human articular link (normal)	Human articular link (osteoarthritic)	Bovine articular link
Residues/1000 residues			
Aspartic acid	105	107	127
Threonine	61	60	45
Serine	74	74	52
Glutamic acid	101	97	82
Proline	61	67	48
Glycine	98	90	99
Alanine	87	78	77
Valine	57	67	73
Methionine	7	6	2
Isoleucine	40	36	33
Leucine	90	103	86
Tyrosine	42	32	62
Phenylalanine	46	46	58
Histidine	30	37	24
Lysine	42	49	58
Arginine	60	51	72

Fig. 2 CL-Sepharose 2B filtration of proteoglycan aggregates.

present in the initial osteoarthritic cartilage extract. This result is not surprising in view of reports from other laboratories showing the greater extractability of proteoglycans from osteoarthritic cartilage.⁷ That link protein is not also present in increased quantities is interesting and may mean that this protein is either more extractable than cartilage proteoglycans or more susceptible to degradation in the osteoarthritic extract. There have been reports of an increase level of proteolytic enzymes¹⁸ in extracts of osteoarthritic cartilage, which could account for degradation of link.

The purpose of this study was to establish whether

the observed decrease in proteoglycan aggregate formation observed with extracts isolated from osteoarthritic cartilage was a result of a qualitative and/or quantitative change in the link protein. The results presented here suggest that these proteins isolated from osteoarthritic cartilage have similar functional and physical properties to link protein from normal articular cartilage. In addition the amounts of link protein extractable from normal and diseased cartilage are nearly identical.

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