COLLAGEN POLYMORPHISM: TWO MOLECULAR SPECIES IN PIG INTERVERTEBRAL DISC

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1. Introduction

Several molecular species of collagen that appear to be tissue-specific, have now been identified in vertebrates [1]. Thus the collagen $[\alpha 1(II)]_3$ of hyaline cartilage is genetically distinct from the more ubiquitous collagen, $[\alpha 1(I)]_2\alpha 2$, of skin, bone and tendon [2]. The mammalian intervertebral disc consists of three anatomical parts; the annulus fibrosus, the nucleus pulposus and the cartilaginous endplates of the vertebral bodies [3]. The annulus fibrosus is often classified as fibrocartilage [4], and shows similarities to both cartilage and tendon in structure. However, the molecular nature of the collagens in the annulus and nucleus has not been investigated in detail, although a recent analysis of the small proportion of collagen that could be solubilised from whale nucleus pulposus by pronase, indicated that it was similar to $[\alpha 1(II)]_3$ of cartilage [5]. The major difficulty in examining the collagen of the disc biochemically, is its extreme insolubility in solvents normally used to extract collagen from other tissues.

In order to understand fully the changes in structure that may occur in human disc as they degenerate with ageing and why some discs are susceptible to prolapse, it is essential that disc collagen, which is the main tensile element, is characterised at the molecular level. In the present study the collagens of the annulus and nucleus of young pigs were solubilised by digestion with cyanogen bromide (CNBr) and characterised by analysing their CNBr-peptides. The results show that the fibrous annulus contains two molecular species of collagen $[\alpha 1(I)]_2\alpha 2$ and $[\alpha 1(II)]_3$ with a molar ratio of 4:1 respectively, whilst the gel-like nucleus contains only $[\alpha 1(II)]_3$.

2. Materials and methods

2.1. *Tissue preparation*

Skin, larynges and lumbar intervertebral discs from 6–9 month old pigs were used. The nucleus pulposus of discs at this stage of maturity was gel-like and was easily separated from the fibrous annulus. Samples of annulus were sliced from the discs carefully avoiding the outer ligamentous regions and the cartilaginous endplates. The tissues were extracted in 4 M guanidine HCl to remove non-collagenous material and the collagenous residues were washed thoroughly with water and freeze-dried. About 3 g wet weight of nucleus pulposus was recovered from the lumbar discs of one spine, yielding about 30 mg of collagen. Several grams of collagen were recovered from the corresponding annuli.

Guanidine HCl-soluble and insoluble collagens from dermis, and guanidine HCl-insoluble collagen from laryngeal cartilage were also prepared. Collagen samples were digested with CNBr by a conventional procedure as previously described [6].

2.2. *Chromatography of CNBr-peptides on CM-cellulose*

The larger CNBr-peptides of the collagens were fractionated by chromatography on CM-cellulose essentially by the method described by Miller [7], and monitored for hexose content by an automated procedure [8].

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2.3. *Chromatography of CNBr-peptides on Phosphocellulose*

The small CNBr-peptides derived from the collagen of skin, cartilage and annulus fibrosus were resolved by column chromatography on phosphocellulose at pH 3.6 [9] and desalted on a column of Bio-Gel P-2 (BioRad Laboratories) prior to amino acid analysis.

3. Results and discussion

Overall amino acid analyses of collagens in the annulus and nucleus revealed hydroxylysine contents of 15 residues and 23 residues respectively, per 100 residues of hydroxyproline, compared with 21 residues per 100 hydroxyproline residues in collagen of pig laryngeal cartilage. The value for the nucleus collagen is similar to that recently reported for collagen of whale nucleus pulposus [5].

The various α chains in the tissues were identified and their relative proportions measured by analysis of their CNBr-peptides. Over 95% of the collagen in the tissue samples was recovered as soluble peptides after digestion with CNBr. The results therefore refer to the total collagen in the matrices of the annulus and nucleus.

The elution profile of the CNBr-digest of annulus collagen from CM-cellulose was similar to that of skin collagen by UV-absorbance at 230 nm, but more closely resembled that of cartilage collagen by hexose content (fig. 1). This finding indicated that the annulus probably contained a mixture of the two collagen types $[\alpha 1\ (I)]_2\alpha 2$ and $[\alpha 1\ (II)]_3$. To confirm that CNBr-peptides from α 1 (I), α 1 (II) and α 2 chains were present, all of the large peptides from the CM-cellulose chromatogram were purified and their sizes determined by molecular sieve chromatography on a column (1.6 cm × 150 cm) of Bio-Gel agarose (1.5 m, 200–400 mesh, Bio-Rad Laboratories) (not shown) [6]. Their elution positions and amino acid analyses in comparison with published compositions of CNBr-peptides from pig skin $[\alpha 1\ (I)]_2\alpha\ 2$ [10], and bovine and human cartilage $[\alpha 1\ (II)]_3$ [11] established their identity (Eyre, unpublished). For example, the peptide α1(II)CB8 (nomenclature of Miller [11]) was isolated from the chromatogram of the annulus digest depicted in fig. 1 and its molecular size (about 14 000 molecular weight) and amino

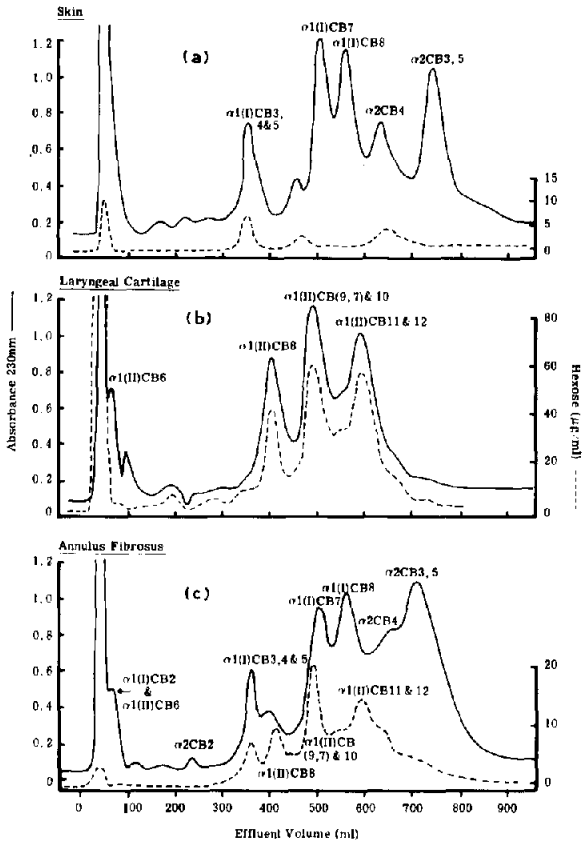

Fig. 1. Chromatography of CNBr-digests of pig collagens on CM-cellulose. The column (2 cm × 15 cm) was eluted at 42°C with a linear gradient of NaCl (0–0.15 M) in 1 litre of 0.02 M Na citrate–citric acid, pH 3.6, and 10 ml fractions were collected. a: 200 mg CNBr-digest of guanidine HCl-soluble skin collagen. b: 180 mg CNBr-digest of laryngeal cartilage collagen. c: 200 mg CNBr-digest of annulus fibrosus collagen. The hexose contents of the initial CNBr-digests were: a) 0.7%; b) 11.4% and c) 1.8% galactose equivalents.

acid composition, which included two residues of glucosylgalactosylhydroxylysine, established its identity. The peptides in all of the major peaks shown in the chromatograms of fig. 1 were analysed similarly. The results were entirely consistent with the annulus containing a mixture of $[\alpha 1(I)]_2\ \alpha 2$ and $[\alpha 1(II)]_3$ collagens at a molar ratio of about 4:1 respectively.

Analysis of the small CNBr-peptides in the collagen digests provided a convenient method for determining

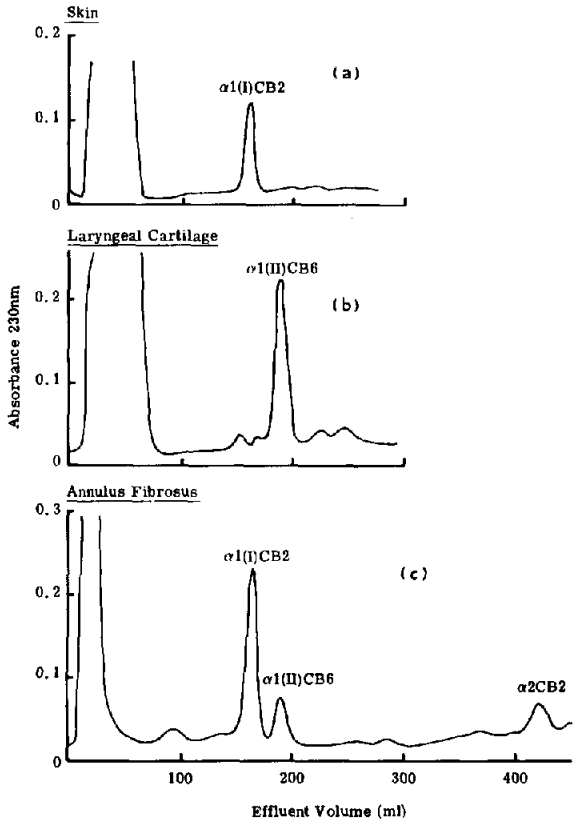

Fig. 2. Chromatography of CNBr-peptides from pig collagens on phosphocellulose. The column (1.5 cm × 10 cm) was eluted at 42°C with a linear gradient of NaCl (0–0.3 M) in 800 ml of 1 mM Na formate–formic acid, pH 3.6, and 5 ml fractions were collected. a: CNBr-peptide, α1(I)CB2, from 100 mg of guanidine HCl-soluble skin collagen. b: CNBr-peptide, α1(II)CB6, from 200 mg of laryngeal cartilage collagen. c: 100 mg total CNBr-digest of annulus fibrosus collagen.

accurately the relative proportions of the two species of collagen. The homologous peptides, α1(I) CB2 and α1(II) CB6 from pig skin and cartilage collagens were eluted in different positions when chromatographed on phosphocellulose (fig. 2), and their amino acid compositions were shown to be identical with those of their counterparts from human collagens (table 1) [11,13,14]. The two peptides were clearly distinguished by having aspartic acid and valine exclusive to α1(II)CB6 and leucine to α1(I)CB2. The annulus digest contained both α1(I)CB2 and α1(II)CB6 (fig.2). Furthermore, the small peptide α2CB2, characteristic of skin collagen, was also obtained thus allowing the quantitation of all the three possible chains in the two types of collagen, i.e. α1(I), α1(II) and α2. From the chromatogram shown in fig. 2c, 0.253 μm of α1(I)CB2, 0.128 μm of α2CB2 and 0.089 μm of α1(II)CB6 were recovered as measured by amino acid analysis. Thus, the molar ratio of α1(I)CB2:α2CB2 was 2:1, the expected value for molecules of $[\alpha 1(I)]_2\ \alpha 2$ collagen. The molar ratio of the peptides α1(I)CB2 to α1(II)CB6, enabled the relative proportions of $[\alpha 1(I)]_2\ \alpha 2$ and $[\alpha 1(II)]_3$ to be calculated, and was determined on three separate digests of tissue from one spine and a fourth digest of tissue from another spine. The results were remarkably consistent with $[\alpha 1(II)]_3$ ranging from 18.5–21. 5% of the total collagen in the annulus.

The small amount of collagen available from the nucleus pulposus did not permit such extensive analysis. However, by using a scaled-down version of the CM-cellulose column, the nature of the collagen in the nucleus could be determined (fig. 3). Thus the profile of CNBr-peptides was indistinguishable from that of the $[\alpha 1(II)]_3$ collagen of laryngeal cartilage,

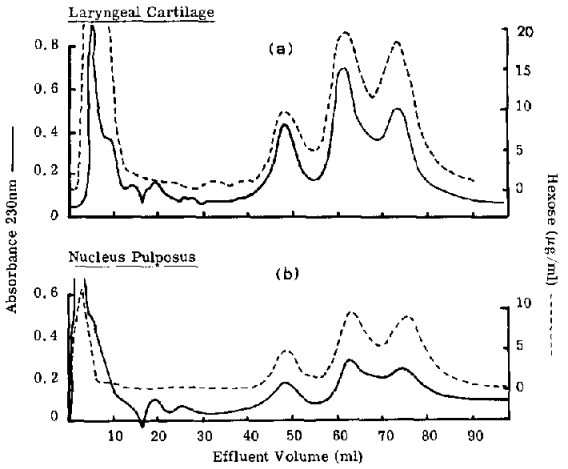

Fig. 3. Chromatography of CNBr-digests of collagens from pig nucleus pulposus and cartilage on a small CM-cellulose column (0.9 cm × 6 cm), which was eluted at 42°C with a linear gradient of NaCl (0.02–0.15 M) in 150 ml of 0.02 M Na citrate–citric acid, pH 3.6. Fractions of 1.5 ml were collected. a) 10 mg of the CNBr-digest of laryngeal cartilage collagen. b) 10 mg of the CNBr-digest of nucleus pulposus collagen. The hexose contents of the digests were a) 11.4% and b) 6.1% galactose equivalents.

Table 1
Amino acid composition of small CNBr-peptides from collagen of pig skin, cartilage and intervertebral disc.†

	α1(I)CB2			α1(II)CB6				α2CB2	
	Skin	Annulus fibrosus	(Human skin [13])	Cartilage	Annulus fibrosus	Nucleus pulposus	(Human cartilage [11])	Annulus fibrosus	(Human skin [13])
4-Hyp	5.3	5.7	5.5	4.1	4.6	4.1	4.0	3.3	2.7
Asp	–	–	–	1 (1.0)	1 (1.1)	1 (1.1)	1 (1.0)	2 (1.8)	2 (2.0)
Thr	–	–	–	–	–	–	–	1 (0.9)	–
Ser	2 (2.0)	2 (1.9)	2 (1.8)	1 (1.0)	1 (1.2)	1 (1.2)	1 (1.0)	2 (1.9)	2 (1.9)
Glu	4 (4.0)	4 (4.0)	4 (3.9)	4 (4.0)	4 (4.2)	4 (4.5)	4 (4.2)	2 (1.8)	1 (1.2)
Pro	6.7	6.0	6.0	5.8	5.4	6.0	5.9	3.9	3.1
Gly	12 (12.0)	12 (12.0)	12	11 (11.0)	11 (11.0)	11 (11.0)	11	11 (11.1)	10
Ala	2 (2.0)	2 (2.1)	2 (2.1)	2 (2.0)	2 (2.2)	2 (2.5)	2 (2.1)	2 (2.0)	3 (3.2)
Val	–	–	–	1 (1.0)	1 (1.1)	1 (1.2)	1 (1.0)	1 (1.0)	1 (1.0)
Leu	1 (1.1)	1 (1.1)	1 (1.0)	–	–	–	–	1 (1.1)	1 (1.0)
Phe	1 (1.1)	1 (1.1)	1 (1.0)	1 (1.0)	1 (1.0)	1 (1.0)	1 (1.0)	–	–
Arg	1 (1.1)	1 (1.2)	1 (1.1)	1 (1.0)	1 (1.1)	1 (1.0)	1 (1.0)	3 (2.8)	3 (2.8)
Hse*	1 (0.9)	1 (0.8)	1 (1.0)	1 (0.9)	1 (0.9)	1 (0.9)	1 (1.0)	1 (1.0)	1 (0.9)
Total	36	36	33	33	33	33	33	33	30

The figures in parentheses are the measured values from which the nearest integers are derived where appropriate.

† Determined using a Locarte amino acid analyser, making the usual corrections for losses during hydrolysis [12].
* includes homoserine lactone.

both by UV-absorbance at 230 nm and hexose distribution. Peptides from an α1(I) chain could not be detected. Amino acid analysis of the CNBr-peptide eluting immediately after the void peak gave a composition that was clearly α1(II)CB6 with no evidence for α1(I)CB2 (table 1). Therefore it is concluded that at this stage of maturity essentially all of the collagen in the nucleus is $[\alpha 1(II)]_3$. This result is consistent with the appearance of the cells in the nucleus, which in the adult tissue have been likened to chondrocytes [3].

4. Conclusion

The present findings show that the collagenous framework of the annulus fibrosus contains two genetic species of collagen. Because the annulus is obviously a composite structure histologically, consisting of concentric fibrous lamellae interspersed by a relatively homogeneous ground substance, it is tempting to predict how the two types of collagen are distributed. Thus $[\alpha 1(I)]_2\,\alpha 2$ is probably contained in the coarse collagen fibres of the lamellae and $[\alpha 1(II)]_3$ may be restricted to fine fibrils in the gel-like intralamellar and interlamellar regions. The presence of $[\alpha 1(II)]_3$ alone in the gelatinous nucleus supports this concept.

The method that we have used to measure the relative proportions of the two collagen species in pig discs should prove applicable to other types of cartilage that may also contain mixed collagen populations. Indeed, in an extension of the present work $[\alpha 1(I)]_2\,\alpha 2$ and $[\alpha 1(II)]_3$ collagens have been identified and quantitated in the annulus fibrosus and nucleus pulposus of human discs (Eyre and Muir, in preparation).

Acknowledgements

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