

Immunolocalisation of type VI collagen in the intervertebral disc

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Abstract

Type VI collagen has been isolated from many connective tissues, including the intervertebral disc. Distribution of this collagen, however, varies considerably within different tissues. In adult mammalian nasal and articular cartilage it is localised preferentially in the matrix immediately surrounding the cell. Intervertebral discs from various species and of various ages were studied and a similar pericellular localisation was found. When antisera to type VI collagen were used staining was seen around the cells of all sections of intervertebral disc, being particularly prominent in the nucleus pulposus. Staining on or around the cells was also seen in the adjacent cartilaginous end plate and bone.

Type VI collagen is ubiquitous, being found in tissues in which the major collagen is type I¹⁻³ or type II collagen.⁴⁻⁶ Both types I and II collagen occur in the intervertebral disc.⁷ The basic monomer of type VI collagen is a 105 nm long triple helix with a large globular domain at each end. The monomers associate laterally into overlapping dimers and tetramers and form larger filaments or microfibrils by end to end aggregation.^{2,8} The microfibrils are stabilised by intramolecular and intermolecular disulphide bonding. It has been suggested that the function of this type of collagen might be to anchor components such as cells, blood vessels, or nerves to the surrounding matrix.³ In articular cartilage type VI collagen has been found throughout the matrix but is more concentrated around the chondrocytes,³ forming part of the fibrous capsule which contains the cell within the pericellular matrix.⁹ Wu *et al* showed that type VI collagen is a significant component of the intervertebral disc, accounting for up to 20% of the total collagen in calf nucleus pulposus and 5-10% of the collagen in the annulus fibrosus.⁷ No studies, however, have reported on the localisation of this collagen within the intervertebral disc. In this study we used immunohistochemical techniques to determine the distribution of type VI collagen within the intervertebral discs of rat, ovine, bovine, and human specimens.

Materials and methods

PREPARATION AND CHARACTERISATION OF TYPE VI COLLAGEN ANTISERUM

Type VI collagen was prepared from pepsin digests of bovine uterus and purified by differential salt precipitation and by dialysis against

phosphate buffers.¹⁰ The type VI collagen was pure as assessed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. To exclude possible traces of other collagens the preparation was dissolved in 4 M guanidinium chloride, 50 mM TRIS/HCl, pH 7.4, heated at 45°C for 30 minutes, and chromatographed on a Sepharose CL-4B column equilibrated in the 4 M guanidinium chloride buffer. The fractions eluting at and just after the void volume, containing a mixture of highly purified short and long forms² of type VI collagen, were dialysed against 0.1 M acetic acid and freeze dried, as previously described.⁹

An antiserum to pure type VI collagen was raised in rabbits by subcutaneous injection of 500 µg protein in 0.5 M NaCl, 50 mM TRIS/HCl, pH 7.4, emulsified with an equal volume of complete Freund's adjuvant. Further injections of 500 µg protein emulsified with incomplete Freund's adjuvant were given at two weekly intervals. Rabbits were bled two weeks after the third injection. The specificity of the antiserum was assessed by the direct enzyme linked immunosorbent assay (ELISA) technique using native collagens prepared by limited pepsin digestion of bovine nasal cartilage (types II, IX, and XI collagens) and bovine placenta (types I, III, IV, and V collagens) and fibronectin prepared from bovine plasma. The antiserum showed a positive ELISA reaction against bovine type VI collagen up to a dilution of 1:50 000. No cross reactivity was detected by ELISA with the other collagens and fibronectin at dilutions greater than 1:100. This antiserum has been used in several studies on canine, human, and bovine tissues.^{5,9,11,12}

COLLECTION OF SAMPLES

Intervertebral discs were obtained from the lumbar spine of a 7 month old sheep, of 7 week and 7 month old rats, and of 17 and 18 month old heifers. Thirty one human discs from 12

Details of human intervertebral discs

Case No	Age	Sex	Level	Cause of death
1	21	M	L4-5	RTA*
2	28	M	L3-S1	Fall of 30 m
3	60	M	L4-S1	Ischaemic heart disease
4	66	F	L4-S1	Coronary arteriosclerosis
5	67	F	L4-S1	CA* pancreas
6	73	M	L4-S1	Not known
7	73	F	L4-S1	CA pancreas
8	74	F	L4-S1	Stroke, coronary heart failure
9	75	M	L1-3	Pneumonia THR* ?osteoarthritis
10	82	F	L4-S1	Peritonitis
11	83	F	L4-S1	Pulmonary embolism THR
12	85	F	T12-S1	Pituitary tumour

*RTA=road traffic accident; CA=carcinoma; THR=total hip replacement.

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Accepted for publication
30 October 1990

subjects, ranging in age from 21 to 85 years (mean (SD) 65 (21) years), were obtained as soon as possible after death (table). The mid-sagittal slice was removed and material was taken from the annulus fibrosus and nucleus pulposus together with the adjacent cartilage end plate and bone (fig 1). The material was rapidly frozen in hexane at -80°C and stored under liquid nitrogen until required.

IMMUNOFLUORESCENT STAINING

Sections ($6\ \mu\text{m}$ thick) were cut on a Bright's rotary cryostat and stained for type VI collagen by indirect immunofluorescence or immunoperoxidase.¹³ The antiserum to type VI collagen was used at a dilution of 1:250 for immunofluorescence and 1:5000 for immunoperoxidase labelling. In one experiment it was used at 1:1000 with immunoperoxidase label (see 'Results'). Normal serum and phosphate buffered saline or TRIS buffered saline controls were included in each experiment. The fluorescein labelled or peroxidase labelled antisera were used at a dilution of 1:50 and all incubations were carried out for 30 minutes at 37°C .

ENZYME PRETREATMENT

Before staining, the sections were subjected to one of the following enzyme treatments:

(a) *Hyaluronidase* (ovine testicular, Sigma type V, 1250 U/mg) 2 mg/ml in 0.025 M NaCl, 0.05 M sodium acetate buffer adjusted to pH 5.0; incubated for 120 minutes at 37°C . This was the regimen used throughout the study, apart from that discussed under 'Results: effect of enzyme pretreatment'.

(b) *Chondroitinase ABC* (Seikagaku Kogyo from ICN Biochemicals, UK) 0.25 U/ml in 0.1 M TRIS acetate buffer at pH 7.6; incubated for 90 minutes at room temperature.

(c) *Hyaluronidase+chondroitinase 2* mg/ml hyaluronidase, incubated for one hour at room temperature, washed in TRIS acetate buffer, and incubated for a further 90 minutes at room temperature with 0.25 U/ml chondroitinase ABC.

(d) *Collagenase 1* (type III, Advance Bio-factures) 30 U/ml in 0.05 M TRIS and 0.01 M calcium acetate, pH 7.2; incubated for 30 minutes at room temperature.

(e) *Collagenase 2* (type III, Advance Bio-factures) 30 U/ml in 0.05 M TRIS and 0.01 M calcium acetate, pH 7.2; incubated overnight at room temperature, with fresh solution applied 2×30 minutes and 1×60 minutes at 37°C .

(f) *No enzyme treatment* For immunofluorescence all sections were fixed in freshly prepared 4% paraformaldehyde in phosphate buffered saline, pH 7.4, for 10 minutes at room temperature. For peroxidase staining all sections were fixed in 10% formaldehyde in TRIS buffered saline, pH 7.4, for 10 minutes at room temperature. Fluorescence was viewed on a Leitz Dialux 20 microscope fitted with incident illumination and Phloempak 2.4 filter unit.

Adjacent sections were stained with Ehrlich's haematoxylin and eosin.

Results

EFFECT OF ENZYME PRETREATMENT

All sections of the intervertebral disc that were pretreated with hyaluronidase showed positive staining with antiserum to type VI collagen, regardless of whether the tissue was derived from rat, sheep, cattle, or humans or from the annulus fibrosus or nucleus pulposus.

Similar staining patterns were seen after preincubation with other enzymes, including chondroitinase ABC, a combination of hyaluronidase and chondroitinase ABC, and two regimens of collagenase treatment. The positive staining with collagenase treated sections confirmed the specificity of the antiserum for type VI collagen as this collagen resists digestion unless it is first reduced. In sections treated with the collagenase 2 regimen, staining with antiserum to type I collagen was obliterated in adjacent bone tissue.

When there was no enzyme pretreatment the results differed between species. In rat and sheep disc some staining was still present, though to a lesser degree. In contrast, sections of human disc showed no staining.

STAINING PATTERN IN THE DISC

There was a distinct pericellular distribution, with positive staining to anti-type VI collagen being seen around most, but not all, of the cells in every sample. In all the species the degree of staining increased from the outer annulus fibrosus inwards, being most obvious in the nucleus pulposus. Often, the cell membrane, together with a concentric ring beyond that, was positive (fig 2A). The zone between these two circles was also positive in some cases (fig 2B). In some specimens there was a series of concentric, positively stained rings (fig 2C). In the bovine tissue there was much generalised staining of the interterritorial matrix in addition to the pericellular region, particularly in the nucleus (fig 3).

These were the typical patterns seen when the primary antiserum was used at a concentration of 1 in 5000 with immunoperoxidase. If used at a concentration of 1 in 1000, however, the staining pattern differed slightly. In the human disc some of the interterritorial matrix and cells themselves were stained, in addition to the

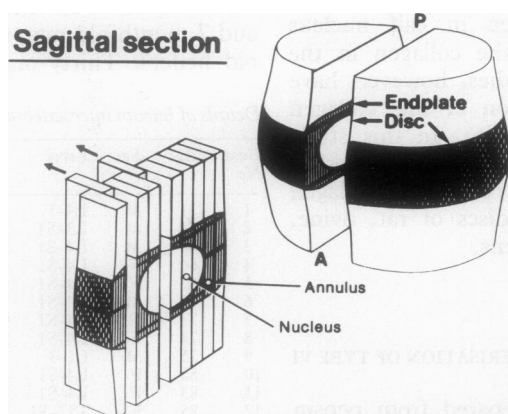


Figure 1 Schematic diagram of sampling technique used for human intervertebral discs.

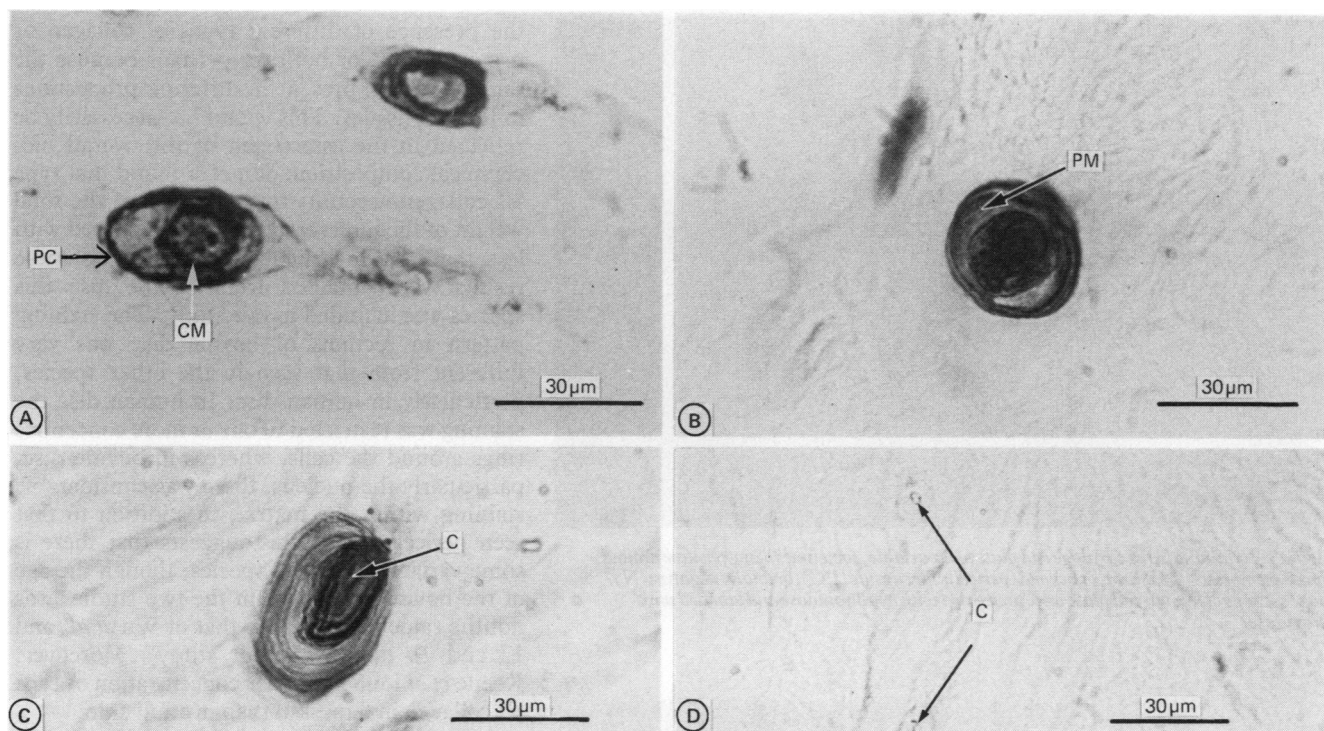


Figure 2 Pericellular staining with antiserum to type VI collagen in the nucleus of the human intervertebral disc is located (A) in the area of the cell membrane (CM) and a concentric ring beyond (pericellular capsule, PC). (B) The area between these two may also be positive (pericellular matrix, PM). (C) In some cases a series of positively stained concentric rings around the cell (C) can be seen. (D) No such staining was seen around cells (C) where normal rabbit serum was substituted for antiserum. All sections were predigested with hyaluronidase and labelled with peroxidase.

pericellular staining seen at 1:5000 dilution (fig 4).

close to the cells was positive (fig 5). There was some variation in the degree of staining throughout the depth of the end plate.

STAINING PATTERN IN THE CARTILAGINOUS END PLATE

There was also a pericellular staining pattern in the end plate, but in this tissue only a region

STAINING PATTERN IN BONE

Most specimens included the adjacent vertebral bone. In these cases positive staining was seen in the osteoid of the bone, together with many osteocytes within the mineralised matrix (fig 6). The mineralised matrix itself, however, was negative. This pattern remained the same even after decalcification with 0.2 M HCl for one hour.

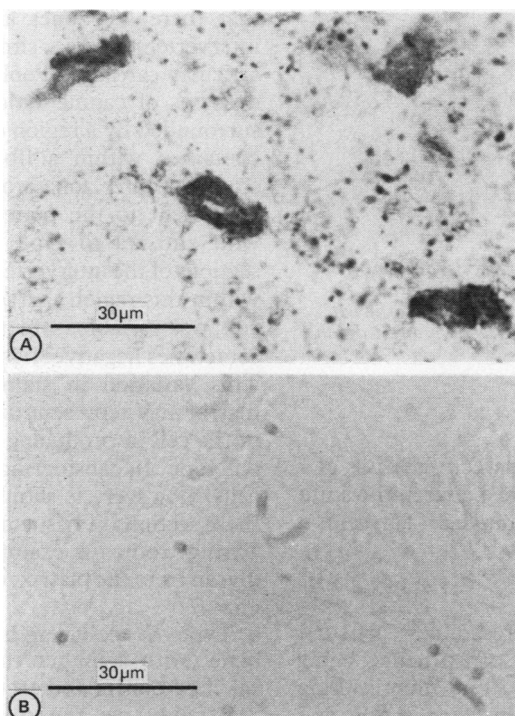


Figure 3 Matrix and pericellular staining in the bovine nucleus pulposus, the section having been pretreated with hyaluronidase and labelled with peroxidase. (B) Control using normal serum.

CHANGE WITH AGE

No change in either the degree or distribution of

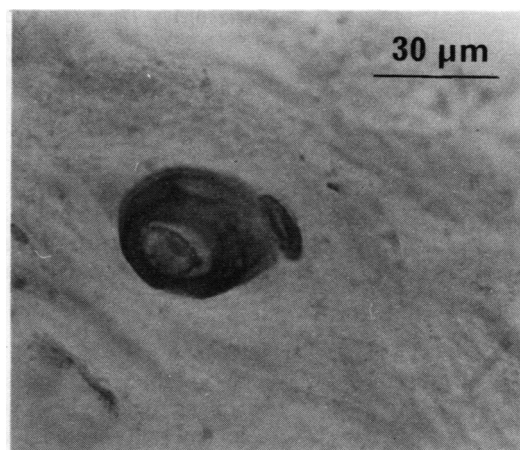


Figure 4 Weak cell and interterritorial matrix staining in the nucleus of human disc with primary antiserum used at a dilution of 1:1000 (cf fig 2 (B)), same specimen stained at 1:5000 dilution. Sections were first digested with hyaluronidase and then labelled with peroxidase.

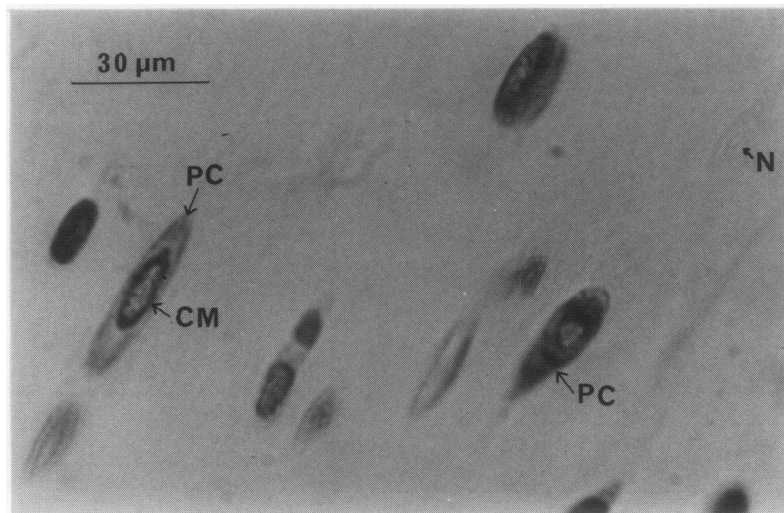


Figure 5 Staining in the cartilage end plate was variable, sometimes being positive around the cell membrane (CM) and, or beyond (pericellular capsule, PC), or totally negative (N). These sections of human end plate were pretreated with hyaluronidase and labelled with peroxidase.

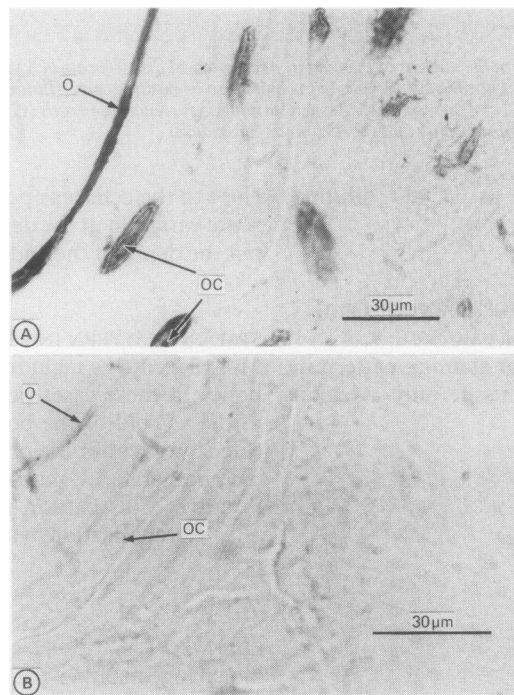


Figure 6(A) The osteoid (O) and osteocytes (OC) in bone were positively labelled with anti-type VI collagen and peroxidase. (B) A similar area on a control section of human bone using normal serum. Sections were initially digested with hyaluronidase.

staining was discernible, between the discs of 7 week and 7 month old rats or between the adult human discs within the age range 21–85 years.

Discussion

The intervertebral disc resembles articular cartilage, with the main constituents being collagen and proteoglycan, but the resulting tissues are very different in organisation and mechanical properties. It is now well known that there are numerous types of collagens and proteoglycans. One reason for the variation in properties of cartilage and disc is perhaps due to

the presence of different types of collagen or proteoglycans, or both, or perhaps because the same types are present in differing proportions in the two tissues. This would not necessarily be reflected in the assessment of the overall biochemical composition. Wu *et al* found that type VI collagen accounts for about 5% of the total weight of the intervertebral disc, compared with less than 1% in articular cartilage.⁷ As these results were obtained from bovine disc this species was included in our study. The staining pattern in sections of bovine disc was very different from that seen in the other species, particularly in human disc. In human disc the staining was restricted to one or more concentric rings around the cells, whereas in bovine disc, particularly the nucleus, there was considerable staining within the matrix, in addition to that seen pericellularly. This suggests that there is some variation between species, though the age of the bovine specimens in the two studies was not the same (3 months in that of Wu *et al*⁷ and 17 and 18 months in our study). Moreover, Keene *et al* found a higher concentration of type VI collagen in fetal skin than in adult skin.³

A pericellular localisation for type VI collagen has been seen in nasal and articular cartilage.^{3,4,9} Indeed this type of collagen seems to have a function similar to that of fibronectin with the ability to adhere to cell membranes within tissues. There are several Arg-Gly-Asp (RGD) tripeptide sequences within the triple helical region of all three α chains of type VI collagen.¹⁴ However, only the RGD sequences in the $\alpha 2$ (VI) and $\alpha 3$ (VI) chains appear to be functional.¹⁵ Perhaps these enable the cell to be located and fixed within the matrix. It is therefore not surprising to find a pericellular distribution within disc tissue as well.

Staining patterns seen in this study indicate that there are zones around the cells of the intervertebral disc similar to those seen in articular cartilage. Poole *et al* described chondrocytes of canine articular cartilage as being surrounded by a region of pericellular matrix all contained within a fibrous capsule.¹⁶ In our study an outer zone around the cells, probably equivalent to the pericellular capsule, stained with antisera to type VI collagen in all the sections of the intervertebral disc (fig 2). Staining within this region, between the capsule and the cell, was variable, sometimes being positive and sometimes negative (figs 2B and A respectively). This variation in staining of the pericellular matrix may represent different levels of activity of the cell in producing type VI collagen at any one time. In considering these zones around the cells, however, it should be remembered that these sections of tissue may be prone to artefacts arising from, for example, leaching of proteoglycan from the matrix, or the plane of sampling, etc.

Type VI collagen has also been found in bone, with a higher concentration in the uncalcified matrix and around the osteocytes.¹⁷ In this study we found strong staining in the osteoid and apparently around or over the osteocytes. This latter appearance might have been due to poor resolution of pericellular staining. Sections in the study of Becker *et al*

were viewed at a greater magnification than those in our study.¹⁷

For several years there have been reports of unique banded or beaded filamentous structures occurring in various connective tissues, in addition to the usual cross banded collagen fibrils. These structures have also been reported in the intervertebral disc.¹⁸⁻²¹ Cornah *et al* described these banded structures (with a repeating period of 100 nm) in the nuclei of both rabbits and scoliotic human discs.¹⁸ They formed a complete corona around the chondrocyte. Buckwalter *et al* also found such structures (but with a period of 85 nm) in all human nuclei, both those obtained at surgery and at necropsy.²¹ These structures are now accepted as being lateral aggregates of type VI collagen microfibrils,²² but whether these are present in vivo or arise artefactually during electron microscopy is not known. The typical pericellular pattern seen in all discs stained with anti-type VI collagen in this study is further evidence indicating that these banded structures are indeed due to type VI collagen within the tissue.

We thank the Arthritis and Rheumatism Council for financial support, Susan Newall and Helena Evans for excellent technical assistance, Roger Evans for provision of bovine material, and Dr V C Duance for provision of antiserum to type I collagen.

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