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Localization of type IX collagen in chondrons isolated from porcine articular cartilage and rat chondrosarcoma

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Summary

Chondrocytes, each with their pericellular matrix bounded by a fibrous capsule, can be extracted singly or in groups from both mature pig articular cartilage and chondrosarcoma tissue. These structures, termed chondrons, are thought to anchor the chondrocytes in the matrix and protect them from the compressive forces experienced when articular cartilage is under load. The capsule of these chondrons contains both type II and type IX collagens and is composed of fine fibrillar material, unlike the large banded fibres of type II collagen found in the rest of the matrix. This suggests a role for type IX collagen in regulating the diameter of type II fibres to produce the fine fibrillar structure of the chondron capsules.

Introduction

Chondrocytes in the middle and deep layers of adult articular cartilage are each enclosed within a specialized microenvironment consisting of a proteoglycan-rich pericellular matrix surrounded by and integrated with a fibrous felt-like lamina, the pericellular capsule (Meachim & Stockwell 1979; Poole *et al.*, 1984, 1985, 1987, 1988; Clark, 1985). The combined unit of the chondrocyte, its pericellular matrix and capsule have been collectively called the chondron after Benninghoff (1925) who used the term to describe the chondrocyte and its specialized microenvironment in hyaline cartilages (Szirami, 1969; Wobst *et al.*, 1980, Murray, 1985; Poole *et al.*, 1987). Chondrons can be isolated intact by high-speed homogenization from horse nasal septum (Szirami, 1969) and from canine articular cartilage of the tibial plateau (Poole *et al.*, 1988). The integrity of the chondron structure, and thus its extractability, is dependent on the type of cartilage, and in the case of articular cartilage, on the zone of cartilage used. The most well-defined and easily extracted chondrons are derived from the deep zone of articular cartilage where the chondrocytes are arranged in columns of varying number of cells which are maintained intact on extraction (Poole *et al.*, 1987, 1988). Chondrons containing single cells are obtained from the middle zone of articular cartilage. It is not possible to extract intact chondrons from the superficial layer where the cells are horizontally flattened and have less clearly defined pericellular matrices. The morphology of the chondrocytes and chondrons in the different zones is thought to reflect their different experience of mechanical loading. Thus the superficial zone is comparatively resistant to compression due to the direction of the collagen fibres while in the middle and deep zones the compressive forces cause more deformation and the chondrons are correspondingly better defined, suggesting a protective role.

It has previously been suggested that the capsule of the chondron may comprise minor collagen types such as type IX collagen (Poole *et al.*, 1984, 1985; Duance *et al.*, 1985). Evidence for this to date has been circumstantial since the capsule appears to be constructed of fine fibrillar material and type IX collagen is known to form fine unbanded fibres *in vitro* (Duance *et al.*, 1985). Using monoclonal and polyclonal antibodies to type IX collagen on sections of cartilage, some studies have shown an increased incidence of type IX collagen located around the lacunal rim which corresponds to the capsule of the chondron (Duance *et al.*, 1982; Ricard-Blum *et al.*, 1982; Evans *et al.*, 1983).

However, other immunolocalization studies on

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embryonic and adult avian sternal cartilages show no such differentiation in type IX or type II collagen distribution (Vaughan *et al.*, 1985; Irwin *et al.*, 1985; Muller-Glauser *et al.*, 1986). Rather, they revealed a uniform distribution of both collagen species throughout the matrix (Vaughan *et al.*, 1985; Irwin *et al.*, 1985) and suggested that type IX may interact with type II collagen.

There is evidence that additional collagen types are located in the capsule surrounding the chondrocytes including type V, type VI and type XI (Gay *et al.*, 1981; Ricard-Blum *et al.*, 1982; Grant *et al.*, 1987) but more detailed studies are required to verify these observations.

Here we describe the isolation of chondrons from articular cartilage of young and old pigs and from the rat chondrosarcoma. The immunolocalization of type IX and type II collagen in the capsule of the chondron is also described and the relevance of these studies to the structure of different cartilage types is discussed.

Materials and methods

Chondron preparation

Cartilage was resected from proximal and distal superior radioulnar joint surfaces of adult and immature pigs and stored in Dulbecco's Modified Eagles Medium at 4° C. The cartilage sample was finely diced, suspended in 20 ml of fresh medium and homogenized in a Polytron tissue homogenizer for 15–30 min at approximately 1000 rev. min^{-1} (Poole *et al.*, 1988).

After homogenization, cartilage chips were allowed to settle and the flocculent supernatant was retained. The remaining cartilage chips were rehomogenized for a further 15 min. Four successive homogenizations yielded 80–100 ml of flocculent homogenate, which was filtered through successively finer nylon Nybolt filters as previously described (Poole *et al.*, 1988). The chondrons used in this study were washed from the 50 and 100 μm^2 pore size filters, and transferred to microfuge tubes prior to further washing and pelleting at 400 *g* for 10 min.

Chondrons were prepared from the comparatively soft chondrosarcoma tissue from rats by a more gentle procedure. The outer membrane of the tumour was removed and the tissue gently sieved through Nybolt nylon monofilament filters with 200, 40 and 20 μm^2 pore sizes and washed through with Dulbecco's tissue culture medium or phosphate-buffered saline (PBS). The resulting suspension of chondrons was then washed twice in PBS before examination of wet-mounted slides by phase contrast light microscopy or use in immunolocalization experiments.

Antibodies and specificity

The murine monoclonal antibody to type IX collagen was produced by standard procedures using type IX collagen extracted from the rat chondrosarcoma (Duance *et al.*, 1984) as antigen. Purified monoclonal antibody was concentrated from tissue culture supernatant by adsorption on a mouse IgG–Sepharose column.

The polyclonal antibody to type II collagen was raised in rabbits against type II collagen extracted from pig articular cartilage and is the same as that described in Duance *et al.*, (1982). A monoclonal antibody raised against pig IgG2 was used in some control experiments.

Specificity of the type IX and type II antibodies was confirmed by localization on Western blots.

Immunolocalization

Chondrons were labelled in suspension with either a monoclonal antibody to type IX collagen or a polyclonal antibody to type II collagen and some preparations were double labelled. The chondrons were thoroughly washed between steps by repeated centrifugation (300 *g*, 10 min) and resuspension in PBS. Second antibodies conjugated to fluorescein or Rhodamine (Sigma) were added to the suspensions and excess was removed by thorough washing in the same way. Wet-mounted preparations of labelled chondrons were examined in a Leitz fluorescence microscope. In some preparations the chondrons were treated with bovine testicular hyaluronidase (Sigma) prior to antibody labelling. Hyaluronidase was dissolved in PBS (1 mg in 1 ml) and the isolated chondrons were treated for 1 h at 25° C.

Electron microscopy

Transmission electron microscopy was performed on chondrons isolated from rat chondrosarcoma prepared as above and centrifuged gently (400*g*, 10 min) to form a soft pellet. This material was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h, postfixed in 1% OsO_4 and then dehydrated sequentially in 10–100% ethanol.

Samples were embedded in epoxy resin, sectioned and stained with uranyl acetate using standard procedures.

Results

The antibodies to type IX and type II collagen were shown to be specific for these antigens (Fig. 1), although occasionally the polyclonal antibody did show some slight cross-reactivity with type IX collagen. Chondron extraction procedures were performed on cartilage samples from a total of five radioulnar joints and one tibial plateau from mature pigs. Up to 50 isolated chondrons were examined from each radioulnar homogenate, while tibial homogenates yielded poor numbers of intact isolated chondrons. The morphology of isolated chondrons examined was consistent with chondron organization in intact tissue (Duance *et al.*, 1982). They included single chondrons, linear chondron columns and chondron clusters of varying size. Single, linear and clustered chondrons were generally free of adherent collagen fibres (Fig. 2a) while the large chondron groups (Fig. 3a) were usually surrounded by or associated with a frayed collagenous matrix. These chondrons were labelled strongly with the monoclonal antibody to type IX collagen (Figs 2b and 3b) and also with the polyclonal antibody to type II collagen (Fig. 2c). Prior treatment with hyaluronidase slightly improved the

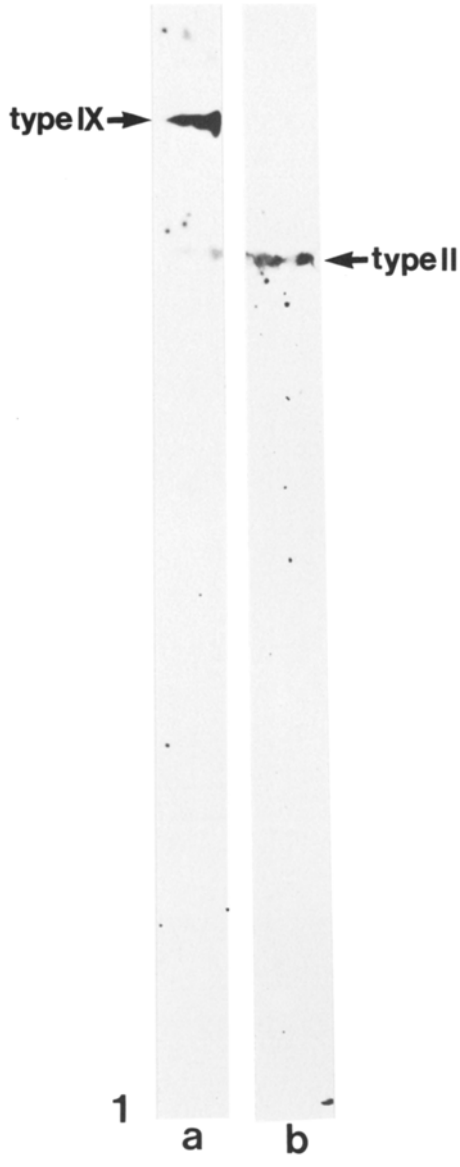


Fig. 1. Western blot of mixed antigens pig type II collagen + rat type IX collagen (each extracted as described in the text) and transferred to nitrocellulose paper after electrophoresis on a sodium dodecyl sulphate/polyacrylamide gel (Laemmli system, 7.5% running gel and 3% stacking gel). Localized with (a) monoclonal antibody to type IX collagen and (b) polyclonal antibody to type II collagen, followed by alkaline phosphatase conjugated second antibodies, and visualized by using α-naphthyl phosphate (1 mg ml^{-1}) and 4-amino-diphenylamine diazonium sulphate (1 mg ml^{-1}) (Sigma).

antibody labelling of the pig cartilage chondrons. No labelling was observed with the control antibody.

Similarly, chondrons from the rat chondrosarcoma contained one, several or larger numbers of chondrocytes. Often each cell in a group appeared to be surrounded by its own capsule and then by another surrounding several cells (Fig. 4a). This could be seen most clearly by fluorescence microscopy after antibody labelling with the antibody to type IX collagen (Fig. 4b). The control antibody did not label these chondrons.

The hyaluronidase treatment did not significantly affect this labelling but appeared to cause a loss of turgidity and some aggregation of the rat chondrosarcoma chondrons.

Attempts to prepare chondrons from articular cartilage of immature pigs resulted in the isolation of quite large groups of cells within cartilage chips (Fig. 5a). No single or columnar arrangements of cells within well-defined chondrons as seen in the older animal were observed. These groups of cells, when stained with anti- (type IX collagen) and anti- (type II collagen) after hyaluronidase treatment, revealed that although the cells were still surrounded by capsules, they were often ruptured with the resulting loss of cellular material (Fig. 5b and c).

Identical results were obtained whether the preparations were singly or doubly labelled.

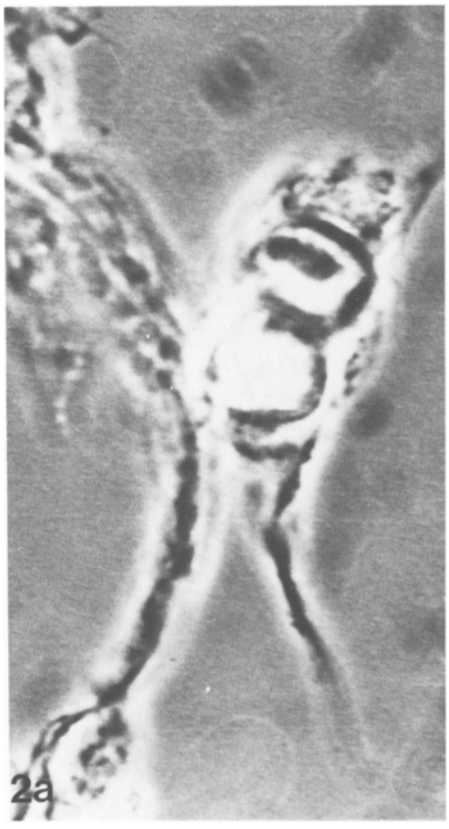
Transmission electron microscopy of the pelleted chondrons from the rat chondrosarcoma revealed the fine fibrous structure of the chondron, becoming denser in the region of the capsule (Fig. 6). No large banded fibrils are seen in these preparations.

Discussion

Chondrons as defined structural units within the cartilage can now be extracted from pig articular cartilage. It is interesting, however, that single or small groups of chondrons can be isolated from cartilage of adult but not young animals. In the same way that the integrity of the chondrons in the three zones of articular cartilage appears to correlate with the distribution of mechanical stress (see the Introduction), so the amount of mechanical stress to which the cartilage has been subjected, reflected in the age of the animal, may influence the development of the chon-

Fig. 2. A double chondron from pig articular cartilage (× 650). (a) Phase contrast; (b) labelled with monoclonal antibody to type IX collagen and a Rhodamine-conjugated second antibody; (c) labelled with polyclonal antibody to type II collagen and a fluorescein-conjugated second antibody. (Double labelled).

Fig. 3. A chondron group from pig articular cartilage (× 650). (a) Phase contrast; (b) labelled with monoclonal antibody to type IX collagen and Rhodamine-conjugated second antibody.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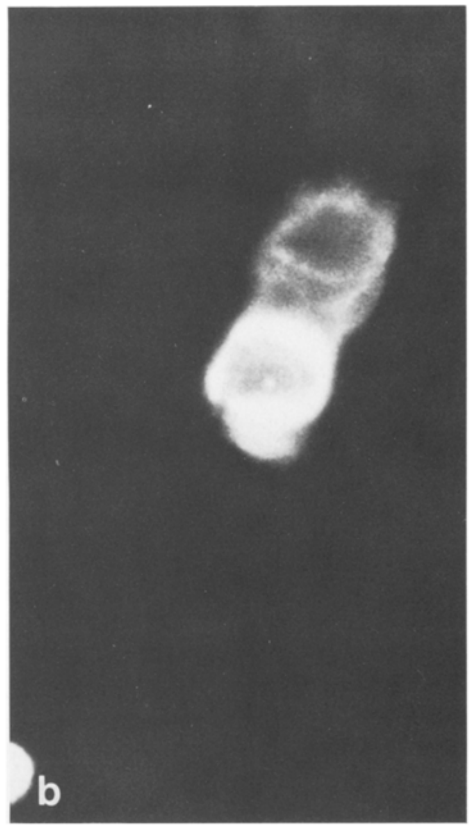
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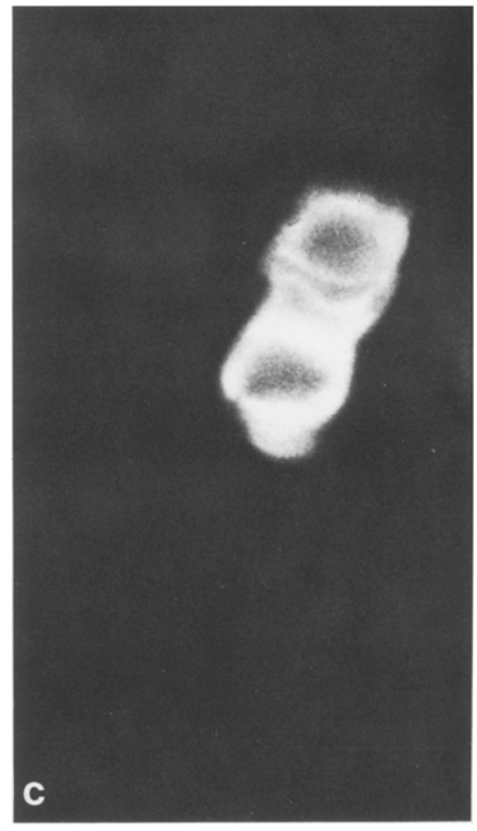
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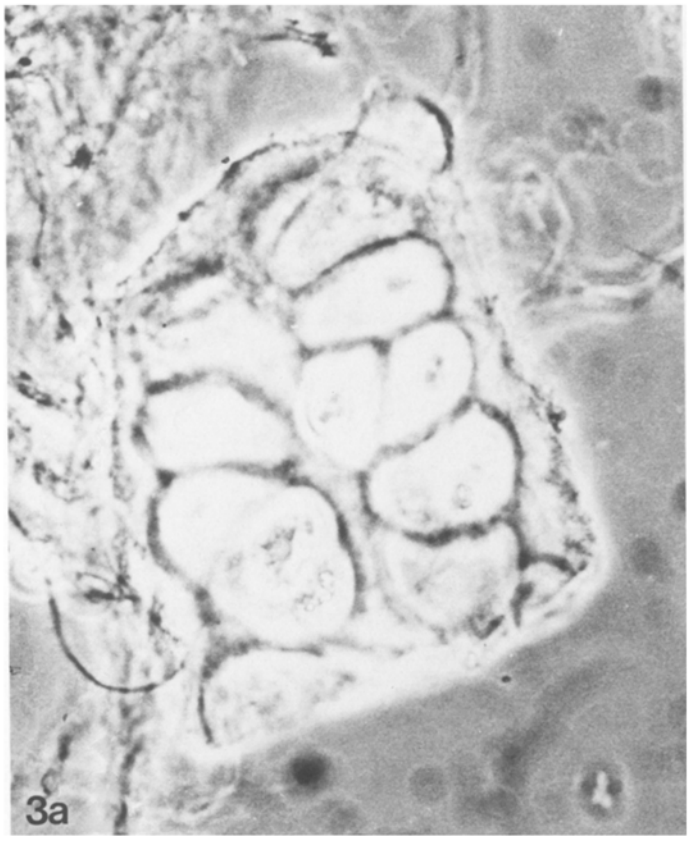
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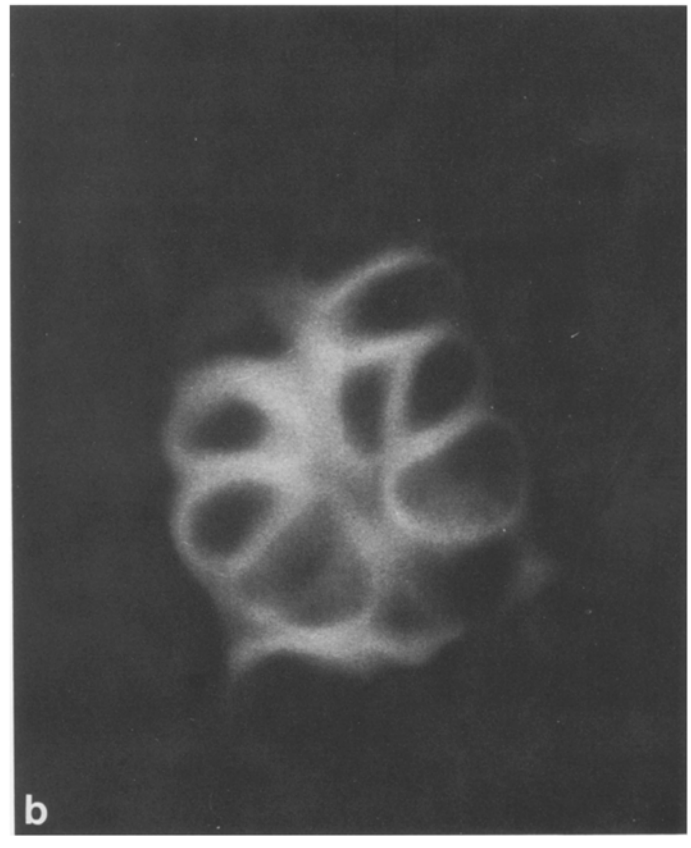
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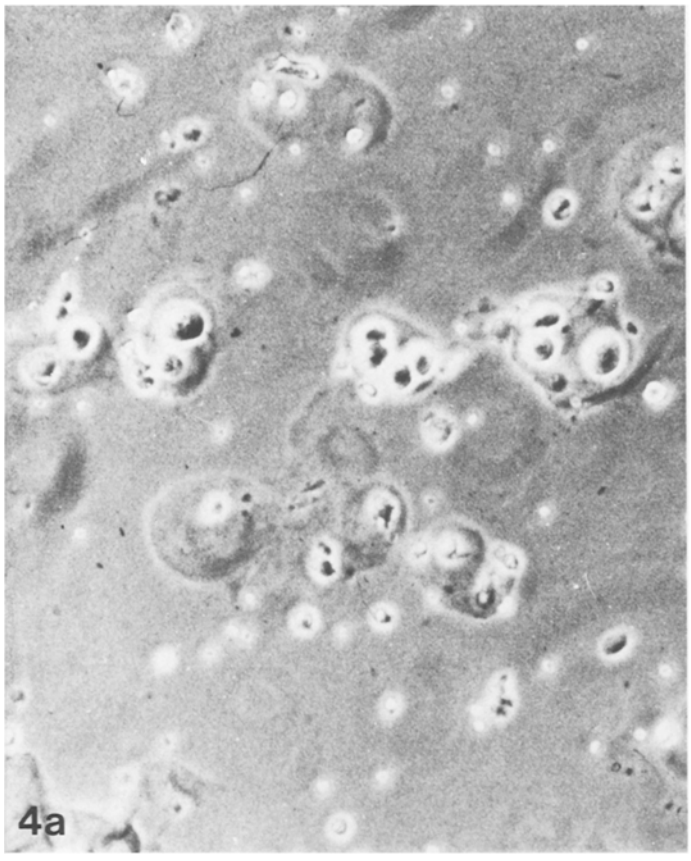
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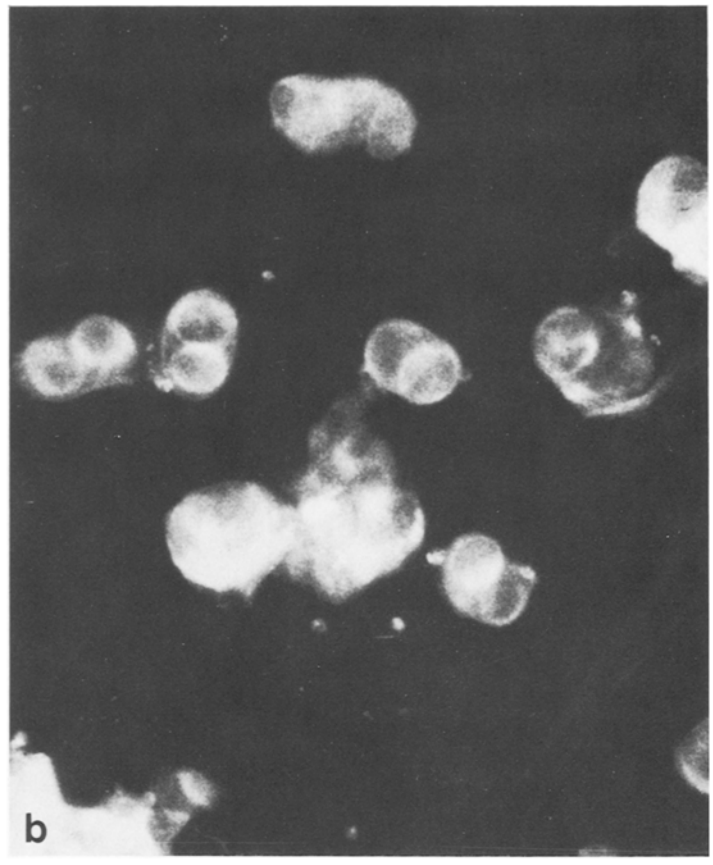
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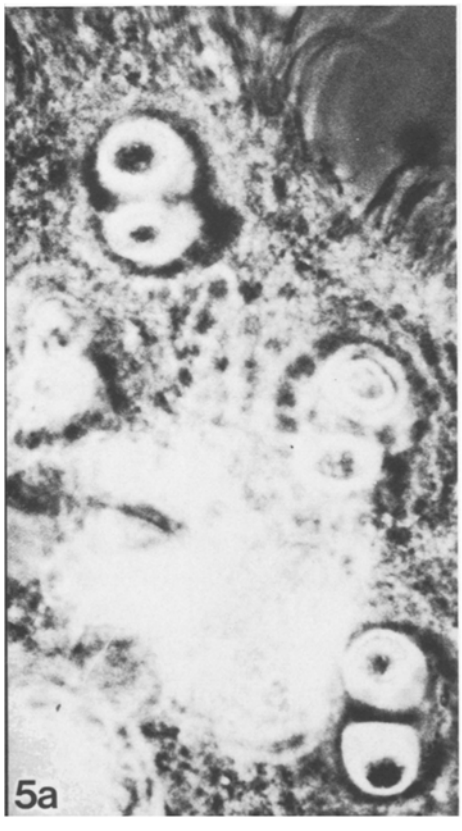
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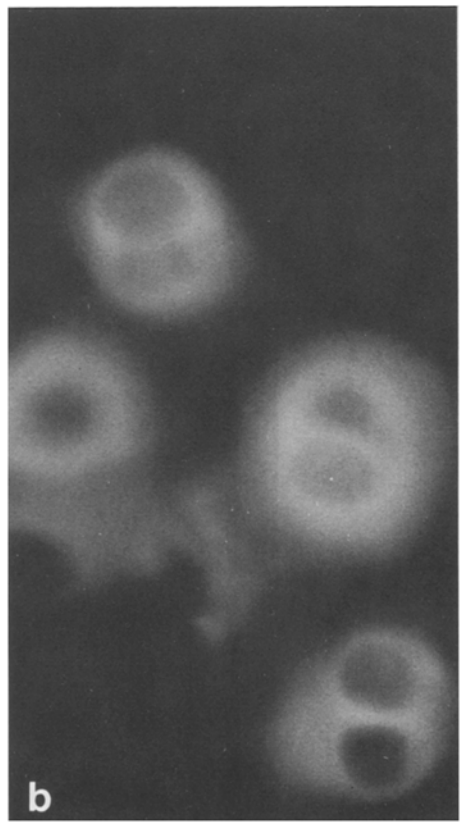
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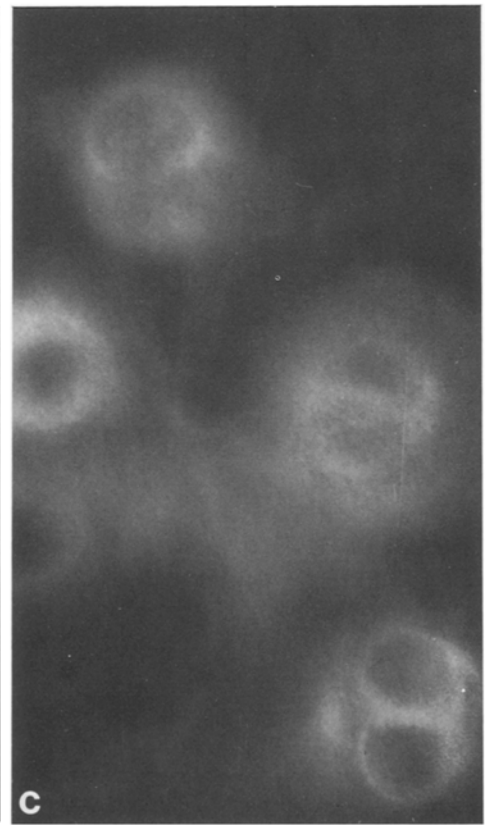
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dron as an entity and therefore affect its extractability.

In the rat chondrosarcoma, which can be described as a young tissue and one that is not subject to any mechanical stress, the chondrons can be isolated intact but the method used is a much more gentle procedure involving no homogenization.

The presence of type IX collagen in the capsule of the chondron has been clearly demonstrated. This agrees with previous immunofluorescence studies on sections of pig articular cartilage in which type IX collagen appears to be localized preferentially around the chondrocytes, or more accurately, the perilacuna rim.

The chondrons were also labelled with antibody to type II collagen, indicating that they are not composed exclusively of type IX collagen. It is possible that type IX collagen may prevent the type II collagen from forming large fibres, limiting their size to the fine fibrillar material of the chondron observed by transmission electron microscopy. Indeed, fibrillogenesis experiments *in vitro* have shown that type IX can modulate the fibril diameter of the type II collagen (Wotton *et al.*, 1988). Gold-labelling studies (Hartmann *et al.*, 1983; Wotton *et al.*, 1988) have revealed an even distribution of type IX collagen along non-striated small-diameter fibrils with little or no staining of larger striated fibrils. However, no preferential location of type IX was found at fibre intersections, a finding reported by Muller-Glauser *et al.*, (1986).

In larger chondron groups, individual chondrons remained embedded within a chip of extracellular matrix. Proteoglycan degradation with testicular hyaluronidase and subsequent removal prior to immunolocalization with the antibody revealed an intense reaction against type IX collagen in the area of the capsule, with a weaker fluorescence reaction in the intervening matrix. These results suggest that type IX collagen is not exclusively localized in the capsule but extends outwards into the adjacent matrix where it appears to be continuous with other chondrons in the group, and may indeed play a role in holding such groups together.

This finding is consistent with the immunolocalization of type IX collagen in chick sternal cartilage. Such cartilage is not subject to the same kind of mechanical stress as articular cartilage and, in young tissue especially, would not be expected to produce such well-defined chondrons. Type IX collagen would thus be less clearly discerned as surrounding the cells, and, in a tissue containing so many chondrocytes, would appear to have a more even distribution. Thus, the variation in structure, function and age of the different cartilage used in each of these studies are the likely causes of the disparate results on type IX collagen distribution. Previously, the differences found in the distribution of type IX collagen have been attributed to variations in the hyaluronidase treatment (Irwin *et al.*, 1985) required to remove matrix proteoglycans which are thought to mask antigenic sites on the collagen molecules (Duance *et al.*, 1982; Ricard-Blum *et al.*, 1982; Evans *et al.*, 1983). However, in the experiments reported here, immunolocalization was achieved without prior hyaluronidase treatment, particularly in chondrons that were released free of adherent matrix. It is possible that the homogenization and washing procedures remove significant amounts of proteoglycans from around the chondrons which are then free to present their entire surface to antibody challenge.

The functional role of type IX collagen, or the significance of its interaction with type II collagen is still largely unknown. We suggest that type IX and type II collagen and possibly other minor collagen species together form a finely porous capsule around mature articular cartilage chondrocytes. This capsule protects the integrity of the chondrocyte/pericellular matrix complex by providing a compliant, but inelastic, barrier during compressive deformation. The interaction between type IX and type II collagens may also play a role in anchoring the chondrons in the matrix during compressive loading, preventing a gradual accumulation of chondrons at the margins of routinely compressed areas of the joint surface.

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Fig. 4. Chondrons from rat chondrosarcoma. (a) Phase contrast (× 400); (b) labelled with monoclonal antibody to type IX collagen and a Fluorescein-conjugated second antibody.

Fig. 5. Chondrons embedded in a cartilage chip from a young pig (× 650). (a) Phase contrast; (b) labelled with monoclonal antibody to type IX collagen and a Rhodamine-conjugated second antibody; (c) labelled with polyclonal antibody to type II collagen and a Fluorescein-conjugated second antibody. (Double labelled).

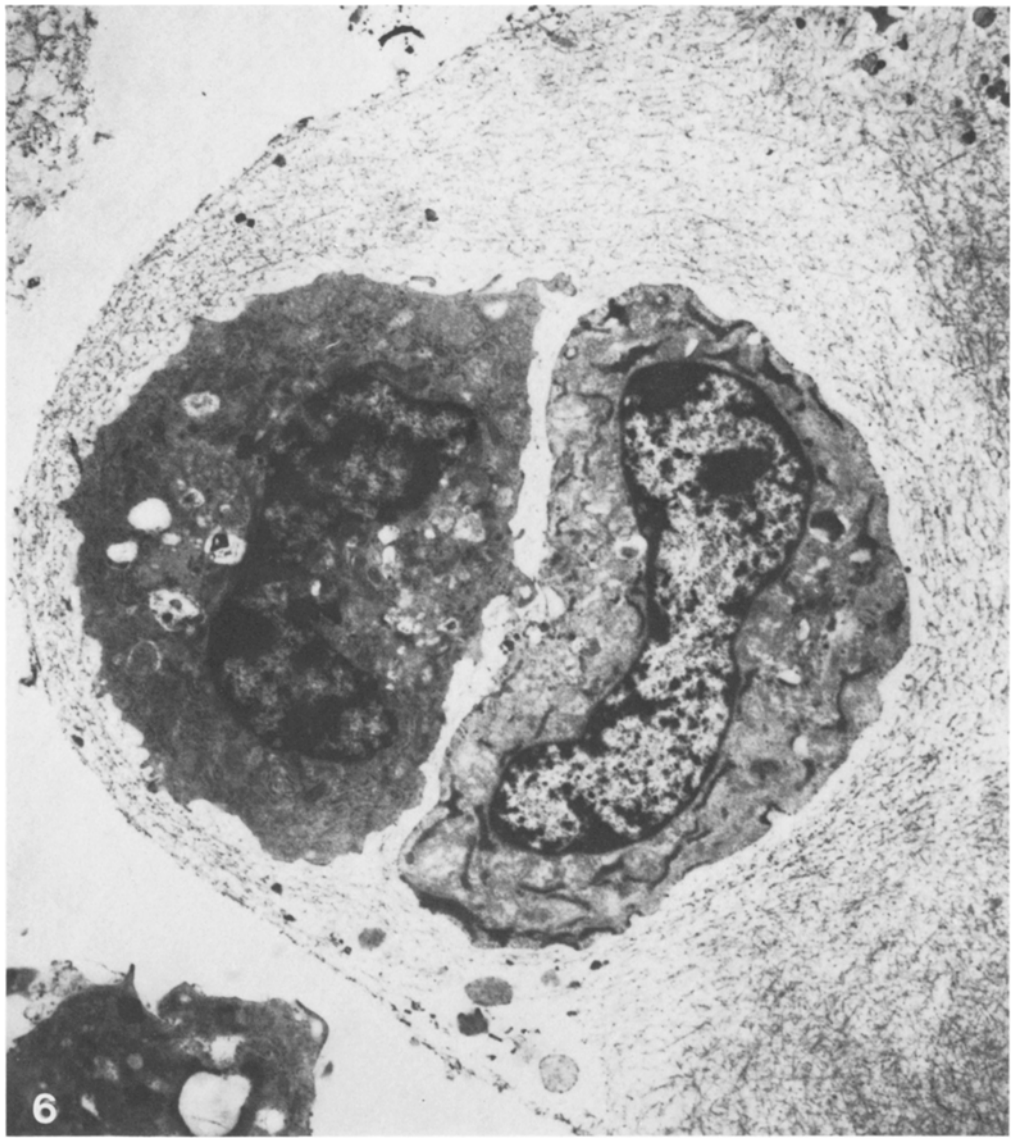

Fig. 6. Electron micrograph of cross section through a chondron from rat chondrosarcoma (× 6K).

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