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Type III collagen in normal human articular cartilage

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Summary

Type III collagen in normal human articular cartilage has been detected biochemically and its location in a diffuse area around the chondrocytes demonstrated by immunofluorescence. It can be found pericellularly throughout the depth of the cartilage and is evident in specimens ranging in age from 17 to 81 years.

Introduction

Type III collagen is present in a number of soft connective tissues such as blood vessel walls, skin, muscle and placenta (Gay & Miller, 1978). It is also a feature of damaged tissues which are attempting repair such as in wound healing of the skin, in fibrotic conditions and even of tissues not usually considered to contain any type III collagen such as articular cartilage in severe osteo-arthritis (Gay & Miller, 1978). It is often found in association with type I collagen and an increased ratio of type III to type I is an indication of a healing process. The collagen fibres in such repair tissue are of smaller diameters than the normal and are thought to be mixed fibres of type I and III collagen (Fleischmajer *et al.*, 1981).

Type II is the major type of collagen found in normal articular cartilage. However, we now know that articular cartilage is an extremely complex tissue not least in its collagen composition. Three types of collagen in addition to type II, namely types VI, IX and XI are certainly present (Mayne, 1986; Eyre *et al.*, 1987b) and types I, V and X have also been reported (Duance, 1983; Furuto *et al.*, 1991; Gannon *et al.*, 1991; Eyre *et al.*, 1992; Wardale & Duance, 1993). These are 'minor' collagens in the sense that they are present in comparatively small amounts but they may have important functions; for example, type IX collagen appears to have a role in regulating the fibril diameter of type II collagen (Wotton *et al.*, 1988) to which it is now known to be covalently cross-linked (Eyre *et al.*, 1987a). Other functions may be suggested by differences in their locations within the tissue, their distributions with age and their associations with chondrocytes of different phenotypes. Examples include: type VI, which appears to be strongly cell-associated (Poole *et al.*, 1988; Duance & Wotton, 1991), type IX collagen, which is widely distributed in the matrix of young tissue but is confined to the pericellular regions in mature cartilage (Wotton *et al.*, 1991; Duance & Wotton, 1991), and type X collagen, which appears to be produced only by the large hypertrophic chondrocytes found in growth plate cartilage and in the calcifying region of articular cartilage (Eyre *et al.*, 1992).

Following our finding that type III collagen is present in both normal human intervertebral disc and in the cartilaginous endplates which are adjacent to it (Roberts *et al.*, 1991a, b), we have examined normal human articular cartilage for evidence of type III collagen. In this study we have used both immunofluorescence and biochemical techniques to demonstrate the presence of type III collagen, its location and distribution with age.

Materials and methods

Specimens

Human articular cartilage samples of various ages were kindly provided by Dr M. Bayliss (Kennedy Institute of Rheumatology) and were taken from the femoral condyles of patients who had undergone amputations above the knee following a diagnosis of osteosarcoma. Cartilage samples from this source are generally considered to be 'normal' and these samples appeared grossly normal with no visible signs of degradation. Full-depth cartilage specimens were snap-frozen in liquid nitrogen-cooled iso-pentane and stored at −80°C until use.

Immunolocalization

Cryostat-frozen sections of 5 μm thickness were treated with bovine testicular hyaluronidase (Sigma) at a concentration of 2 mg ml^{-1} in phosphate-buffered saline (PBS), pH 7.4, at room temperature, overnight. After extensive washing in PBS, a type III collagen-specific primary antibody raised in goat was applied at a dilution of 1:100 in PBS for 4 h at room temperature. This was followed by further washing and the application of a fluorescein isothiocyanate-conjugated second antibody specific for goat IgG (Sigma), used at the manufacturer's recommended dilution. Sections were thoroughly washed in PBS and mounted in Citifluor AF1 (Agar Scientific) prior to fluorescence observation on a Leitz Dialux microscope. The antibody to type III collagen is a polyclonal antiserum raised against type III collagen which had been pepsin-extracted from human placenta. The antiserum is the same as that used previously (Roberts *et al.*, 1991a, b) and has been extensively checked by ELISA and Western blotting for cross-reactivity to other collagen types. Non-immune goat antiserum was used as a primary antibody control.

Immunoblotting

After sectioning, the remainder of each sample was treated with bovine testicular hyaluronidase in PBS at 2 mg ml^{-1} at room temperature overnight, washed in PBS and then digested with cyanogen bromide (CB) in 70% formic acid at 30°C for 36 h. The digests were diluted 20-fold with distilled water and centrifuged in a bench centrifuge to remove any remaining solid material. The supernatants were dialysed against water, aliquots removed for hydroxyproline analysis and the remainder freeze dried. The CB-insoluble material from each sample was taken up in SDS-PAGE sample buffer, and aliquots again removed for hydroxyproline analysis. In only one sample did a residue remain after this treatment and this was also retained for hydroxyproline analysis. CB peptides of pepsin-derived type III collagen from human placenta were also prepared by the same method for use as a standard. All the CB peptide preparations were examined by SDS-PAGE (Laemmli, 1970) and Western blotting (Towbin *et al.*, 1979). Further identification was carried out using the antibody to human type III collagen, as described above, followed by an alkaline phosphatase second antibody which had been absorbed against human serum proteins (Sigma). The alkaline phosphatase substrate used was a mixture of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and Nitro Blue Tetrazolium (Sigma).

Total collagen contents of the various fractions were carried out by automated hydroxyproline analysis (Woessner, 1976) following hydrolysis at 110°C in 6 N HCl for 20 h.

Results

Type III collagen was found in all the specimens of normal human articular cartilage examined by immunofluorescence. Figure 1 shows the localization of the type III in three specimens from patients of different ages, 17, 44 and 81 years. In the younger specimens a diffuse area of labelling is present in the pericellular regions and is evident throughout the depth of the cartilage (Fig. 1A, D). The labelling appears to become less diffuse with increasing age of the sample (Fig. 1, D–F) but is still apparent around the cells throughout the cartilage depth (Fig. 1, A–C). Articular cartilage from older people is characterized by a degree of autofluorescence in the interterritorial matrix which can be seen in the specimen from the 81-year-old patient (arrows Fig. 1C, F). Autofluorescence of this kind appears rather yellow and is therefore readily distinguished from the bright green FITC fluorescence which in this case is confined to the pericellular location of the type III collagen.

The presence of type III collagen was confirmed biochemically by the immunoblotting of CB-digested cartilage from the same specimens, which revealed peptides characteristic of type III, specifically CB5, CB4 and CB6 + 3 (Fig 2, E–G). These were, however, not detectable by Coomassie Brilliant Blue staining of the gel itself which showed only the predominant type II CB peptides (Fig. 2, A–C). On the blot the type II peptides do not cross-react with the antibody to type III collagen and appear as blank outlines. The αI(III) CB8 peptide visible in the blot of standard type III peptides (lane H) is masked in the cartilage digests by the vast excess of the αI(II) CB8 visible on the Coomassie Blue-stained gel (lanes A–C). The amount of type III CB peptides detected appeared to be greatly reduced in the oldest specimen (Fig. 2E) despite the equivalent amounts of type II peptides visible on the gel in all three specimens (Fig. 2, A–C). This is consistent with the lower specific labelling of type III collagen in this specimen (Fig. 1C, F). The effectiveness of the CB digestion varied with the age of the cartilage. The digestion period had been extended since adult human tissues are known to be very insoluble. Under these conditions the 17- and 44-year-old specimens were 100% solubilized by CB under the conditions used and the 81-year-old tissue was 89% solubilized. Subsequent SDS treatment brought a further 7% into solution, leaving 4% as an insoluble residue. Hydroxyproline analyses revealed an apparent reduction in the amount of collagen present in the oldest specimen, about 36% (relative to dry weight of tissue) compared to about 75% in the younger specimens. Although meaningful quantifications are not possible with the limited number of samples, the hydroxyproline results did enable approximately similar amounts of collagen to be loaded onto the gels.

Discussion

Our results show that type III collagen is present in small amounts in normal human articular cartilage. Although it has been reported in osteo-arthritic cartilage where it has usually been assumed to be associated with attempted repair mechanisms, it has not previously been considered to be a constituent of normal hyaline cartilage. Certainly until the advent of immunoblotting procedures there was no reason to suspect its presence since it cannot be detected by Coomassie Brilliant Blue staining of gels after SDS-PAGE. Indeed, these results indicate type III

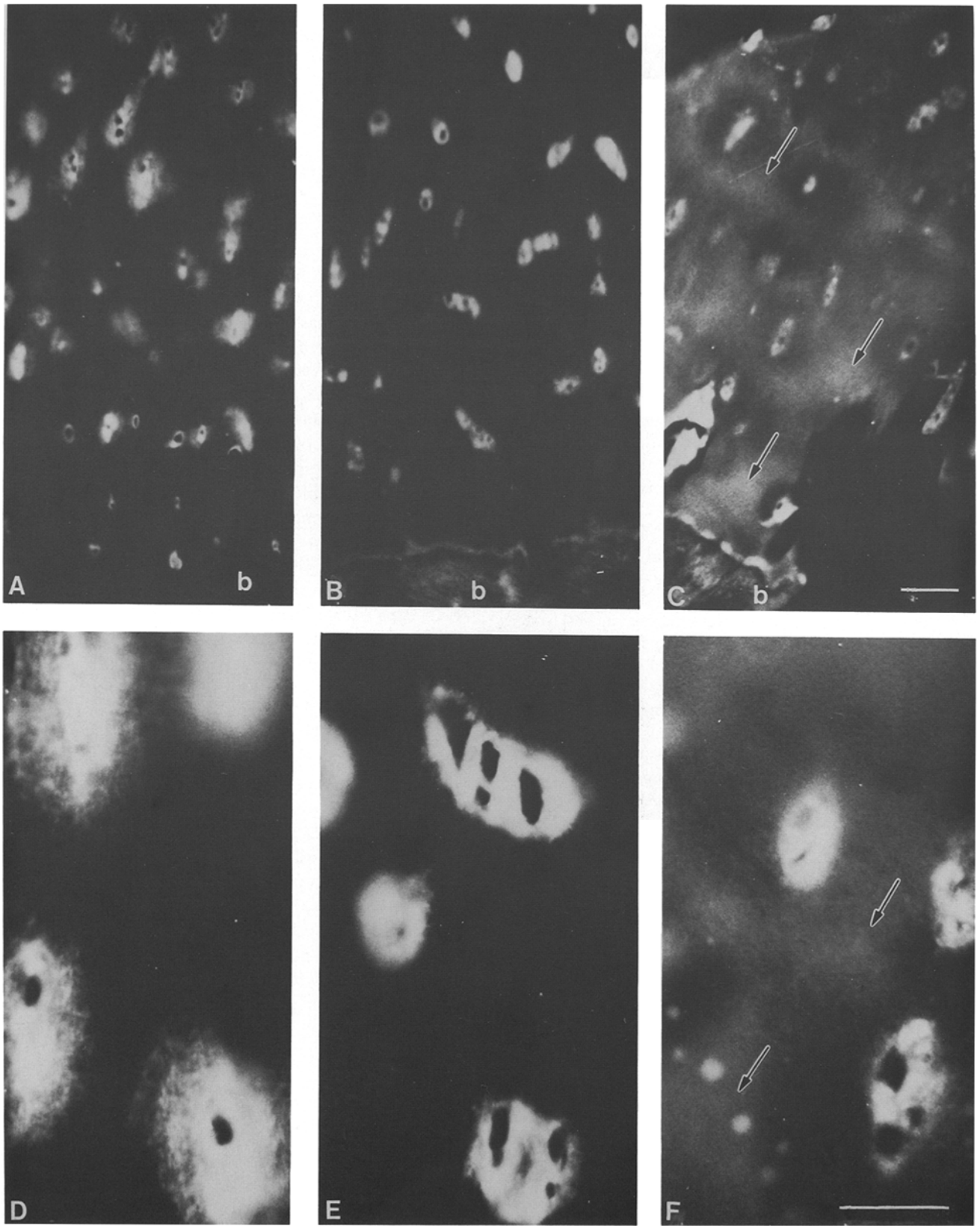

Fig. 1. Immunofluorescent localization of type III collagen in normal human femoral condyle articular cartilage: A and D = 17 years; B and E = 44 years; C and F = 81 years. A, B and C are deep and middle areas of cartilage. b = bone. Bar = 100 μm. D, E and F are pericellular regions. Bar = 50 μm. Arrow = autofluorescence.

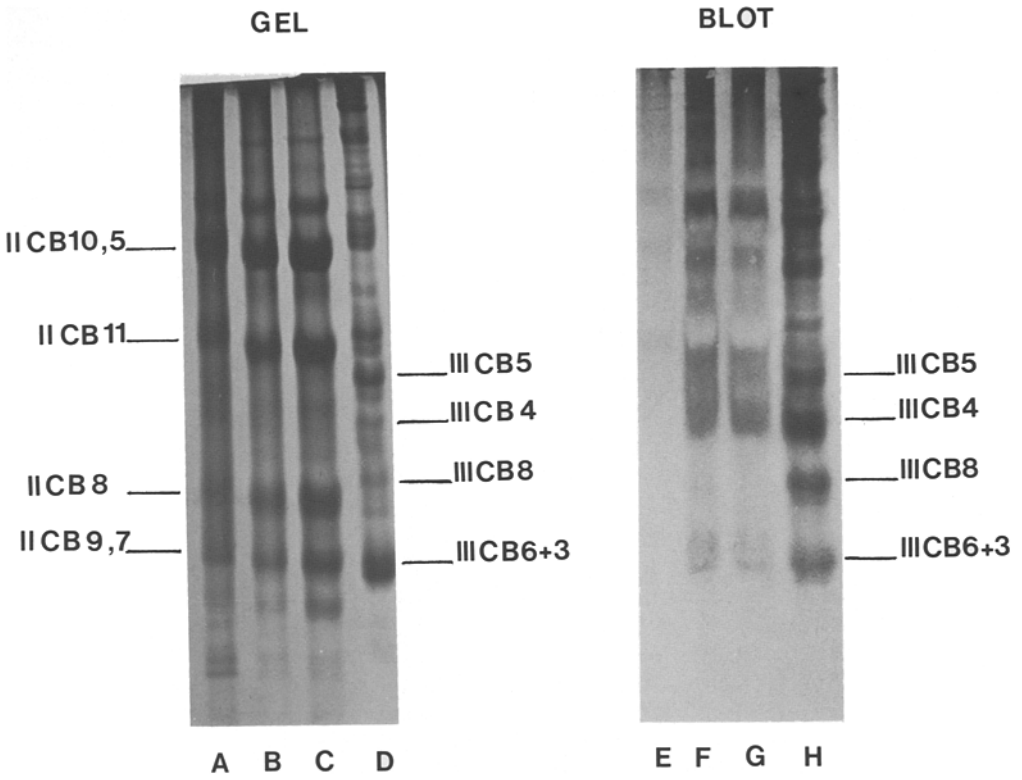

Fig. 2. SDS-PAGE (A–D) stained with Coomassie Blue, and immunoblots (E–H) of CB peptides from normal human femoral condyle articular cartilage (A and E = 81 years; B and F = 44 years; C and G = 17 years) and from standard type III collagen (D and H). Immunoblots localized with antibody to human type III collagen.

collagen represents probably less than 1% of the total collagen present.

The localization of the type III collagen to a diffuse area around the chondrocytes is consistent with the results of a previous study in which we found that type III collagen in the normal adult intervertebral disc is also confined to a diffuse area around the chondrocytes. These areas may correspond to the chondron capsules. The amount of type III collagen that was detectable both immunohistochemically and biochemically appears to be significantly reduced in the oldest specimen. The distribution of type III seems to differ from that of type IX collagen in cartilage, in that the type III localization is more diffuse and is present in older samples than type IX collagen which is difficult to detect even immunohistochemically after the age of about 40 years. It is interesting that both types IX and III collagens are thought to regulate fibril diameters of types II and I collagen respectively in different tissues. It is not known if type III collagen could also perform this function in articular cartilage in the absence of type I collagen which although it has been detected in articular cartilage of some species seems to be confined to the superficial zones.

Aigner *et al.* (1993) have reported the presence of type III collagen in osteo-arthritic cartilage in the absence of type I collagen. Unlike Aigner *et al.*, however, we found type III collagen around the chondrocytes throughout the depth of not only osteo-arthritic cartilage (results not shown) but also, as shown here, normal cartilage. Aigner *et al.* found type III collagen only around the cells in an area which they called the upper mid zone. For these studies they used *in situ* hybridization and immunolocalization with an antibody to procollagen type III. One explanation for the difference in the findings may be that Aigner *et al.*, in their detection of mRNA and use of an antibody to the procollagen, may have located an area of cartilage where chondrocytes were actively synthesizing new collagens, whereas our antibody to pepsin-derived type III collagen detects both newly synthesized and previously laid down collagen. From their results Aigner *et al.* hypothesized that, in osteo-arthritic cartilage, there may be a phenotypically different population of chondrocytes which, in addition to type II collagen, are capable of producing type III which is not associated with type I synthesis. Our results suggest that all the chondrocytes may be capable of producing type III collagen at some stage in the normal tissue. However, it is possible that only those chondrocytes in a particular zone of the cartilage are capable of responding to the challenge of osteo-arthritic changes by renewed synthesis. Further studies are in progress to better define the role of type III collagen in articular cartilage.

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