Type VI collagen of the intervertebral disc

Biochemical and electron-microscopic characterization of the native protein

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The collagen framework of the intervertebral disc contains two major fibril-forming collagens, types I and II. Smaller amounts of other types of collagen are also present. On examination of the nature and distribution of these minor collagens within bovine disc tissue, type VI collagen was found to be unusually abundant. It accounted for about 20 % of the total collagen in calf nucleus pulposus, and about 5 % in the annulus fibrosus. It was discovered by serially digesting disc tissue with chondroitin ABC lyase and *Streptomyces* hyaluronidase that native covalent polymers of type VI collagen could be extracted. Electron micrographs of this material prepared by rotary shadowing revealed the characteristic dimensions of tetramers and double tetramers of type VI molecules, with their central rods and terminal globular domains. Molecular-sieve column chromatography on agarose under non-reducing non-denaturing conditions gave a series of protein peaks with molecular sizes equivalent to the tetramer, double tetramer and higher multimers. On SDS/polyacrylamide-gel electrophoresis after disulphide cleavage, these fractions of type VI collagen all showed a main band at M_r 140 000 and four lesser bands between M_r 180 000 and 240 000. On electrophoresis without disulphide cleavage in agarose/2.4 % polyacrylamide only dimeric (six chains) and tetrameric (12 chains) forms of type VI molecules were present. The ability to extract all the type VI collagen of the tissue in 4 M-guanidinium chloride, and absence of aldehyde-mediated cross-linking residues on direct analysis, showed that, in contrast with most matrix collagens, type VI collagen does not function as a covalently cross-linked structural polymer.

INTRODUCTION

At least 11 genetically distinct collagens are present in vertebrate tissues. They have been tentatively assigned to three groups (Miller, 1985) on the basis of their molecular conformations: group 1, types I, II, III, V, XI ($1\alpha 2\alpha 3\alpha$), all of which form banded fibrils of 67 nm repeat period; group 2, types IV and VII, the molecules of which are of similar length or are larger than those of group 1 but have interruptions and/or bear terminal globular extensions on their triple-helical domains; group 3, types VI, VIII, IX and X, which are short-helix molecules featuring various globular extensions and helical interruptions. The term 'minor collagens' is often used to describe those collagens, including types V, XI, VI, IX and X, that appear to be co-distributed in small amounts along with types I, II or III, the main fibril-forming species.

The extracellular matrix of the intervertebral disc is formed by a unique combination of collagens and other macromolecules. It shows similarities in gross texture and composition to both hyaline cartilage and fibrocartilage (Eyre, 1979). Collagen and proteoglycans are the main constituents, though several other matrix proteins are present, most as yet poorly characterized. The two main collagen types of the disc, types I and II, are distributed radially in opposing concentration gradients (Eyre & Muir, 1976). Type I collagen dominates in the coarse-fibre bundles that form the lamellae of the outer annulus, and type II collagen gradually increases in concentration towards the centre until it is the main collagen forming the random fibrous network of the

nucleus pulposus. Two minor types of collagen, types IX and XI ($1\alpha 2\alpha 3\alpha$), have also been reported in small amounts in the disc (Ayad *et al.*, 1981, 1982).

On attempting to map the complete range of collagen types and their spatial distribution within the bovine disc, both the annulus fibrosus and nucleus pulposus were found to be remarkably rich in type VI collagen, a protein found in small amounts in dermis, tendon, blood vessels and other connective tissues. We here report the isolation of an intact native form of type VI collagen from the bovine intervertebral disc, and its identification by biochemical properties, general appearance and specific dimensions obtained by electron microscopy.

EXPERIMENTAL

Tissue preparation

Lumbar discs from 3-month-old-calf spines supplied by a local abattoir were dissected into nucleus pulposus and annulus fibrosus. Samples of fibrocartilaginous knee meniscus and Achilles tendon were also taken from 3-month-old calves for comparison. Tissue was cut into small pieces, washed at 4 °C with 0.15 M-NaCl followed by distilled water, both containing the proteinase inhibitors, 2 mM-phenylmethanesulphonyl fluoride, 2 mM-EDTA, 5 mM-benzamidine and 10 mM-N-ethylmaleimide, and then freeze-dried. In other experiments, the washed tissues frozen in liquid N₂ were pulverized in a Waring blender and freeze-dried.

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Glycosidase pretreatment

Dried tissues (100 mg each) were treated with chondroitin ABC lyase (EC 4.2.2.4) (Seikagaku Kogyo Co., Tokyo, Japan) (0.125 unit/ml) in 4 ml of 0.05 M-Tris/HCl/0.06 M-sodium acetate buffer, pH 8.0, containing 2 mM-phenylmethanesulphonyl fluoride, 2 mM-EDTA, 5 mM-benzamidine and 10 mM-*N*-ethylmaleimide at 22 °C (room temperature) for 24 h. After removal of the supernatant, the residues were treated with *Streptomyces* hyaluronidase (EC 4.2.2.1) (Seikagaku Kogyo Co.) [5 TRU (turbidity-reducing units)/ml] in 4 ml of 0.05 M-sodium acetate buffer, pH 6.0, containing 0.15 M-NaCl and the same proteinase inhibitors at 22 °C for 24 h. These treatments have been shown to remove most of the proteoglycans from the collagen network of cartilage by first removing the chondroitin sulphate chains and then depolymerizing the hyaluronic acid and so releasing the truncated proteoglycan subunits (Chun *et al.*, 1986). The glycosidase extracts were filtered through Whatman no. 541 filter paper, and portions of the filtrate were analysed directly by SDS/polyacrylamide-gel electrophoresis.

Peptic digestion and salt fractionation of collagens

Whole tissue, and the washed tissue residues after extraction by glycosidases or 4 M-guanidinium chloride (Wu & Eyre, 1984), were digested with pepsin (EC 3.4.23.1) (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.) at an enzyme/substrate ratio of 1:50 (w/w) for 24 h in 3% (v/v) acetic acid at 4 °C. Solubilized material was fractionated into major and minor collagen classes by selective salt precipitation at 0.9 M-NaCl and 2.0 M-NaCl. In some experiments the residue after hyaluronidase digestion was extracted in 4 M-guanidinium chloride in 0.05 M-Tris/HCl buffer, pH 7.4, containing proteinase inhibitors for 24 h at 4 °C, before washing and pepsin digestion.

Molecular-sieve chromatography

Hyaluronidase extracts of the annulus fibrosus, which contained native forms of type VI collagen, were chromatographed directly without concentration under non-denaturing conditions on a column (1.5 cm × 160 cm) of Agarose A50m (100–200 mesh) (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The column was equilibrated and eluted with 0.05 M-sodium acetate buffer, pH 6.0, containing 0.15 M-NaCl at room temperature.

SDS/polyacrylamide-gel electrophoresis

All collagen fractions, including type VI collagen in direct tissue extracts and column effluents, were electrophoresed in 1.5 mm-thick SDS/8%-polyacrylamide slab gels according to the method of Laemmli (1970).

Agarose/polyacrylamide composite gels were prepared by including 0.5% agarose in the SDS/2.4% polyacrylamide as suggested by Peacock & Dingman (1968).

Electron microscopy

A solution of native type VI collagen (0.1 mg/ml), diluted directly from a hyaluronidase extract of annulus fibrosus, was dialysed against 0.2 M-ammonium acetate buffer, pH 6.0, at 4 °C, then sprayed on to freshly cleaved mica discs, dried *in vacuo* and rotary-shadowed with approx. 0.1 µg of tungsten/cm² as described by Slayter

(1976). Dark-field electron microscopy was carried out on the resulting specimens as described previously (Slayter *et al.*, 1985).

Cross-link analysis

Purified intact forms of type VI collagen extracted by hyaluronidase were treated with NaBH₄ in 0.1 M-sodium phosphate buffer, pH 7.0, and hydrolysed in 6 M-HCl for 24 h at 108 °C (Eyre, 1987). A procedure was followed to enrich any cross-linking amino acids by molecular sieving the hydrolysate on a column (1.3 cm × 60 cm) of Bio-Gel P2 eluted by 10% (v/v) acetic acid (Eyre & Oguchi, 1980; Eyre, 1987). The pooled fraction that would contain cross-linking amino acids was assayed for borohydride-reduced components (Eyre, 1987) and non-reducible mature pyridinoline residues (Eyre *et al.*, 1984a) on an amino acid analyser that employed ion-exchange column chromatography and ninhydrin detection for the former, and on reverse-phase h.p.l.c. and fluorescence detection for the latter.

RESULTS

Molecular types of collagen from peptic digestion

The pepsin-solubilized minor collagens from annulus fibrosus were qualitatively similar in genetic type to those from fibrocartilaginous meniscus (Fig. 1a). Meniscus, however, differs from annulus fibrosus in containing only a trace of type II collagen (Eyre & Wu, 1983; Eyre *et al.*, 1984c). Analysis of the 0.9 M-NaCl precipitate from the peptic digest of annulus fibrosus showed mostly type I collagen with some type II collagen, as judged by the CNBr-cleavage peptide profile (results not shown). This fraction accounted for about 95% of the solubilized collagen. The 2.0 M-NaCl precipitate contained type V collagen and several lower-*M_r* polypeptides that later were identified as components of type VI collagen (Fig. 1a). The peptic digest of nucleus pulposus showed a similar profile of collagen types to that of hyaline cartilage. Type II collagen was the main constituent of the 0.9 M-NaCl precipitate, and two other cartilage-specific minor collagens, type XI (1α2α3α) and the disulphide-bonded type IX collagen, were present in the 2.0 M-NaCl precipitate (Fig. 1b).

In addition to these collagens, the pepsin digests of annulus and nucleus both contained a disulphide-bonded protein of high *M_r* in the 2.0 M-NaCl precipitate, which before reduction ran at the top of the 8% separating gel (results not shown). After disulphide reduction it dissociated to yield three polypeptides with apparent *M_r* of 58000, 64000 and 70000, based on globular protein standards (Figs. 1a and 1b). Similar molecular species were recovered from meniscus and tendon (Fig. 1a). Their mobilities resembled those of the SC3, SC1 and SC2 chains respectively of type VI collagen released by mild peptic digestion (Jander *et al.*, 1984; Gibson & Cleary, 1985). This preliminary evidence for type VI collagen being abundant in the intervertebral disc was confirmed by characterizing the intact form of type VI collagen extracted by hyaluronidase or 4 M-guanidinium chloride.

Extraction of type VI collagen by *Streptomyces*-hyaluronidase digestion

When hyaluronidase extracts of annulus fibrosus and nucleus pulposus were analysed by SDS/8%-polyacryl-

Fig. 1. SDS/polyacrylamide-gel electrophoresis of pepsin-solubilized fractions from calf annulus fibrosus and nucleus pulposus

Tissues were treated with glycosidases to remove proteoglycans and then digested with pepsin. Pepsin-solubilized material was fractionated into major and minor collagen classes by selective salt precipitation at 0.9 M-NaCl and 2.0 M-NaCl. Samples were analysed in 8% gels under reducing conditions. Collagen fractions in panel (a) were derived from annulus fibrosus (lanes 1 and 2), meniscus (lanes 3 and 4) and Achilles tendon (lanes 5 and 6), and in panel (b) from articular cartilage (lanes 7 and 8) and nucleus pulposus (lanes 9 and 10). Lanes 1, 3, 5, 7 and 9, 0.9 M-NaCl fractions; lanes 2, 4, 6, 8 and 10, 2.0 M-NaCl fractions. The pepsin-derived fragments of type IX collagen seen in (b) were larger than usual (Wu & Eyre, 1984), because of the low ratio of pepsin to substrate and hence milder digestion conditions.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of the intact chains of type VI collagen extracted from annulus fibrosus and nucleus pulposus by *Streptomyces hyaluronidase*

Portions of the hyaluronidase extracts were mixed directly with an equal volume of sample buffer at double normal concentration (2% SDS/20% glycerol/0.1 M-Tris buffer, pH 6.8), heat-denatured and analysed on an 8% slab gel. Lane 1, Achilles tendon; lanes 2 and 3, nucleus pulposus; lanes 4 and 5, annulus fibrosus. Disulphide bonds in the samples in lanes 3 and 5 were reduced with dithiothreitol before electrophoresis.

amide-gel electrophoresis under non-reducing conditions, high- M_r protein barely penetrated the 3% stacking gel, and very little type I or type II collagen chains were present (Fig. 2). After reduction of disulphide bonds, the polymeric protein dissociated into a prominent 140000- M_r band and a cluster of four bands in the M_r range 180000–240000. Qualitatively similar electrophoretic

profiles were given by equivalent extracts of Achilles tendon (results not shown), and are typical of those reported for the intact tissue forms of type VI collagen (Jander *et al.*, 1984; Gibson & Cleary, 1985). Densitometry of the stained gels indicated that the four higher- M_r components accounted for about 40% of the total mass of the type VI collagen polypeptides from annulus

Fig. 3. SDS/polyacrylamide-gel electrophoresis of native extracts of type VI collagen after treatment by pepsin

Hyaluronidase extracts from nucleus pulposus and annulus fibrosus, which were enriched in the intact forms of type VI collagen (Fig. 2), were dialysed against 0.1 M-acetic acid and freeze-dried. Samples were digested with pepsin in 3% acetic acid at 4 °C and analysed on an 8% polyacrylamide gel under non-reducing (lanes 2 and 4) and reducing (lanes 3 and 5) conditions. Lane 1, Bio-Rad protein standards: myosin (M_r 200 000), β -galactosidase (M_r 116 000), phosphorylase *b* (M_r 92 500), bovine serum albumin (M_r 66 200) and ovalbumin (M_r 45 000). Lanes 2 and 3, nucleus pulposus; lanes 4 and 5, annulus fibrosus. Pepsin-derived chains of type VI collagen are labelled SC1, SC2 and SC3 according to the nomenclature of Jander *et al.* (1984).

fibrosus, and about 30% of that from nucleus pulposus. Pepsin treatment of the non-reduced intact type VI collagen yielded 58 000–70 000- M_r components after disulphide reduction (Fig. 3, lanes 3 and 5), which appeared to be identical in mobility with those recovered directly from the tissue by peptic digestion as shown in Fig. 1. Before disulphide reduction, the pepsin-treated type VI barely penetrated the 8% separating gel (Fig. 3, lanes 2 and 4). The chondroitin ABC lyase extracts of annulus fibrosus and nucleus pulposus contained no type VI collagen when run electrophoretically under the same conditions as the hyaluronidase extracts (results not shown).

Tissue concentrations of type VI collagen

To assess the total amount and the extractability, without resorting to proteinases, of type VI collagen, disc tissue after glycosidase treatment was extracted with 4 M-guanidinium chloride, and then the washed residue was digested with pepsin. The relative amount of type VI collagen in each fraction was estimated by electrophoresis.

The results showed that hyaluronidase solubilized about 30–40% of the type VI collagen of calf annulus fibrosus and nucleus pulposus. The remainder was all extracted by 4 M-guanidinium chloride, since no type VI fragments were seen in the final peptic digest. The hyaluronidase extract was highly selective for type VI

collagen, which made up about 90% of the protein stained by Coomassie Blue on polyacrylamide-gel electrophoresis. Type VI accounted for about 50% of protein in the subsequent 4 M-guanidinium chloride extract. It was estimated that type VI accounted for about 5% of the dry weight of both nucleus pulposus and annulus fibrosus, or 15–20% of the total collagenous protein in the nucleus and 5–10% of that in the annulus.

Rotary-shadowing electron microscopy

The molecular morphology of type VI components in a hyaluronidase extract of annulus fibrosus was viewed by rotary-shadowing electron microscopy (Fig. 4). The images and dimensions obtained from them differed in detail from those obtained from pepsin-solubilized type VI collagen (Furthmayr *et al.*, 1983), but closely resembled those of the intact forms of type VI collagen isolated from aorta, ligament and neurofibroma as reported by Jander *et al.* (1984) and Engel *et al.* (1985), except that here individual molecular tetramers were the commonest component (Fig. 4a). Some double tetramers and multimer forms were evident (Figs. 4b and 4c).

Table 1 summarizes the mean measurements of various dimensions of the type VI structures observed, including total length, central rod length, central rod width, diameters of inner and outer globules and length of the globular overlap domain formed between tetramer units. The observed tetramer unit is about 145 nm long, with a 59 nm × 11 nm central rod and two pairs of globular structures distributed at each end. The inner and outer globular pairs are connected to each other by short, relatively thin (approx. 1 nm), rod-like segments. In most structures the individual globular domains of the outer pairs are separated (Fig. 4), though in some they appeared confluent at either one or both ends of the tetramer. The globular domains of the inner pair were usually more closely associated or confluent; only a few rotary-shadowed images revealed any separation between them and or of the individual dimer strands that formed the central rod. Occasionally, a fine strand was seen protruding from the end of a multimer (Figs. 4d and 4e), with a diameter consistent with it being a strand of hyaluronic acid, and similar to extended thin flexible strands found above (Figs. 4f and 4g) in these preparations.

Polymers appear to form from the tetramers by end-to-end interchange between the regions consisting of outer thin rods and terminal globules. The schematic drawing incorporated in Table 1 illustrates two of the possible modes of interaction evident from the electron micrographs. The connecting linkage did not appear to be very rigid or consistent at the level of resolution available in the method. In different images the outer globules were variously placed in the gap between the inner globules of the two tetramers placed close to but staggered from the inner globules of the other tetramer. The distance across the total junction of globular units of adjacent tetramers was fairly constant at 54 nm. The length of the periodic repeat in type VI multimers is about 113 nm on the basis of these linear dimensions.

Agarose molecular-sieve chromatography

Fig. 5 shows the elution profile at hyaluronidase-extracted type VI collagen on a Bio-Rad A50m agarose column under non-reducing non-denaturing conditions. Several components were partially resolved. Proteins

Fig. 4. Electron micrographs of rotary-shadowed native molecular aggregates of type VI collagen prepared by *Streptomyces-hyaluronidase* extraction of annulus fibrosus

Selected tungsten-shadowed type VI collagen replicas with dark-field imaging: (a) tetramers; (b) double tetramers; (c) triple tetramers; (d) and (e) double and triple tetramers respectively with fine strains at ends; (f) and (g) fine strains found in the preparation that may be hyaluronic acid. The bar represents 100 nm.

under the peak at $K_{av} = 0.40$ had an estimated M_r of 2000000. This component appeared to be twice the molecular size of the type VI dimer (Gibson & Cleary, 1985), and was therefore judged to be the tetramer of the type VI collagen molecule. Material under the peak at

$K_{av} = 0.21$, and in the void-volume peak, were most probably the double tetramer and higher multimers respectively. These results are consistent with the direct images by rotary-shadowing electron microscopy showing mostly tetramers and double tetramers with a few

Table 1. Various parameters of annulus fibrosus type VI collagen measured by electron microscopy

Native preparations of type VI collagen (*Streptomyces*-hyaluronidase extract) were examined as tungsten-shadowed replicas by electron microscopy. The following dimensions were measured as defined in the schematic drawing accompanying the Table: A, total length of individual tetramer; B, central rod-zone length; C, central rod-zone width; D, width of terminal globule; E, width of internal globule; F, width of the globular interchange domain in aggregated tetramers as indicated in the schematic drawing (two possible models for the globular interchange domain are shown). The column headings refer to: Peak, the most frequent size; Mean, the average size; and Number, the number of molecular images measured.

Fig. 5. Molecular-sieve chromatography of native type VI collagen molecular aggregates under non-reducing and non-denaturing conditions

A sample (2 ml) of type VI collagen extracted from annulus fibrosus by hyaluronidase was chromatographed directly on a column (1.5 cm $\times$ 160 cm) of 2% agarose beads (Bio-Rad A50m) at room temperature eluted with 0.05 M-sodium acetate buffer, pH 6.0, containing 0.15 M-NaCl at a flow rate of 9 ml/h. Fractions (3.6 ml) were collected; material in each fraction was analysed by electrophoresis in SDS/8% polyacrylamide gel under reducing conditions. The electrophoretic profiles shown in the inset are placed in their appropriate position on the chromatogram. The relative elution positions of thyroglobulin (M_r 669000) and apoferritin (M_r 443000) are indicated.

larger aggregates in such extracts. Electrophoresis of material from the various column fractions showed that all consisted of a prominent 140000- M_r polypeptide and four fainter polypeptides (M_r 180000–240000) when run in the presence of SDS after reducing disulphide bonds with dithiothreitol (Fig. 5). Together the results indicate that all these polypeptides are fundamental components of the basic molecular subunit of type VI collagen.

The selective extraction of type VI collagen by hyaluronidase, but not by chondroitinase, suggested that type VI collagen and hyaluronate may be bound to each other in the tissue, directly or indirectly. Aggregation experiments were therefore performed, with portions of an extract of type VI collagen, mixed and equilibrated with various concentrations of added hyaluronic acid (from 0.5–3.0 mg/ml), and chromatographing the mix-

Fig. 6. SDS/agarose/polyacrylamide-gel electrophoresis to determine the size distribution of disulphide-bonded polymers of type VI collagen chains

Addition portions of the samples run in lanes 2 and 4 of Fig. 2 were analysed on an SDS/0.5% agarose/2.4% polyacrylamide composite gel under non-reducing conditions. Lane 1, annulus fibrosus; lane 2, nucleus pulposus. The relative mobility of the reference protein (thyroglobulin, M_r 669 000) is indicated. The other arrowhead identifies the position of a thyroglobulin dimer.

ture on the agarose A50m column. All mixtures showed essentially the same elution profile as that shown in Fig. 5, with no shift in relative peak sizes (results not shown). This indicated that the hydrodynamic size of type VI collagen was not affected by equilibrating polymeric hyaluronic acid with the native collagen preparation. However, since the type VI collagen for this experiment was extracted by hyaluronidase digestion, it could be argued that short hyaluronate oligosaccharides still firmly occupied binding sites on the type VI subunits, so preventing any interaction with the added polymeric hyaluronate.

Agarose/polyacrylamide-gel electrophoresis

In order to obtain information on the molecular size of the type VI collagen native polymers, hyaluronidase-extracted type VI collagen was analysed by electrophoresis in SDS/0.5%-agarose/2.4%-polyacrylamide composite gels under non-reducing conditions. Two bands with M_r values of about 1 000 000 and 2 000 000 were observed (Fig. 6). Their sizes suggested that they represented the disulphide-bonded dimer and tetramer of the basic type VI collagen molecule. No band having an apparent M_r expected for the individual molecule could be found, even when a sample was run on a gel at a slightly higher percentage of polyacrylamide. When excised from the gel, reduced with dithiothreitol and re-run on an SDS/8%-polyacrylamide gel, both of these disulphide-bonded structures gave a similar type VI pattern, with a prominent 140 000- M_r band and the

cluster of four bands in the M_r range 180 000–240 000 (results not shown).

Cross-link analysis

Purified type VI collagen extracted by hyaluronidase was analysed for the presence of borohydride-reducible cross-links and non-reducible pyridinoline cross-links that are present in many types of collagen. Despite the observation of tetramers and multimers in the preparation, hydroxylysino-leucine, dihydroxylysino-leucine and hydroxy-pyridinium (hydroxylysylpyridinoline and lysylpyridinoline) cross-links were not detected (< 0.03 residue of hydroxylysino-leucine or dihydroxylysino-leucine/collagen molecule and < 0.001 residue of hydroxylysylpyridinoline or lysylpyridinoline/collagen molecule). The analyses could also rule out the presence of the desmosine and isodesmosine cross-linking residues found in elastin. This result and the protein's complete extractability without the use of proteinases indicate that type VI collagen is not polymerized by aldehyde-mediated cross-links or any other form of covalent bonds, in contrast with all other known forms of fibrillar collagen of the extracellular matrix (Eyre *et al.*, 1984b; Wu & Eyre, 1985).

DISCUSSION

The extracellular matrix of the intervertebral disc is proving to be a complex mix of collagen and proteoglycan components. Although in many ways combining features of both hyaline cartilage and fibrocartilage, the matrix also has unique biochemical properties.

Nucleus pulposus has a similar collagen phenotype to that of hyaline cartilage, with type II collagen as the main constituent and type XI ($1\alpha 2\alpha 3\alpha$) and type IX collagens as the minor collagens. Annulus fibrosus contains types I and II collagens in its fibrous framework, and both types V and XI are probably there as minor fibrillar collagens. The present findings show that, in addition to these collagens, the annulus fibrosus is unusually rich in the protein called type VI collagen, which accounts for about 5% of the tissue dry weight (compared with $< 1\%$ in articular cartilage and 1–2% in meniscus; J.-J. Wu & D. R. Eyre, unpublished work). In the nucleus pulposus, in which collagen makes up about 30% of the dry weight on the basis of the hydroxyproline content, type VI accounts for about 20% of the collagenous protein.

Type VI collagen, best classed as a short-helix group 3 collagen molecule (Miller, 1985), is widely distributed among soft connective tissues, including aorta, placenta, uterus, skin, ligament, tendon and cornea (Chung *et al.*, 1976; Furuto & Miller, 1980, 1981; Jander *et al.*, 1981, 1983; Abedin *et al.*, 1982; Odermatt *et al.*, 1983; Furthmayr *et al.*, 1983; Knight *et al.*, 1984; Trueb & Bornstein, 1984; Gibson & Cleary, 1985; Engel *et al.*, 1985; Ayad *et al.*, 1985; Zimmermann *et al.*, 1986). It is believed to be the major component of the microfibrillar structures seen in the matrices of these tissues (Von der Mark *et al.*, 1984; Engel *et al.*, 1985).

In previous studies, polymeric and intact-chain forms of type VI collagen have been extracted from tissues by using strong denaturing solvents, either with or without disulphide-bond reduction (Jander *et al.*, 1984; Knight *et al.*, 1984; Trueb & Bornstein, 1984; Gibson & Cleary, 1985; Ayad *et al.*, 1985). An important point to emerge

from the present work is the ability to extract an intact form of type VI collagen from tissues by using *Streptomyces* hyaluronidase under non-denaturing conditions. Tissues were first treated with chondroitin ABC lyase to remove chondroitin sulphate from the proteoglycans, at pH 8.0 to minimize cleavage of hyaluronate. Then *Streptomyces* hyaluronidase was used to depolymerize the hyaluronic acid. Type VI collagen was recovered selectively and in quite pure form in the hyaluronidase extract, suggesting that the protein may be intimately associated with the proteoglycan complex. However, the possibility that type VI collagen might be bound directly to hyaluronic acid in the tissue was not supported by the failure of hyaluronate to influence the hydrodynamic size of type VI collagen when these molecules were mixed and equilibrated before agarose chromatography. The results nevertheless still indicate some form of interaction between type VI collagen and a component of the proteoglycan complex, since type VI seems to be released from the tissue only when the complex is depolymerized by hyaluronidase. An alternative explanation might be that type VI aggregates are physically restricted from diffusing within the matrix by the proteoglycan/hyaluronate polymer, and only become freely diffusible when this is disrupted. It should be noted that, under the conditions used (pH 8.0), chondroitin ABC lyase (supplied by Seikagaku Kogyo Co.), which extracts no type VI collagen, has a low activity against hyaluronic acid. Experiments on articular cartilage have shown that this enzyme can degrade and remove over 80% of the chondroitin sulphate from this tissue, leaving 90% of the proteoglycan cores with their attached keratan sulphate chains, and with the link proteins, still aggregated on hyaluronate and retained within the cartilage (Chun *et al.*, 1986).

It has been consistently observed that intact forms of type VI collagen from cell cultures and tissue extracts contain polypeptides in the M_r range 180 000–240 000, in addition to the main component of M_r 140 000 (Hessle & Engvall, 1984; Gibson & Cleary, 1985; Ayad *et al.*, 1986). A polypeptide of M_r 240 000 was thought to be a precursor or a cross-linked form of the 140 000- M_r chain (Trueb & Bornstein, 1984; Gibson & Cleary, 1985). However, peptide mapping after CNBr digestion or V8-proteinase digestion showed that the four components of M_r 180 000–240 000 resolved in the present study are variants of a single chain that differ in length, and that appear to be quite distinct from the 140 000- M_r chain(s) (J.-J. Wu & D. R. Eyre, unpublished work). This observation supports an earlier suggestion that the 240 000- M_r polypeptide is a distinct chain component of the type VI collagen molecule and is not a derivative or cross-linked form of the 140 000- M_r component (Ayad *et al.*, 1986; Engvall *et al.*, 1986).

Electron microscopy after rotary shadowing of pepsin-solubilized type VI collagen has revealed that the individual type VI molecule consists of a 105 nm triple-helical domain with a globular domain at each end (Furthmayr *et al.*, 1983). These molecules aggregate into antiparallel staggered dimers, two of which in turn align in register to form the basic tetrameric subunit of type VI collagen. The molecular tetramers can form fibrillar structures by an end-to-end polymerization (Furthmayr *et al.*, 1983). The exact interaction sites and the mechanism of the aggregation between tetramers to form polymers is not clear. Disulphide bonding or other

covalent bonding can be ruled out, since electrophoresis of the intact type VI polymers in an agarose/polyacrylamide composite gel in the presence of SDS, but without reduction, showed only two discrete molecular sizes, dimers and tetramers.

The electron micrographs (Fig. 4) and gel-filtration results (Fig. 5) all show that the native form of type VI collagen extracted from disc tissue under non-denaturing conditions and without proteolysis exists largely in the form of tetramers. Double tetramers are also prominent, but there are few larger polymers. Similar observations have been made on type VI collagen made by cultured fibroblasts (Engvall *et al.*, 1986), in that tetramers and some multimers were the prominent forms in the medium and cell layer. It was concluded that the type VI collagen molecules assembled into the tetrameric units before being secreted from the cell (Engvall *et al.*, 1986).

The electron-microscopic measurements presented on the various parameters of the type VI collagen tetramer (Table 1) confirm and extend those originally reported for the pepsin-extracted protein (Furthmayr *et al.*, 1983). It is important to note that in the present work the protein was exposed to neither proteinases nor denaturants, and that the molecules were shadowed with tungsten, not platinum. The former more closely images the native protein.

Recent electron-microscopic findings with immunogold localization established that lateral aggregates of the filamentous form of type VI collagen are responsible for the 100–110 nm banded structures that have been seen in cultures of skin and tendon fibroblasts (Bruns, 1984; Bruns *et al.*, 1986). It therefore seems likely that banded aggregates of type VI collagen, which the present results establish as a prominent protein of the nucleus pulposus, are responsible for the banded structures of 110 nm period that have been noted as a puzzling and consistent feature in electron micrographs of nucleus pulposus (Cornah *et al.*, 1970; Buckwalter *et al.*, 1979).

Though previous biochemical studies have all isolated type VI collagen from tissues in which type I collagen dominates, the present findings show that type VI can be abundant also in a tissue that is based on a type II collagen extracellular matrix. The native polymeric form of type VI collagen and its spatial relationships with type I and type II collagen fibrils and the proteoglycan domain of the extracellular matrix remain to be determined. It seems, however, that, despite its ability to form aggregates as viewed by electron microscopy, type VI collagen has extraction properties and a lack of extensive cross-linking that fit it more in the realm of a matrix glycoprotein that is intimately associated with or entrapped in the proteoglycan complex than of a cross-linked collagen microfibril that provides tensile strength.

This work was supported by grants from the National Institutes of Health (U.S.A.) (GM14237 to H. S. S. and AR36794 to D. R. E.). We acknowledge the assistance of Ms. Rosilyn Ford in portions of this work and Ms. Ginny Apone for preparing the manuscript.

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Received 12 February 1987/4 June 1987; accepted 7 August 1987