Variation in quantity and extractability of the 148-kilodalton cartilage protein with age

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The occurrence of non-collagenous matrix proteins was studied in samples of tracheal cartilage from steers of different ages. The amounts of the 148 kDa cartilage protein in 4M-guanidinium chloride extracts and in subsequent trypsin digests of the extraction residues were determined by radioimmunoassay. Surprisingly, the 148 kDa-protein antigenicity was not changed by tryptic digestion, even though the protein was extensively degraded. The amount of the 148 kDa protein increased dramatically with age, both in the guanidinium chloride extract and in the subsequent tryptic digest, and reached maximal values at about 3 and 8 years respectively. The increase of the guanidinium chloride-soluble pool preceded that of the trypsin-digestible pool, possibly indicating a metabolic relationship. The ratio between the trypsin-digestible and guanidinium chloride-soluble pools increased continuously, and at 12 years of age close to 90% of the total 148 kDa protein detected was insoluble in guanidinium chloride. At all ages, the bulk of the cartilage collagen was insoluble both to extraction with guanidinium chloride and to tryptic digestion. The decreasing extractability of the 148 kDa protein was therefore not secondary to changes in the solubility of the collagen network. Other cartilage proteins, such as the link proteins and the 36 kDa protein, showed much smaller quantitative variations of a different character.

Cartilage fulfils a number of important functions, being a part of the skeletal system and essential for its growth and development. Therefore much interest has been centred around the structure and composition of its major extracellular-matrix components, collagen and proteoglycans (for references see Bornstein & Traub, 1979; Heinegård & Paulsson, 1984). In recent studies, characterization has been extended to some predominant non-collagenous proteins present in cartilage.

One of these, the 148 kDa protein, is a glycoprotein composed of three similarly sized subunits, joined by disulphide bonds (Paulsson & Heinegård, 1981). Another, the 36 kDa protein, is also glycoprotein in nature and consists of a single polypeptide, rich in leucine (M. Paulsson, Y. Sommarin & D. Heinegård, unpublished work). Whereas the 36 kDa protein appears to be uniformly present in most or all bovine cartilages, the

148 kDa protein is very prominent in some, i.e. tracheal and nasal cartilage, but cannot be detected in guanidinium chloride extracts either of articular cartilage or of the intervertebral disc (Paulsson & Heinegård, 1982). Both these proteins are synthesized by the chondrocytes (Paulsson *et al.*, 1983). The 148 kDa protein appears to be associated with proteoglycans in the matrix, indicated by the co-fractionation with these molecules when isolated by non-denaturing procedures (Paulsson & Heinegård, 1979).

In a number of studies it has been shown that the composition and concentration of cartilage collagen and proteoglycans changes with the aging of the animal (for reference see Inerot & Heinegård, 1982). More recently it was demonstrated that the variation in proteoglycan structure is largely due to gradual changes in the proportions between two distinct populations of aggregating proteoglycans (Inerot & Heinegård, 1983). Such changes could have a considerable influence on the physical properties of cartilage and do therefore provide a possibility of understanding the maturation and aging of the tissue in molecular terms.

In the present work we extend those obser-

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vations by characterizing the changes occurring among prominent non-collagenous proteins in tracheal cartilage during the aging process.

Experimental

Extraction and fractionation of cartilage proteins

The samples of bovine tracheal cartilage were those described previously (Inerot & Heinegård, 1983). They were from two male fetuses, 9 months of gestational age, and from steers 11, 22, 41, 75, 78, 96, 126 and 141 months of age (one animal of each age). Tracheal rings had been dissected free from soft tissues and perichondrium shortly after slaughter of the animals, frozen in liquid N₂ and ground in a Wiley mill.

Samples of frozen cartilage (200 mg) were extracted for 24 h at 3°C in 4 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors 10 mM-EDTA, 0.1 M-6-aminohexanoic acid, 5 mM-benzamidinium chloride and 10 mM-N-ethylmaleimide. The extraction residues were pelleted by centrifugation at 3000 g_{av.} for 15 min, re-extracted in the same buffer and then washed by repeated suspension in water.

The residues were suspended in 0.2 M-sodium phosphate buffer, pH 8.0, containing 0.5 mg of trypsin [1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated, from bovine pancreas; Sigma Chemical Co., St. Louis, MO, U.S.A.]/ml and incubated at 37°C for 48 h under gentle agitation. Trypsin activity was then inhibited by the addition of phenylmethanesulphonyl fluoride to a final concentration of 10 mM. After a brief incubation at room temperature, the trypsin-resistant residue was pelleted by centrifugation, as described above. The residue was extracted with guanidinium chloride, as above, washed with water and finally dissolved by incubation overnight at 60°C in 2.5 ml of 1 M-NaOH. The guanidinium chloride extract of the trypsin-digested residue contained only minute amounts of protein and was therefore later discarded.

In a separate experiment other portions (250 mg) of the cartilage samples were extracted with 5 ml of the guanidinium chloride extraction buffer, as described above, and then briefly resuspended in 2.5 ml of the extraction buffer. The residues were dissolved in 1 M-NaOH for determination of hydroxyproline contents, and the pooled supernatants were fractionated by direct CsCl-density-gradient centrifugation. Solid CsCl and additional extraction solvent was added to give a density of 1.36 g/ml in a final volume of 13.5 ml. The samples were centrifuged in a Beckman 70.1 Ti angle rotor at 40000 rev./min (r_{av} , 6.1 cm, 109000 g) for 60 h at 18°C. The top third of the gradient was separated by using a Beckman tube-slicer. Under the

conditions used this fraction will contain all cartilage proteins and virtually no proteoglycans (Heinegård *et al.*, 1981).

Radioimmunoassay

The amounts of 148 kDa protein were determined by use of the radioimmunoassay described previously (Paulsson & Heinegård, 1982). Briefly, the procedure involves iodination of the protein by the chloramine-T method (Greenwood *et al.*, 1963) and dissolving the ¹²⁵I-antigen and the samples and standards to be assayed in 0.1% bovine serum albumin/0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4. Excess sodium dodecyl sulphate is bound in mixed micelles by the addition of a 10-fold excess of Triton X-100. The assay is then performed with the use of a double-antibody system. All samples were assayed in triplicates and at three different dilutions.

Before assay the samples had to be transferred to the sodium dodecyl sulphate-containing solvent. For extracts containing guanidinium chloride this was accomplished by using precipitation with ethanol as described elsewhere (Paulsson *et al.*, 1983). The precipitates were dissolved and diluted in the sodium dodecyl sulphate-containing buffer used for radioimmunoassay. Samples from the tryptic digests were made 0.1% with regard to ovomucoid, to ensure complete inhibition of trypsin, and 0.4% with regard to sodium dodecyl sulphate, before dilution in the radioimmunoassay buffer.

The proteins extracted with guanidinium chloride were also analysed in the radioimmunoassay for the 148 kDa protein after tryptic digestion. Samples of the extracts were precipitated with ethanol (Paulsson *et al.*, 1983), and the precipitates were suspended in a volume of 0.2 M-sodium phosphate buffer, pH 8.0, containing 0.5 mg of trypsin/ml, identical with the original volume of the sample. Digestion and subsequent handling of the digests were the same as described for the residues.

Samples for the determination of link protein were pretreated and dissolved in an identical manner and assayed by using the same procedure as in the 148 kDa protein assay, but with an anti-serum specific for the link proteins (Wieslander & Heinegård, 1979, 1980).

Gel chromatography

The size of the antigenic fragments of the 148 kDa protein obtained after tryptic digestion was compared by gel chromatography on a column (0.8 cm × 140 cm) of Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden) eluted with 0.4% sodium dodecyl sulphate/10 mM-sodium

phosphate buffer, pH 7.4. Fractions of volume 1.1 ml were collected. Samples chromatographed were purified 148 kDa protein, either not treated or digested with trypsin (1 mg of 148 kDa protein/ml of trypsin solution, conditions as above), and tryptic digests both of the guanidinium chloride-extractable protein and of the guanidinium chloride-insoluble residue from steers approximately 2 years of age. The samples, containing 50 µg of the purified protein or 250 µl of tryptic digests of tissue protein, were made 0.4% with respect to sodium dodecyl sulphate by addition of a concentrated stock solution.

Gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Neville (1971), but with the use of 1.5 mm-thick slab gels. The gels were T 8.0% × C 2.5%, 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 for 2 h at 37°C, to reduce disulphide bonds, or not treated before electrophoresis. Each sample represented an equal amount of tissue, corresponding to 25 µg of hydroxyproline, as determined in the extraction residues. Gels were stained for protein with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.). Apparent molecular masses were determined by comparison with reference proteins ('high-molecular-weight' and 'low-molecular-weight' standard proteins; Pharmacia Fine Chemicals).

Hydroxyproline determination

Residues were dissolved by incubation overnight at 60°C in 2.5 ml of 1 M-NaOH. The samples were neutralized by the addition of 0.75 ml of 5 M-acetic acid. Portions of the guanidinium chloride extracts were precipitated with ethanol (Paulsson *et al.*, 1983), briefly dissolved in 1 M-NaOH and neutralized with 5 M-acetic acid, as with the residues. Samples from the tryptic digests required no pretreatment. Hydrolysis was done in 6 M-HCl at 100°C for 24 h, and the contents of hydroxyproline were determined by the procedure of Stegeman & Stalder (1967).

Results and discussion

Solubilization and quantification of the 148 kDa protein

In previous studies the 148 kDa protein has been solubilized from the tissue by extraction with 4 M- or 5 M-guanidinium chloride. Even though such

solvents do extract considerable amounts of the protein from bovine tracheal and nasal cartilages (Paulsson & Heinegård, 1982), it has not been known if all of the 148 kDa protein present in the tissue is recovered. When planning the present study, aiming at identifying changes occurring among cartilage non-collagenous proteins with aging of the animal, we therefore wanted to determine if additional 148 kDa protein remains in the residue after extraction with guanidinium chloride. We found that tryptic digestion of extraction residues releases large amounts of protein fragments that react in the radioimmunoassay for the 148 kDa protein. Surprisingly, such a digest, as well as a digest of the purified protein, completely inhibited the binding of ¹²⁵I-labelled 148 kDa protein to the antibodies, showing that all antigenic determinants of the intact protein were still present, although no 148 kDa protein could be demonstrated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (results not shown). Control digests containing only trypsin did not affect the assay, when treated with the same combination of proteinase inhibitors as the sample.

To determine if the quantitative response in the assay obtained with the tryptic peptides was similar to that of the intact protein, proteins present in guanidinium chloride extracts of tracheal cartilage were analysed with or without prior trypsin digestion of the samples. The conditions (1:100 relation between enzyme and the wet weight of the original cartilage sample, 48 h of incubation at 37°C) were chosen to ensure limit digestion. The inhibitions obtained with and without trypsin treatment were almost identical, and the sample dilutions gave slopes closely similar to that of the standard curve (Fig. 1). It was concluded that the tryptic peptides could be quantified without any changes in the assay conditions and that the values obtained with digests were representative for the amount of intact 148 kDa protein originally present.

In a separate experiment the sizes of the antigenic fragments obtained from guanidinium chloride-soluble and guanidinium chloride-insoluble 148 kDa protein were compared by gel chromatography on a column of Sephacryl S-300 (superfine grade) (Fig. 2). In both cases tryptic digestion gave rise to a single major antigenic fragment that had an identical elution position whether originating from guanidinium chloride-soluble or from guanidinium chloride-insoluble 148 kDa protein. Thus it appears that the radioimmunoassay quantifies an identical structure in tryptic digests of guanidinium chloride extracts and of the insoluble residues. The relative proportion between the values for the two kinds of

Fig. 1. Inhibition in the 148 kDa-protein radioimmunoassay by guanidinium chloride-extracted proteins, digested (○) or not digested (●) with trypsin

Dilutions of samples from two extracts of bovine tracheal cartilage, containing large and small quantities of 148 kDa protein respectively, are compared with a dilution curve of purified intact 148 kDa protein (■). Inhibition is expressed as B/B_0 , where B is the radioactivity (c.p.m.) bound in sample determinations and B_0 is the radioactivity bound in the absence of competing antigen. For full experimental details see the text.

samples can therefore be expected to be representative.

Variation in quantity of the 148 kDa protein with age

The amounts of 148 kDa protein in 4M-guanidinium chloride extracts and in tryptic digests of the extraction residues were determined for tracheal cartilage samples from steers ranging from 0 to 12 years of age (Fig. 3). The values given for the guanidinium chloride extracts were determined after tryptic digestion of ethanol-precipitated samples to ensure that the data on extracts and extraction residues would be altogether comparable, but samples of extracts were also assayed without digestion and gave values in good agreement. The amount of 148 kDa protein measured were calculated and expressed as a ratio to the hydroxyproline content of the whole tissue sample. The values obtained can then be considered as an

Fig. 2. Sephacryl S-300 (superfine-grade) gel chromatography of the 148 kDa protein and tryptic digests thereof

For the preparation of samples and other experimental details see the text. The amount of 148 kDa-protein reactivity was determined by using the radioimmunoassay, with intact 148 kDa protein as a standard. Samples were: (a) intact 148 kDa protein; (b) tryptic digest of the 148 kDa protein; (c) tryptic digest of guanidinium chloride-extractable proteins from bovine tracheal cartilage; (d) tryptic digest of the insoluble residue obtained after guanidinium chloride extraction of bovine tracheal cartilage.

expression of the tissue concentration of 148 kDa protein, as the collagen is relatively constant and provides a measure of the tissue mass.

Fig. 3. Variation in contents of the 148 kDa protein in bovine tracheal cartilage with age

(a) The amounts of the 148 kDa protein detected in guanidinium chloride extracts (▲) and in tryptic digests of the extraction residues (○) and the total detectable amount of the protein (●) were determined by radioimmunoassay of tryptic peptides and related to the total amount of hydroxyproline present in the tissue samples. (b) The ratio between the trypsin-digestible and guanidinium chloride-soluble pools of the 148 kDa protein was calculated from the data given in (a). One sample (78 months of age) consistently gave an aberrant partition between guanidinium chloride-soluble and guanidinium chloride-insoluble protein. The data points for this sample are given within parentheses. For full experimental details see the text.

Comparatively small amounts of 148 kDa protein were present in the samples from the youngest animals (Fig. 3a). The guanidinium chloride-soluble as well as the trypsin-digestible pools of the protein thereafter increase markedly with age, to reach maximal amounts at 3–4 years and 6–10 years respectively. Both pools again decrease at greater ages. The ratio between the trypsin-digestible and the guanidinium chloride-soluble pools of the 148 kDa protein increases continuously (Fig. 3b), the changes being most pronounced between 3 and 8 years of age.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of cartilage proteins

The variation of the quantity of the 148 kDa protein in the guanidinium chloride extracts could

be confirmed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 4). Before electrophoresis the protein fraction was partially purified by direct dissociative CsCl-density-gradient centrifugation to remove proteoglycans, which otherwise cause the stacking gel to collapse. The 148 kDa protein is easily identified because of its trimeric structure (Paulsson & Heinegård, 1981), which results in a shift in mobility between samples treated (Fig. 4a) or not treated (Fig. 4b) with 2-mercaptoethanol. Some material remains at the position of the non-reduced 148 kDa protein even after reduction. As this band shows similar variation in intensity as do the 148 kDa protein subunits, it could bear some relation to this protein. Among other previously described proteins, the 36 kDa protein shows a somewhat increasing staining intensity during growth and maturation and then remains rather constant. Interestingly, its mobility changes slightly at early ages.

Quantification of link proteins

The amount of link proteins is more difficult to assess from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, because of their lesser abundance and their polymorphism. They were therefore separately quantified by radioimmunoassay and related to the original tissue mass, determined as the total hydroxyproline content of the cartilage sample (Fig. 5). A slight increase occurs with increasing age, but the changes are much less pronounced than those observed with the 148 kDa protein and are more similar to those estimated for the 36 kDa protein. The increase in contents of link proteins is of similar magnitude to that of the proteoglycan contents previously described (Inerot & Heinegård, 1983), which indicates that the ratio between link proteins and proteoglycans remains relatively constant in the tissue.

Solubility of collagen

An increasing stability of the collagen network could lead to entrapment of other molecules with ensuing decreased extraction yields. Therefore the distribution of collagen between the guanidinium chloride extract, the tryptic digest and the trypsin-resistant residue was determined and plotted as a function of age (Fig. 6). The bulk of the collagen remains insoluble at all ages. The guanidinium chloride-soluble and trypsin-digestible pools constitute a somewhat larger fraction in early years, but the differences are minute when compared with the changes in quantity and solubility of the 148 kDa protein and probably do not have any bearing on these parameters.

Fig. 4. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of guanidinium chloride-extractable proteins from bovine tracheal cartilage of different ages

(a) Samples reduced with 2-mercaptoethanol. (b) Samples not reduced. Each sample corresponds to 25 μ g of hydroxyproline as determined in the extraction residue. For full experimental details see the text.

General discussion

The apparently complete insensitivity of the antigenicity of the 148 kDa protein to tryptic

digestion is remarkable, particularly as the protein is being extensively degraded. One possible explanation would be that the oligosaccharides present on the protein are major antigenic determinants.

Fig. 5. Variation in contents of link protein in guanidinium chloride extracts of bovine tracheal cartilage with age. The amounts of link protein were determined by radioimmunoassay and related to the amount of hydroxyproline in the tissue samples. For full experimental details see the text.

Fig. 6. Variation in the recovery of collagen in the guanidinium chloride extract, the subsequent tryptic digest and the insoluble residue with age.

Collagen in extracts and residues was determined as the amount of hydroxyproline released by acid hydrolysis. For full experimental details see the text.

The fact that the antigenicity is much decreased after reduction and alkylation (Paulsson & Heinegård, 1982) does, however, indicate that the determinants are peptide in nature and probably depend on higher-order structure. The other possible explanation is that the antiserum is only directed against determinants present in one comparatively small trypsin-resistant region of the protein. This seems likely, considering the homogeneity of the trypsin-resistant antigen on gel chromatography (Fig. 2).

Even though large amounts of peptides, reacting in the 148 kDa-protein radioimmunoassay, could be released by tryptic digestion, the tissue could not be completely solubilized with less than incubation in 1 M-NaOH at elevated temperature. It is therefore possible that a portion of the 148 kDa

protein is still not accounted for. The decrease in 148 kDa protein seen at greater ages should therefore be interpreted with some caution.

All values, given in the present text, for amounts of non-collagenous proteins are related to the amount of collagen in the tissue sample, as determined by its content of hydroxyproline. It was not possible to obtain representative values for the wet weights, as the samples were collected over a rather long period of time and loss of water occurs during storage in the freezer. There was, however, a tendency for the samples from the older animals to contain less collagen per weight unit. Therefore a slight increase in amount relative to hydroxyproline could mean a constant amount relative to wet weight.

The guanidinium chloride-soluble pool of 148 kDa protein reaches its maximum at considerably younger age than does the trypsin-digestible pool. An attractive interpretation of the presently available data is that the guanidinium chloride-soluble pool of 148 kDa protein is a transient one, containing relatively recently secreted molecules, some of which will eventually be laid down in the matrix in a manner that renders them resistant to extraction with guanidinium chloride. The mechanism by which the 148 kDa protein becomes unextractable is not known, but it is possible that, in analogy with collagen, it depends on the formation of covalent cross-links.

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