Chondrons from articular cartilage:

I. Immunolocalization of type VI collagen in the pericellular capsule of isolated canine tibial chondrons

C. ANTHONY POOLE¹, SHIRLEY AYAD² and JENNIFER R. SCHOFIELD¹

¹Connective Tissue Research Group, Department of Surgery, University of Auckland, School of Medicine, Private Bag, Auckland, New Zealand
²Department of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, England

Summary

A heterogeneous population of intact chondrons extracted from low-speed homogenates of canine tibial cartilage were stained by indirect immunofluorescence methods with a polyclonal antibody to type VI collagen. In each of the four chondron groups examined, anti-(type VI collagen) antisera was concentrated in the capsule immediately adjacent to the chondrocyte complex. A constant but weaker fluorescent reaction persists in 'tail-like' extensions common to single and double chondrons and in the medial connections between adjacent chondrons in linear columns and aggregated clusters. Frayed collagen bundles typical of chondron preparations did not react with the antibody. Similarly, chondrons reacted with normal rabbit serum, or treated by type VI collagen extraction procedures, showed no staining reaction. The differential localization of type VI collagen in the pericellular capsule is discussed in relation to the maintenance of the chondron's integrity and to the protection of the chondrocyte during dynamic compressive loading.

Key words: articular cartilage, chondrons, type VI collagen.

Introduction

Recent studies have successfully isolated intact chondrons from low-speed homogenates of adult canine tibial cartilage (Poole et al. 1986, 1988a) and established the chondron as a true morphological entity in articular cartilage. Chondrons have long been considered to function as the primary metabolic unit in hyaline cartilages (Benninghoff, 1925; Szirmai, 1969; Wobst et al. 1980). In articular cartilage, they consist of a chondrocyte, its pericellular matrix, and a compacted filamentous capsule thought to contain minor cartilage collagens (Poole et al. 1984, 1985, 1987).

In the first of a series of experiments on the pericellular distribution of minor collagens, chondrons extracted from porcine radio-ulnalar cartilage and rat condrosarcoma were reacted with an antibody to type IX collagen (Poole et al. 1988b). The localization of this collagen species in the pericellular capsule suggests a close functional inter-relationship between chondron components and the dominant fibrillar network of articular cartilage, which could be important in tethering the chondron and maintaining the position of the chondrocyte within the matrix (Poole et al. 1988a,b). These observations are also consistent with the recent data showing covalent pyridinoline crosslinks between type II and type IX collagens (Eyre et al. 1987a).

This report establishes the presence of an additional minor collagen species, type VI collagen, as an integral component of isolated canine tibial chondrons.

Materials and methods

Sample collection

Fresh cartilage samples were resected at the tidemark from the medial and lateral tibial plateaux of 10 mature, healthy,
crossbred dogs (2–6 years; 20–37 kg) killed under supervi-
sion at a local Pound. Resected samples were immediately
transferred to an ice-cold sterile solution of Dulbecco's
modified Eagle medium (DMEM) prepared from dried
dry powder (Gibco, Grand Island, New York), and buffered
with sodium bicarbonate (44 mM). The total sample, weigh-
ing approximately 1 g, was finely diced and suspended in
20 ml fresh sterile DMEM at 4°C in preparation for hom-
genization. Occasionally, samples of intact cartilage were
retained for histology and comparison of chondron structure
in intact versus extracted tissues.

Chondron extraction
Homogenization was carried out using a Polytron grinder
(Kinematica GMBH, Lucerne, Switzerland) fitted with a
PTA 10S aggregate and accurately controlled by a RECO 302
programmable regulator at selected speeds ranging from 4000
to 10 000 revs min⁻¹.

All samples were treated by serial homogenization. Under
this regime the cartilage samples were homogenized for
5–15 min at the selected speed. The homogenate was briefly
spun at 200 g for 30 s to sediment large cartilage chips and the
flocculent supernatant was collected. The cartilage sediment
was then washed and agitated with 10 ml fresh media, the
supernatant was again briefly spun at 200 g for 30 s and the
flocculent supernatants were pooled. The remaining cartilage
pellet was resuspended in 20 ml fresh media at 4°C and the
homogenization sequence was repeated until the cartilage
sample was exhausted.

At the completion of serial homogenization the pooled
supernatants were filtered through a series of Nybolt nylon
filters (Swiss Silk, Zurich, Switzerland) ranging in pore size
from 1000 to 100 μm² as described (Poole et al. 1986, 1988a).
Material trapped on the filters (<500 μm²) and the filtered
supernatant were collected and centrifuged at 400 g for
15 min to pellet suspended material, which was then washed
and stored at 4°C in preparation for evaluation by phase-
contrast microscopy and antibody staining.

Preparation of type VI collagen antigen
Type VI collagen was prepared from bovine uterus by pepsin
digestion and purified by differential salt precipitation and by
dialysis against phosphate buffers (Abedin et al. 1982). The
fraction that precipitated at 2 M-NaCl, pH 2–5, and by dialysis
against 0.02 M-Na₂HPO₄, pH 9–2, consisted of pure type VI
collagen as assessed by SDS–polyacrylamide gel electrophoresis
(SDS–PAGE) (Laemmli, 1970). However, in order to exclude possible traces of other collagens (particularly type V
collagen) not detectable by SDS–PAGE, the purified
type VI collagen preparation was dissolved in 4 M-guanidi-
nium-chloride, 50 mM-Tris-HCl, pH 7–4 (4 M-GuCl buffer), heated at 45°C for 30 min and applied to a Sepharose
CL-4B column (1.5 cm × 94 cm) equilibrated with the 4 M-
GuCl buffer. The column was eluted at a rate of 19 ml h⁻¹
and the fractions eluting at and just after the void volume,
which contained the pure type VI collagen, were dialysed
against 0.1 M-acetic acid and freeze-dried.

Preparation of anti-(type VI collagen) antiserum
An antiserum against the pepsinized form of type VI collagen
was raised in female rabbits by subcutaneous injection of
0.5 mg of protein in 0.5 M-NaCl, 50 mM-Tris-HCl, pH 7–4,
emulsified with an equal volume of complete Freund's
adjuvant. Additional injections of 0.5 mg protein emulsified
with incomplete Freund's adjuvant were given at 2-weekly
intervals. The antiserum was collected 2 weeks after the third
injection and showed a positive reaction against type VI
collagen up to a dilution of 1:50 000 as assayed by direct
ELISA (Rennard et al. 1980). No cross-reactivity was
observed with the cartilage types II, IX and XI collagens or
with the non-cartilage types I, III and IV collagens. Weak
cross-reactivity at concentrations greater than 1:100 was
observed with the cartilage types II, IX and XI collagens,
the 7S domain of type IV collagen and fibronectin. However, the crude anti-
serrum, when used at dilutions greater than 1:500, produced
an identical staining pattern to that observed with the
purified antibody (Aplin et al. 1988; also see below).

Purification of anti-(type VI collagen) antiserum
IgG antibodies were isolated from the crude antiserum by
ammonium sulphate precipitation (45 % saturation) followed
by affinity chromatography on protein A–Sepharose equilib-
trated in phosphate-buffered saline (PBS). Bound IgG anti-
bodies were eluted with 0.1 M-glycine-HCl, pH 2–8, and
immediately dialysed against PBS. The IgG fraction was
further purified by affinity chromatography on type V col-
lagen, 7S collagen, fibronectin and finally type VI collagen,
each immobilized on Sepharose 4B. The purified antibody
was specific for type VI collagen and showed no activity
against other collagen types. It reacted preferentially with
the
Fig. 2. Single chondrons viewed by phase-contrast (A,C) and fluorescence microscopy (B,D,E). A–B. Simple chondrons consist of a moderately dense chondrocyte (c), a birefringent pericellular matrix (pm) and a dense refractile pericellular capsule (pc). Affinity-purified antibody reaction was confined to the pericellular capsule, which defines the boundary of the chondrocyte complex (cc). A, ×1835; B, ×1870. C–D. Tapered tails (t) common to single chondrons appear continuous with the pericellular capsule and were often associated with frayed collagen fibres (small arrowheads). Capsular tails stained weakly with crude antiserum, which was concentrated around the chondrocyte complex (cc). C, ×895; D, ×1055. E. In capsular ghosts, the loss of the chondrocyte complex and a uniform staining reaction suggests the capsule collapses during processing. The persistence of a tail in many ghosts indicates that they form an integral component of the capsular structure. ×1210.

\(\alpha 3(\text{VI})\) chain of the pepsinized type VI collagen preparation in immunoblots using the method of Blake et al. (1984).

**Antibody staining**

Samples (1 ml) of filtered chondrons were placed in plastic tubes, centrifuged at 400 g for 10 min and washed in PBS (pH 7.3). Samples were resuspended in 1 ml of PBS (pH 5.5) containing 2 mg (600 units) of ovine testicular hyaluronidase (type IIS, Sigma Chemical Company, Illinois, USA), digested for 2 h at room temperature with constant agitation and finally washed three times and pelleted as described above. Chondrons were resuspended in 1 ml PBS containing anti-(type VI collagen) antiserum at dilutions of either 1:500–1:1000 (crude antiserum), or 1:100–1:1000 (purified antibody), and agitated overnight (14 h) at room temperature. Preparations were again washed and pelleted three times and resuspended in 1 ml PBS containing fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Amersham Australia Pty Ltd, North Ryde, Australia) at dilutions of 1:100. After agitation for 2 h at room temperature, chondrons were finally washed and pelleted, resuspended in 0.5 ml PBS and viewed on a Reichert microscope fitted with epifluorescent optics. Kodak Ektachrome 200 colour transparencies and Ilford FP4 or HP5 black and white film were used for all photography.

For control experiments, samples of fresh homogenate were collected onto cellulose acetate filters (Gelman Scientific, Indiana, USA) to minimize chondron damage during the extraction procedures attempted. In negative controls, an anti-(type VI collagen) antiserum was replaced by normal rabbit serum diluted 1:20 in PBS. Alternatively, type VI collagen was extracted from filter-collected chondrons by reduction, alkylation and digestion at 37 °C with 0.5–1.0 \(\mu\)g ml\(^{-1}\) bacterial collagenase (Sigma Chemical Company, IL, USA) for 60–120 min (Knight et al. 1984). In positive control experiments chondrons on filters were digested with bacterial collagenase without prior reduction and alkylation. All control filters were finally treated with testicular hyaluronidase and processed in a manner similar to that described above.

**Results**

**General comments**

The structure of adult canine tibial cartilage is illustrated in Fig. 1 and the morphology of chondrons typical of each matrix layer identified. The homogenization procedures employed in this study effectively shredded the cartilage matrix and release chondrons in a random fashion. As a consequence, many chondrons were damaged during the process, often resulting in capsular 'ghosts' devoid of their chondrocyte–pericellular matrix complex (hereafter referred to as the 'chondrocyte complex'). The cartilage homogenates examined therefore represent a heterogeneous mixture.
Fig. 3. Complementary sets of double chondrons viewed by phase-contrast (A,C) and fluorescence microscopy (B,D). A–B. In its simplest form two chondrocyte complexes (cc) are confined within a common pericellular capsule (pc). A purified antibody reaction surrounds each chondrocyte complex with a narrow medial boundary of reduced fluorescence (arrowheads) between each chondron. A, ×1554; B, ×1710. C–D. The spatial separation of each chondrocyte is defined by the length of the medial capsular segment (arrowheads). Phase-dense tails (t) often taper from one pole of double chondrons and constantly show a reduced reaction to crude antiserum, which was concentrated in the capsule around each chondrocyte complex (cc). C, ×1430; D, ×1520.

of chondrons from all levels of the matrix, capsular ghosts and teased collagen debris.

Chondrons were divisible into four broad categories: single chondrons, double chondrons, multiple linear columns (three or more chondrons) and chondron clusters/matrix 'chips'. Fresh samples of each homogenate were extensively photographed by phase-contrast microscopy and then matched with equivalent examples of labelled chondrons from the same homogenate. It is important to note however, that the images of chondrons presented in these figures represent whole-mount preparations and, as such, the central portion of the capsule is seen en face and thus appears less intensely stained than the margins of the chondron, which are viewed on edge and appear to fluoresce more intensely (Fig. 2). As a consequence, the space occupied by the chondrocyte complex was well-defined in intact examples but was lost in collapsed capsular ghosts (Fig. 2E).

**Single chondrons**

Single chondrons represent the smallest chondron unit identified and were commonly found in the middle layers of intact tibial cartilage (Fig. 1). In its simplest form (Fig. 2A,B) the capsule appeared as an ovoid 'bladder' around the cell and stained intensely with purified antibody (Fig. 2B).

A feature common to many single chondrons was the presence of a tapered 'tail' that extends from one pole of the capsule (Figs 1, 2C,D). These phase-dense tails showed a wide variation in length and were often interwoven with frayed collagen bundles (Fig. 2C). In equivalent examples stained with crude antiserum the most intense reaction was found in the capsule immediately adjacent to the chondrocyte complex, while a less-intense but well-defined reaction persists along the length of the tapered tail (Fig. 2D). Pericellular channels (Poole *et al.* 1984, 1985, 1988a) were regularly observed during this study and also stained with antibody (results not shown).

**Double chondrons**

Double chondrons were common to canine tibial homogenates (Fig. 3) and were typically found in the middle-to-deep layers of intact tissue (Fig. 1). In their simplest form (Fig. 3A) the double chondrons consist of two chondrocyte complexes spatially separated but linked together by a common capsular sheath (Fig. 3A).
Fig. 4. Multiple chondron columns: A. in phase-contrast a linear column of five chondrons is connected by a common capsular sheath (arrowheads). Frayed collagen bundles (cf) persistently radiate from multiple linear columns. ×970. B. Following challenge with crude antiserum, discrete double and treble chondrons appear to join at a weakly stained interface (arrowhead) to form a column of five chondrons. Radial collagen fibres show no reaction to the antiserum. ×965.

Discrete type VI collagen antibody staining was evident around each chondrocyte complex, producing a clear separation between the two chondrons in the unit (Fig. 3B). A similar staining pattern persists in examples where the chondrocytes were more widely separated (Fig. 3C,D), with a weaker fluorescent reaction in the medial connection between the chondrons (Fig. 3D). Tapered tails also present in double chondrons showed a constant but weak reaction to the crude antiserum (Fig. 3D).

Chondron columns

Multiple chondron columns consist of three or more chondron units organized in a linear array typical of those seen in the deep layers of intact tibial cartilage (Figs 1, 4). Larger columns usually contain combinations of smaller chondron units linked together in series (Fig. 4A). This was particularly evident after antibody staining when, in the example illustrated (Fig. 4B), discrete double and treble chondron units appeared to join at a clearly defined interface to produce a column of five. Again, type VI collagen was concentrated in the pericellular capsule with a weaker reaction between adjacent chondrons (Fig. 4B). Frayed collagen bundles typical of linear columns were sensitive to collagenase digestion (Poole, personal observation) and showed no cross-reactivity with crude antiserum (Fig. 4B).

Clusters and chips

Clusters consist of randomly organized groups of single and double chondrons that remain aggregated during the homogenization procedure. An intense fluorescent reaction was common to the capsule surrounding each chondrocyte complex, with a weaker stippled reaction linking individual chondrons in the cluster (Fig. 5A).

Chips represent the largest of the filtrates collected and varied from 200 and 500 μm in diameter. These large cartilage fragments usually contained several antibody-positive chondrons (Fig. 5B) aligned and spatially organized in parallel, with radial collagen fibres typical of the deep layer matrix.

The superficial layer represents a small proportion of the total sample homogenized (see Fig. 1) and was usually identified as large, flat chips collected on the coarser nylon filters (>500 μm) not used in this study. Occasionally, however, small surface layer fragments containing circular, disc-shaped chondrocytes were found in the labelled preparation and showed an intense fluorescent reaction around each chondrocyte complex (Fig. 5C). In contrast to chips from the deep layer, a weak fluorescent reaction persisted in the fibrous matrix that separates chondrons in superficial layer chips (cf. Fig. 5B,C).

Controls

In control experiments, chondrons collected onto cellulose filters and stained with crude antiserum reacted intensely (Fig. 6A), while comparable samples treated with normal rabbit serum showed a complete absence of chondron staining (Fig. 6B). Similarly, samples that were reduced, alkylated and treated with bacterial collagenase to remove type VI collagen showed no reaction to the crude antiserum (Fig. 6C). In contrast, pre-digestion of filter-collected chondrons with collagenase alone, which is sufficient to remove all other cartilage collagens but not native type VI collagen (Abedin et al. 1982; Jander et al. 1984), showed a strong antibody reaction in the capsule of the chondron (Fig. 6D). Moreover, the total absence of matrix staining in all but the most superficial layer samples confirms the lack of cross-reactivity with the type II collagen, which predominates in larger cartilage fractions (see Figs 4 and 5).
Fig. 5. Chondron clusters and matrix chips stained with crude antiserum: A. In a cluster of seven single chondrons, the capsule surrounding each chondrocyte complex are stained intensely, while a weaker fluorescent reaction connects each chondron in the cluster (arrowheads). ×890. B. Large matrix chip from the deep layer showing a number of antibody-stained chondrons aligned parallel to the radial collagen fibres of the matrix that show no reaction. Non-aligned chondrons (arrowheads) were located within frayed collagen bundles adhering to the dominant matrix chip. ×220. C. Flat chips of matrix thought to originate from the superficial layer contained disc-shaped chondrocytes surrounded by bright fluorescence. A weaker reaction persists in the collagenous matrix separating these chondrons, suggesting that type VI collagen has a wider matrix distribution in the superficial layers than in the deep layers (cf. Fig. 5B). ×260.

Discussion

Techniques for the extraction and isolation of chondrons from low-speed homogenates of mature canine tibial cartilage have recently been introduced (Poole et al. 1986, 1988a) and are currently being developed to examine the detailed structure, composition and function of this fundamental cartilage unit. While it must be conceded that many chondrons are damaged during the homogenization procedures, comparison of extracted chondrons with those found in intact cartilage (Fig. 1) confirms that basic chondron morphology is retained following homogenization. Recent trials on chondrocyte viability have also shown that a small but significant proportion of chondrons retain their metabolic status after the homogenization procedures described (Poole, personal observation). We therefore believe the antibody staining reactions presented conclusively identify type VI collagen as an integral component of the capsule in isolated canine tibial chondrons.

Type VI collagen has now been extracted from a wide variety of non-cartilaginous tissues. In these 'soft' tissues it is organized into microfibrils with a characteristic beaded periodicity of 100–110 nm (for review, see Engel et al. 1985; Rauterberg et al. 1986). Yet, despite the ubiquitous distribution of type VI collagen among the connective tissues examined, its presence in hyaline cartilages remains controversial. Using immunochemical techniques Von der Mark et al. (1984) found no trace of anti-(type VI collagen) antibody staining in hyaline cartilage. Similarly, Lisenmayer et al. (1986) reported little or no reactivity in several chick cartilages examined, despite extensive hyaluronidase pretreatment. Ayad et al. (1984) on the other hand reported type VI collagen localized in a pericellular position around adult bovine nasal cartilage chondrocytes, while Engel et al. (1985) described unpublished immunofluorescent data indicating a co-distribution of type VI and type II collagen in the nucleus pulposus. Subsequent biochemical studies have confirmed these observations, showing that type VI collagen forms 20% of the total collagen in young nucleus pulposus (Wu et al. 1987), 1–2% of the collagens in adult bovine articular cartilage (Eyre et al. 1987b) and can be extracted from growth plate cartilage (Ayad et al. 1987).

The discovery of type VI collagen in hyaline cartilages is not surprising, since banded aggregates with a repeating periodicity of 110 nm have previously been identified in the nucleus pulposus of young rabbits and humans (Cornah et al. 1970; Buckwalter et al. 1979) and, on the basis of their comparison with beaded
controls: A. Small samples of homogenate collected onto filters and stained with crude antiserum show the diversity of chondron fragments present in the homogenate. X106. B. Substitution of the crude antiserum with normal rabbit serum reveals a complete absence of chondron staining on the complementary half of the filter shown in A. X106. C. No antibody reaction was evident after reduction, alkylation and bacterial collagenase digestion of filter collected chondrons. X106. D. The capsule of an intact single chondron shows a typical antibody staining reaction after bacterial collagenase digestion. X1600.

Type VI collagen in isolated chondrons

filaments (Bruns, 1984; Bruns et al. 1986), probably represent a native form of type VI collagen in this tissue. Recent ultrastructural studies on human articular cartilage have shown identical banded fibrils preferentially localized in the pericellular capsule of the chondron (Poole et al. 1987), and again these probably represent the intact form of this collagen in articular cartilage.

Type VI collagen is characterized by numerous disulphide bonds, which stabilize its tertiary and quaternary structures and as a consequence render it completely resistant in its native form to bacterial collagenase (Abedin et al. 1982; Jander et al. 1984). It is, however, degraded by this enzyme if the disulphide bonds are first reduced. These properties were exploited in positive and negative control experiments, which indicate (Fig. 6) that the antiserum used was highly specific for type VI collagen. We therefore conclude that type VI collagen in articular cartilage is exclusive to the capsule of the chondron and does not form a general component of the territorial and interterritorial matrices. We did note however that chips of cartilage obviously originating from the superficial layers, showed weak fluorescence in the matrix between densely stained chondrons (Fig. 5C), suggesting that type VI collagen may be more widely distributed in the superficial layers.

The function of type VI collagen is not yet understood. Current speculation suggests that cell-associated type VI collagen may provide an adhesive mechanism for substratum attachment (Carter, 1982; Bruns, 1984; Linsenmayer et al. 1986), while type VI collagen microfibrils associated with striated interstitial collagens may provide an independent fibrillar network involved in the organization and/or stabilization of extracellular matrix components (Gibson & Cleary, 1983; Jander et al. 1984; Linsenmayer et al. 1986; Von der Mark et al. 1984).

The isolated chondrons illustrated showed a constant but differential staining reaction, with higher concentrations of type VI collagen in the capsule immediately adjacent to the chondrocyte complex and reduced concentrations in both the tapered tails and the medial connections between adjacent chondrons. It is therefore possible that type VI collagen could perform a dual role in the chondron. In higher concentration around the cell, type VI collagen could provide a substratum suitable for the functional interaction between the chondrocyte and the pericellular capsule, which is thought to resist the hydrodynamic distention of the chondron during compressive loading (Poole et al. 1984, 1985, 1988a). Conversely, the tail-like extensions and medial connections are thought to tether the chondron and secure the position of the chondrocyte within the matrix (Poole, 1988a,b). The wider distribution of type VI collagen in these structures could therefore be important in organizing and stabilizing the minor cartilage collagens and fine type II collagen fibres previously identified in the cellular microenvironment (see Poole et al. 1987, 1988a,b).

The mechanisms responsible for the formation of double and treble chondrons or their organization into linear columns have not been identified. However, the fluorescent staining pattern common to the capsule of each chondron suggests that individual chondrocytes are responsible for the formation of their own cellular microenvironment, while the continuity of the capsule around multiple chondron columns implies some form of cooperative interaction between chondrocytes within the group. We suggest that chondrocytes at different levels of the matrix would experience a unique set of dynamic mechanical forces and could subsequently develop, in conjunction with neighbouring cells, a protective cellular microenvironment tailored to suit
the individual requirements of the cell and its position within the matrix.

This work was supported and financed by the Medical Research Council of New Zealand (C.A.P., J.R.S.) and by a grant from the Arthritis and Rheumatism Council, England to S.A. We thank Mr Michael Flint and Dr Stephen Skinner for critical comments on the manuscript and Mrs Patricia James for her continued secretarial support.

References


VON DER MARK, H., AUMAILLE, M., WICK, G.,


(Received 5 January 1988 – Accepted, in revised form, 12 April 1988)