Types I and II Collagens in Intervertebral Disc

INTERCHANGING RADIAL DISTRIBUTIONS IN ANNULUS FIBROSUS

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(Received 12 April 1976)

Intervertebral disc is a highly specialized cartilaginous tissue, containing two genetic types of collagen (I and II). Analysis of peptides from a CNBr digest of collagen showed that the proportions of I and II varied gradually and inversely across pig annulus fibrosus, with exclusively type I at the extreme outer edge and exclusively type II in the nucleus pulposus.

Molecular polymorphism of collagen is now well established, with each genetically distinct molecule, type I, II, III or IV, showing tissue specificity (for reviews, see Miller & Matukas, 1974; Gallop & Paz, 1975). However, the molecular features that suit each type of collagen for a particular tissue or matrix are poorly understood. For example, it is suggested that the high content of covalently bound hexose may equip type II collagen for its role in cartilage, although the function of the carbohydrate is still largely unknown (Nimni, 1974). The annulus fibrosus of the intervertebral disc, which is a highly specialized fibrocartilage consisting of about 10-15 fibrous lamellae, was shown to contain two species of collagen in what may be a composite fibrillar matrix. Thus in pig and man the annulus fibrosus contained both type I and type II collagens, whereas the central nucleus pulposus of the disc contained only type II collagen (Eyre & Muir, 1975a). However, nothing is known about the distribution of the two types of collagen within the annulus, nor how such molecular polymorphism is related to the complex lamellar architecture and the unique load-bearing properties of the disc (Szirmai, 1970).

In the present study the relative radial distributions of type I and type II collagens were determined in small serial samples taken from the annulus of a single pig disc. The findings show a smooth change in molecular type, from type I to type II, on going from outer to inner regions. This peculiar distribution clearly reflects a unique recruitment of different connective-tissue macromolecules to the complex structural requirements of the intervertebral disc.

Materials and Methods

Tissue

The outer ligamentous sheath was removed from the spine of a 4-year-old Large White sow and a

transverse cut was made through the middle of the disc between the first and second lumbar vertebrae (L1/L2). A slice (about 0.5 mm thick) of the annulus fibrosus was taken from a lateral anterior segment (see Fig. 1). The slice was teased out on a glass slide to give maximum spread in the radial dimension of the disc, then split into eleven serial samples of roughly equal size. No attempt was made to dissect out individual fibrous lamellae. The samples were dried in a desiccator when each weighed about 1 mg.

The remainder of the annulus fibrosus from the same disc was dissected into two samples: (a) an outer annulus with obvious lamellae, (b) an inner annulus that had less well-defined lamellae and included the region sometimes called the transition zone. Each preparation was freeze-dried.

Analysis

Dry tissue was reacted with CNBr in 70% (v/v) formic acid as previously described (Eyre & Glimcher, 1973; Eyre & Muir, 1975b), the small samples being treated with 5 mg of CNBr in 0.5 ml of formic acid. Digests were diluted with water and freeze-dried.

Collagen fragments in the CNBr digests of serial samples of annulus were analysed by disc electrophoresis in sodium dodecyl sulphate/polyacrylamide gels. Essentially the procedure of Furthmayr & Timpl (1971) was followed, as previously described (Eyre & Muir, 1975b). Peptides from the CNBr digest of collagen ('CNBr peptides') were scanned densitometrically in the stained gels (Eyre & Muir, 1975b) and the relative proportions of types I and II collagens could be assessed from the densitometric profiles as follows. Peak areas for the two peptides, $\alpha 2\text{CB}3,5$ and $\alpha 1(\text{II})\text{CB}10$, which have proved useful markers for types I and II collagens (Eyre & Muir, 1975b), were determined by tracing peaks, cutting out the tracing paper and weighing it. The ratio of

areas under these two peaks was assumed to be proportional to the ratio of their parent collagen molecules, i.e. $\alpha 2\text{CB}3,5/\alpha 1(\text{II})\text{CB}10$ proportional to type I/type II. Triplicate portions from each of the eleven serial samples were run. Samples were also run from a CNBr digest of pig annulus fibrosus in which the proportion of collagen types had previously been determined. This preparation contained 82% type I and 18% type II collagens, determined accurately by isolation and analysis of the small peptides $\alpha 1(\text{I})\text{CB}2$ and $\alpha 1(\text{II})\text{CB}6$ (Eyre & Muir, 1974). For six gels run with this standard, the average ratio of peak areas for the stained peptide bands, $\alpha 2\text{CB}3,5/\alpha 1(\text{II})\text{CB}10$, was $3.9 (\pm 0.2 \text{ s.e.m.})$. This established the

reproducibility of the method and also enabled a calibration factor to be calculated for converting the peptide ratios measured for the serial samples of annulus into approximate molar ratios of type I to type II collagens.

The CNBr digests of the large pooled samples of outer and the inner annulus were chromatographed on columns of CM-cellulose and phosphocellulose as described before (Eyre & Muir, 1974, 1975*b*). Relative proportions of types I and II collagens in these tissue samples were assessed from the amounts of the peptides $\alpha 1(\text{I})\text{CB}2$ and $\alpha 1(\text{II})\text{CB}6$ recovered from the phosphocellulose column and measured by amino acid analysis (Eyre & Muir, 1974, 1975*b*).

Fig. 1. Preparation and electrophoresis of collagen CNBr peptides in serial samples of annulus fibrosus

(a) Diagram showing how the eleven serial samples were dissected from the anterior annulus fibrosus of a single lumbar disc. (b) Disc electrophoresis in sodium dodecyl sulphate/polyacrylamide gels of collagen CNBr peptides by CNBr digestion of the samples identified in (a). About $40 \mu\text{g}$ of each digest was run in 7.5% (w/v) acrylamide gel ($60 \text{ mm} \times 4 \text{ mm}$) for 3 h at 6 mA per tube. Gels were stained with Coomassie Brilliant Blue R and scanned by using a densitometer with a filter giving maximum transmission at 580–600 nm. The proportions of type II collagen, expressed as a percentage of total collagen, were calculated from the densitometry profiles for samples 1, 2, 4, 6, 8 and 10 (shown here), giving 0, 7, 31, 66, 95 and 92% respectively. The profiles of samples not shown agreed entirely with the gradual change in type I/type II ratio across the annulus.

Results and Discussion

The findings establish that the relative proportions of type I and type II collagens change on going from outer to inner regions of annulus fibrosus. Disc electrophoresis of CNBr peptides prepared from the serial sections of annulus clearly showed a smooth gradation in the type I/type II ratio, with only type I collagen detected in the outermost sample and essentially only type II in the innermost region of so-called transition zone (Fig. 1*a*). The central nucleus pulposus of the pig disc was previously found to contain exclusively type II collagen (Eyre & Muir, 1974). In the samples of annulus that contained both types of collagen, their relative amounts could be assessed with reasonable accuracy from the densitometric profiles of the CNBr peptides after gel electrophoresis (Fig. 1*b*). Chromatographic analysis (Fig. 2) of CNBr peptides derived from the inner and outer halves of annulus supported the findings. Thus peptides of type II collagen are richer in hexose than are those of type I collagen, and the elution from CM-cellulose of u.v. absorbance and hexose-containing material indicated that the inner part of the annulus contained a considerably higher proportion of type II collagen than did the outer region. This difference

was confirmed by measuring the relative amounts of types I and II collagens in the two samples of pooled tissue more precisely by the isolation on phosphocellulose and amino acid analysis of the peptides $\alpha 1(\text{I})\text{CB}2$ and $\alpha 1(\text{II})\text{CB}6$ (Eyre & Muir, 1974, 1975*b*). Thus the preparation of inner annulus contained 68% type I, 32% type II, and outer annulus 84% type I, 16% type II, by this method. Previous analyses on whole annulus pooled from several pig discs gave 18–21% type II, 79–82% type I (Eyre & Muir, 1974).

Comparable analyses on human intervertebral disc showed similar gradations of molecular species across the annulus (D. R. Eyre & H. Muir, unpublished work). In man, however, the annulus as a whole contains a considerably higher proportion of type II relative to type I collagen compared with pig annulus (Eyre, 1975), perhaps reflecting a structural adaptation of the disc in the biped to an altered spinal loading.

A question that remains is how the two molecular species of collagen in the annulus are distributed at the fibril level. Each molecular type is probably restricted to its own fibril, but this has yet to be shown, and the level of ultrastructure to which heterogeneity extends in the resulting mixed population of fibrils also re-

Fig. 2. Chromatography on CM-cellulose of CNBr peptides of collagen from inner and outer regions of annulus fibrosus

Conditions were as described previously (Eyre & Muir, 1975*b*). About 20mg of CNBr digest was applied for each chromatogram. (a) Inner annulus; (b) outer annulus. —, E_{230} ; ----, hexose.

mains to be established. Thus it is possible that composite fibres or fibrous sheets are made up of a weave of two fibril populations or, alternatively, tracts of type II fibrils may be interspersed between larger domains that contain only type I collagen. Scanning-electron-microscopic studies of human intervertebral disc, although showing some variation in fibril diameters (Takeda, 1975), do not show two morphologically distinct fibril populations, at least in proportions that would explain the biochemical findings.

How synthesis and deposition of the two species of collagen are controlled is also unknown. For example, they may each be synthesized by a distinct variety of cell, or both produced by a single cell population but regulated independently. Other related questions concern the influence of proteoglycans and glycoproteins on fibrillogenesis and the extent to which components of the extracellular matrix undergo self-assembly. These are fundamental to our understanding of the formation and repair of connective tissues.

Considerable insight into the relative contributions of the various connective-tissue macromolecules to ultrastructure and mechanical function may be gained from studies of composite matrices such as fibrocartilage of the intervertebral disc. A specific role in fibrocartilage for proteoglycans that contain copolymers of dermatan sulphate and chondroitin sulphate, which have been identified in the meniscus (Habuchi, *et al.* 1973), deserves further attention.

Specific antibodies to type I and type II collagens were prepared (Hahn *et al.*, 1974) which, when combined with ferritin for electron optical studies, might

well reveal the precise distribution of each type of collagen amongst fibrils of the annulus, and perhaps show differences between posterior and anterior regions and changes that may accompany aging or degeneration of the disc.

We thank Mr. Roger Brown for technical assistance. We are grateful to the Medical Research Council for the support of D.R.E., and the Arthritis and Rheumatism Council for general support.

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