Radioimmunoassay of the 148-kilodalton cartilage protein

Distribution of the protein among bovine tissues

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A sensitive and specific radioimmunoassay for the 148 kDa cartilage protein (previously referred to as 'cartilage matrix protein') was developed. The protein is insoluble in conventional buffers and was therefore dissolved in sodium dodecyl sulphate. Excess sodium dodecyl sulphate was bound in mixed micelles with Triton X-100 before assay. Antibodies raised against the 148 kDa cartilage protein did not react with other cartilage macromolecules. Neither did they react with guanidinium chloride extracts of a number of non-cartilaginous tissues. The protein, then, is unique for cartilage. It was demonstrated in tracheal, nasal-septum, xiphisternal, auricular and epiphyseal cartilage. Surprisingly, the protein was detected neither in extracts of articular cartilage nor in extracts of the annulus fibrosus or the nucleus pulposus of the intervertebral disc.

The 148 kDa cartilage protein* was first identified in bovine tracheal cartilage, as a protein that co-fractionated with cartilage proteoglycans (Paulsson & Heinegård, 1979). The purified protein was found to contain three polypeptide chains, each having a molecular mass of 52 kDa (Paulsson & Heinegård, 1981). Its oligomeric structure requires intact disulphide bonds.

To understand the physiological function of the 148 kDa cartilage protein, a number of questions will have to be answered. Is it structurally related to any other cartilage macromolecule? Is it present only in cartilage? What is the specificity of its interaction with cartilage proteoglycans? In the study of these problems a method for the assay of the protein is required. An immunoassay method would provide both the necessary specificity and sensitivity. The design of such an assay method is complicated by the insolubility of the 148 kDa protein in solvents other than chaotropic salts and ionic detergents, e.g. sodium dodecyl sulphate. It has, however, been shown that proteins dissolved in sodium dodecyl sulphate may be detected by immunochemical methods, particularly when excess sodium dodecyl sulphate is bound in mixed micelles with a non-ionic detergent, such as Triton X-100 (Converse &

Papermaster, 1975). More recently, Caterson *et al.* (1979) developed a radioimmunoassay method, using a similar principle, in which cartilage link proteins were dissolved in a mixture of deoxycholate and Nonidet P-40.

Experimental

Purification of antigens

The 148 kDa protein was purified from bovine tracheal cartilage and its subunits were prepared by reduction and carboxymethylation as described elsewhere (Paulsson & Heinegård, 1981). Cartilage proteoglycan core protein, hyaluronic acid-binding region and link protein were prepared as described previously (Wieslander & Heinegård, 1979). Type II collagen was prepared from bovine nasal-septum cartilage by using a modification of the procedure of Miller (1972). The 36 kDa protein was isolated from bovine tracheal cartilage (M. Paulsson, Y. Sommarin & D. Heinegård, unpublished work) by using a side fraction obtained in the purification of the 148 kDa protein (Paulsson & Heinegård, 1981).

¹²⁵I-labelling of the 148 kDa cartilage protein

The 148 kDa protein was iodinated by a modification of the chloramine-T procedure of Greenwood *et al.* (1963). A stock solution (0.5 mg/ml) of the protein was made in 6 M-urea/5 mM-Tris/HCl buffer, pH 7.5. A 25 µl portion (containing 12.5 µg of protein) was mixed with 5 µl (0.5 mCi) of [¹²⁵I]iodide and 20 µl of chloramine-T (2 mg/ml) in

*The 148 kDa cartilage protein has previously (Paulsson & Heinegård, 1979, 1981) been referred to as the 'cartilage matrix protein'. An operational nomenclature is now introduced to allow simplified identification of novel non-collagenous cartilage proteins. Each such protein is designated by its molecular mass.

the urea/Tris/HCl buffer. The reaction was stopped after 60 s by addition of 100 μ l of sodium metabisulphite (1 mg/ml) in the urea/Tris/HCl buffer. The sample was diluted with 400 μ l of 1% (w/v) sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.5, and transferred to a column (V_t 10 ml) of Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). The column had been pretreated by elution with 2 ml of 1% (w/v) bovine serum albumin in the sodium dodecyl sulphate/phosphate buffer, and was subsequently eluted with the same buffer not containing bovine serum albumin. The iodinated 148 kDa protein was recovered from the void volume.

Preparation of antisera

Samples (150 μ g) of the 148 kDa protein, suspended in 0.9% NaCl, were used for immunization of rabbits, initially together with Freund's complete adjuvant and for booster after 1 month together with Freund's incomplete adjuvant. The animals were bled twice monthly, starting 1 month after the first booster. Sera were not purified further.

Second antibody was raised in sheep immunized with rabbit immunoglobulin G Fab fragments. The immunoglobulin fraction was isolated by $(\text{NH}_4)_2\text{SO}_4$ precipitation.

Radioimmunoassay protocol

^{125}I -labelled 148 kDa protein, as well as samples to be analysed, were diluted with 0.1% bovine serum albumin/0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4. Samples of ^{125}I -labelled antigen solution (50 μ l containing 2–3 ng of the 148 kDa protein, i.e. 15 000–30 000 c.p.m.) and 50 μ l of test sample solution were mixed. Then 100 μ l of 4% (w/v) Triton X-100/10 mM-sodium phosphate buffer, pH 7.4, and 100 μ l of the appropriate dilution of the first antiserum in diluent buffer (0.5% bovine serum albumin/0.9% NaCl/0.1% NaN_3 /50 mM-sodium phosphate buffer, pH 7.5) were added. After mixing, the tubes were incubated for 20 h at 4°C. Subsequently 100 μ l of the appropriate dilution of the second antiserum in diluent buffer, containing 5% (w/v) poly(ethylene glycol) 6000, was added, and the tubes were incubated for another 20 h at 4°C. The mixture was diluted with 1 ml of the diluent buffer before centrifugation. The supernatant was decanted and the radioactivity of the precipitate was determined. Samples were assayed in triplicate and the average of the determinations was used for calculations.

Gel chromatography of a tracheal-cartilage extract

Bovine tracheal cartilage was extracted with guanidinium chloride as described previously (Paulsson & Heinegård, 1981). A sample of the extract was mixed with 9 vol. of ethanol and incubated

overnight at 4°C to precipitate proteins and proteoglycans. The precipitate was recovered by centrifugation in a bench-top centrifuge and washed twice with ethanol. It was redissolved in 1% sodium dodecyl sulphate/5 mM-sodium phosphate buffer, pH 7.4, and chromatographed on a column (0.8 cm $\times$ 138 cm) of Sepharose CL-4B (Pharmacia Fine Chemicals) eluted with the same buffer. Fractions (1.1 ml) were analysed for protein content with an automated version (Heinegård, 1973) of the Lowry procedure (Lowry *et al.*, 1951), with bovine serum albumin as standard. A sample of each fraction was diluted with 99 vol. of 0.1% bovine serum albumin/0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4, before quantitative determination of the 148 kDa protein by radioimmunoassay.

Preparation of tissue extracts

Samples of the following bovine tissues were obtained from 2-year-old animals directly after slaughter: cartilage from tracheal rings, nasal septum, xiphoid process of the sternum, ear, fetlock joint, knee joint (femoral surface) and intervertebral disc (anulus fibrosus as well as nucleus pulposus); aortic wall; skin; tendon; skeletal muscle; kidney and liver parenchyma; cornea, lens, sclera and vitreous body of the eye. Epiphyseal cartilage was from a calf about 6 months of age. The tissues were carefully dissected to prepare samples as pure and homogeneous as possible, and the pieces were frozen at –60°C until used. While still frozen, the pieces were finely minced with a scalpel.

The wet weight of each sample was recorded, and ice-cold 5 M-guanidinium chloride/5 mM-sodium phosphate buffer, pH 7.4, containing the proteinase inhibitors 5 mM-benzamidine chloride, 0.1 M-6-aminohexanoic acid, 10 mM-EDTA and 10 mM-N-ethylmaleimide, was added in a proportion of 20 ml/g of tissue. Extraction proceeded for 20 h at 4°C under gentle agitation. Unextracted material was removed by centrifugation at 9000 g_{av} for 30 min at 4°C. An extract of the mineralized matrix of adult bovine diaphyseal bone was prepared by Dr. A. Franzén (University of Lund). Ground bone had been pre-extracted, in a manner similar to that described above, to remove material not integral in the mineralized matrix. The matrix was then dissolved by extraction with a solvent as above, but also containing 0.25 M-EDTA (A. Franzén & D. Heinegård, unpublished work).

A 1 ml portion of each extract, corresponding to an equal amount of wet tissue, was mixed with 9 vol. of ethanol and kept at 4°C overnight. The precipitates were recovered by centrifugation in a bench-top centrifuge, briefly dissolved or suspended in 1 ml of 0.5 M-sodium acetate buffer, pH 7.0, and again precipitated with ethanol, as above, to remove

residual guanidinium chloride. These precipitates were dissolved in 5 ml of 0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4. Serial dilutions were made in 0.1% bovine serum albumin/0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4.

Dissociative CsCl-density-gradient centrifugation of cartilage extracts

Measured volumes (2.5–5.0 ml) of the cartilage extracts were adjusted to the density of 1.40 g/ml by addition of solid CsCl and diluted to 13.5 ml by addition of CsCl/extraction solvent of the same density. The samples were centrifuged at 38 000 rev./min (100 000 g , r_{av} , 6.1 cm) in the Beckman 70.1 Ti rotor for 60 h at 18°C. By using a tube slicer, the gradients were cut into a top fraction of 4.7 ml and a bottom fraction of 8.8 ml, corresponding to 35% and 65% respectively of the total volume. Measured samples of the top fractions, corresponding to an equal amount of wet tissue, were precipitated with ethanol, by using the same procedure as for whole extracts. The precipitates were analysed by radioimmunoassay and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Neville (1971), but with 1.5 mm-thick slab gels. The gels were 8.0% T $\times$ 2.5% C, where the first numeral (T) designates the total amount of monomer (% w/v) and the second numeral (C) designates the amount of *NN'*-methylenebisacrylamide, expressed as a fraction (% w/w) of total monomer in the gel casting solution. Identical samples were either treated with 2-mercaptoethanol, to reduce disulphide bonds, or not treated. Gels were stained for protein with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.). Apparent molecular weights were determined by comparison with reference proteins (high-molecular-weight and low-molecular-weight standard proteins; Pharmacia Fine Chemicals).

Results and discussion

Assay conditions

In initial experiments the different parameters of the assay method were tested. Radioimmunoassay in the presence of detergents was tested by using modifications of the assay for link proteins previously described (Wieslander & Heinegård, 1980). The presence of 0.2% sodium dodecyl sulphate/2% Triton X-100 in the antigen solution did not interfere with the assay. With this procedure we could omit the maleylation step, which was previously required to render the link proteins soluble. The assay may also be performed in dilute sodium dodecyl sulphate

without addition of Triton X-100. Preferably, however, Triton X-100 should be included to bind excess sodium dodecyl sulphate in mixed micelles and thereby decrease the risk of denaturation of immunoglobulins.

The sodium dodecyl sulphate/Triton X-100 solvent system worked equally well in the assay for the 148 kDa cartilage protein. The kinetics of the first and second antibody reactions were determined. In both cases maximal precipitation had been achieved at 20 h of incubation. The optimal ratio between first and second antibody was determined. Variations in temperature between 4°C and 24°C did not greatly affect the assay, and incubations were subsequently done at 4°C. With high concentrations of first

Fig. 1. Inhibition in the radioimmunoassay by 148 kDa cartilage protein standards

For full experimental details see the text. (a) Inhibition expressed as B/B_0 versus concentration in sample (logarithmic scale), where B is the radioactivity (c.p.m.) bound in sample determinations and B_0 is the radioactivity (c.p.m.) bound in the absence of competing antigen. (b) Inhibition expressed as $\text{logit}(B/B_0)$ versus $\log(\text{concentration})$ (Rodbard *et al.*, 1969).

antibody, 80–85% of the ^{125}I -labelled 148 kDa protein could be precipitated. A dilution of 1:3200 of the first antiserum gave approximately half-maximal precipitation and was used as a routine in inhibition experiments.

The inhibition curves were symmetrical (Fig. 1a), and half-maximal inhibition was obtained with about 3 ng/sample of competing antigen, a value in good agreement with the amount of iodinated antigen present. By using the logit/log plot procedure of Rodbard *et al.* (1969), a straight line was obtained (Fig. 1b). Such plots were subsequently used to calculate amounts. The assay could be used over a range from 0.2 to 50 ng/sample, even though the accuracy was expectedly higher in the middle portion of the range.

Effect of reduction and carboxymethylation of the 148 kDa cartilage protein

As the procedure for extraction and purification of the 148 kDa protein utilizes the denaturing conditions of 4 M- or 5 M-guanidinium chloride and the molecules are treated with 0.4% sodium dodecyl sulphate before assay, it is unlikely that the antigens are in a native state. The protein is, however, still recognized by the antibodies. It was therefore considered relevant to determine if the more extensively denatured subunits of the 148 kDa protein react with the antibodies raised against the intact protein. Such subunits were prepared by reduction and carboxymethylation (Paulsson & Heinegård, 1981). Compared with the intact protein, they were significantly less capable of inhibiting the binding of iodinated antigen (Fig. 2). The shape of the curve indicates that the subunits compete only for a fraction of the antibodies, and then with a lower affinity. This loss of antigenic determinants may be due to loss of tertiary and quaternary structure on dissociation of the trimeric complex and/or to interference of the carboxymethylated cysteine residues with antigenic sites.

Specificity of the assay method

To determine if the 148 kDa protein is antigenically related to other known cartilage macromolecules, inhibitory capacity in the assay was tested for proteoglycan core protein, proteoglycan hyaluronic acid-binding region, link protein, type II collagen and 36 kDa protein, a protein quantitatively prominent in tracheal cartilage (Paulsson & Heinegård, 1981). None of the preparations gave significant inhibition (results not shown), i.e. more than a 1000-fold excess compared with the amount of 148 kDa protein was required for inhibition. Inhibition at those high concentrations of competing antigen is probably due to minute (less than 0.1%) contaminations of 148 kDa protein antigen in the preparations used.

Fig. 2. Inhibition in the radioimmunoassay by the subunits of the 148 kDa protein (■) and the intact protein (●). For full experimental details see the text. For definition of B/B_0 see Fig. 1 legend.

The specificity was further demonstrated by separating the proteins and proteoglycans obtained in a guanidinium chloride extract of bovine tracheal cartilage by gel chromatography on Sepharose CL-4B (Fig. 3). The 148 kDa protein was eluted as a distinct peak identified by the radioimmunoassay. The proteoglycans were eluted well separated at or close to the void volume, whereas the link proteins and the 36 kDa protein were eluted more retarded. Therefore the assay method appears to be highly specific for the 148 kDa protein, and this protein was found to be antigenically different from other matrix macromolecules.

Tissue distribution of the 148 kDa cartilage protein

Having demonstrated the specificity of the radioimmunoassay, it was then possible to determine the distribution of the 148 kDa protein among bovine tissues. 5 M-Guanidinium chloride, a solvent that efficiently extracts the 148 kDa protein from tracheal cartilage (Paulsson & Heinegård, 1981), was assumed to extract the protein also from other tissues. Proteins were recovered from the extracts by ethanol precipitation and dissolved in the 0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4, used in the assay. Serial dilutions made from extracts of the following non-cartilaginous tissues were tested in the radioimmunoassay (results not shown): bone, aortic wall, skin, tendon, cornea, lens, sclera, vitreous body, skeletal muscle, kidney and liver. None of these

Fig. 3. Sepharose CL-4B gel chromatography of cartilage proteins in 1% sodium dodecyl sulphate/5 mM-sodium phosphate buffer, pH 7.4

For the preparation of the samples and other experimental details see the text. The curves designate total protein (—) and the 148 kDa cartilage protein, as determined by radioimmunoassay (---). The arrow indicates the elution position of purified 148 kDa protein, when chromatographed on the same column. V_0 and V_t indicate the void volume and the total volume respectively.

tissues contained detectable amounts of the 148 kDa protein, by using a procedure with a detection limit of about 1 µg of protein/g wet wt. of tissue. It appears that the 148 kDa protein is unique to cartilage.

Moderate inhibitions were obtained when dilution series of bovine sera were tested, corresponding to about 110 ng and 80 ng of the 148 kDa cartilage protein/ml in foetal and adult serum respectively (results not shown). It is possible that small amounts of the protein are released to the blood. These concentrations, however, are close to the detection limit in the assay. Therefore rather concentrated serum samples were used, and the inhibition could be due to unspecific interference by plasma proteins. In support, no distinct peak of inhibitory activity could be seen when serum was fractionated by ion-exchange chromatography (results not shown).

The cartilages analysed for content of the 148 kDa protein were from tracheal rings, nasal septum, xiphisternal process, articular surfaces of the fetlock and knee joints, epiphysial growth plate, ear and the anulus fibrous and nucleus pulposus of the intervertebral disc. Radioimmunoassay was done on material recovered by ethanol precipitation of 5 M-guanidinium chloride extracts, as described above for the non-cartilaginous tissues, but also on partially purified protein fractions obtained by direct dissociative CsCl-density-gradient centrifugation.

The density-gradient centrifugation was performed to remove proteoglycans and thereby allow parallel comparative analysis by radioimmunoassay and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Under the conditions used, all proteins are recovered in the top third of the gradient (Paulsson & Heinegård, 1981), and the radioimmunoassays of

Fig. 4. Inhibition in the radioimmunoassay by samples from CsCl-density-gradient top fractions prepared from different bovine cartilages

For the preparation of the samples and other experimental details see the text. Samples were from tracheal (a), nasal-septum (b), xiphisternal (c), auricular (d), epiphysial (e) and articular (f) cartilage. Each sample represents an equal wet weight of tissue. For definition of B/B_0 see Fig. 1 legend.

Table 1. Concentration of the 148 kDa protein in bovine cartilages determined with radioimmunoassay

The tissues were extracted with 5 M-guanidinium chloride and the protein fractions were isolated by CsCl-density-gradient centrifugation, as described in the Experimental section.

Cartilage	148 kDa cartilage protein (mg/g wet wt. of tissue)
Tracheal	13.62
Nasal-septum	1.12
Xiphisternal	0.432
Auricular	0.036
Epiphysial	0.003
Articular (fetlock joint)	Not detected
Articular (knee joint)	Not detected
Anulus fibrosus	Not detected
Nucleus pulposus	Not detected

the extracts and of the CsCl-density-gradient top fractions were accordingly in complete agreement. Some of the inhibition curves are depicted in Fig. 4. The amount of the 148 kDa protein in the extracts varies remarkably between the different cartilages. The protein is most enriched in tracheal cartilage (Table 1), but is present in comparatively large amounts also in nasal-septum and xiphisternal cartilages. Cartilage from ear and epiphysial growth plate contain small amounts of the protein, and it could not be detected at all in articular cartilage or in any part of the intervertebral disc (Table 1).

The differential distribution of the 148 kDa protein among cartilages was confirmed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of some of the CsCl-density-gradient top fractions (Figs. 5a and 5b). The reduced subunits of the 148 kDa protein migrate with an apparent molecular weight of 60 000 (Fig. 5a; Paulsson & Heinegård, 1981). A prominent band with this mobility was seen in the sample from tracheal cartilage and, gradually fainter, in the samples from nasal-septum and xiphisternal cartilage. Some of the other samples also gave bands of similar

Fig. 5. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of samples from CsCl-density-gradient top fractions prepared from different bovine cartilages

For the preparation of the samples and other experimental details see the text. (a) Samples reduced with 2-mercapto-ethanol. (b) Samples not reduced. Lane 1, tracheal cartilage; lane 2, nasal-septum cartilage; lane 3, xiphisternal cartilage; lane 4, auricular cartilage; lane 5, epiphysial cartilage; lane 6, articular cartilage; lane 7, annulus fibrosus. Each sample represents an equal wet weight of tissue.

mobility. As the 148 kDa protein is an oligomeric protein, sensitive to reduction, further information could be gained by electrophoresis without prior reduction of samples (Fig. 5b). Under those conditions the 148 kDa protein is seen as a band close to the top of the gel. Again, this band was prominent in the sample from tracheal cartilage, and was present, but fainter, in the nasal-septum and xiphisternal cartilage samples. No material of similar mobility was seen in the other preparations. Samples from articular cartilage and intervertebral disc each contained a unique component of somewhat lower mobility than that of the link proteins. Whereas the content of the 148 kDa protein varies markedly between cartilages, the contents of link proteins and the 36 kDa protein appear to be relatively constant (Fig. 5a), indicating that the variation seen is not due to different extractability. It appears that the variation in 148 kDa protein content among the extracts represents a true difference in tissue content of the protein.

General discussion

According to morphological and gross biochemical analysis, different cartilages share many characteristics. The major molecular constituents, collagen and proteoglycans, are fairly constant from one cartilage to another, even though minor compositional differences are recognized. The formation of aggregates between proteoglycans, hyaluronic acid and link proteins also appears to be a constant feature of cartilage.

It was therefore surprising to find such marked differences between cartilages with regard to the amount of the 148 kDa protein present. The protein is not associated specifically with hyaline, fibrous or elastic cartilages. It is indeed difficult to relate the distribution pattern to other known parameters of cartilage composition and structure. This result, and

the observation of other proteins that appear prominent only in some of the cartilages (Figs. 5a and 5b), raises the question if there is a differentiation among cartilages expressed through their non-collagenous proteins. Some of these proteins, such as the link proteins and the 36 kDa protein (Fig. 5a), would then be common to all cartilages, whereas other proteins, such as the 148 kDa protein, would be subject to variation. Proteins of the second group could have functions specific for that very tissue and might be markers of cartilage differentiation.

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