Volume 234, number 1, 79–82 FEB 06004 July 1988

Type IX collagen: a possible function in articular cartilage

S.F. Wotton, V.C. Duance and P.R. Fryer*

AFRC Institute of Food Research, Bristol Laboratory, Langford, Bristol, Avon BS18 7DY and **Clinical Research Centre, Watford Road, Harrow, Middlesex, England*

Received 29 March 1988

The effect of type IX on in vitro fibrillogenesis of type II collagen indicated that, while not preventing fibrillogenesis, the presence of type IX collagen reduced the size of the type II fibre aggregates. This observation is consistent with the in vivo localisation studies of type IX collagen. Using the immunogold labelling technique, type IX collagen was shown to be located evenly on small fibrils which occur at higher concentration closer to the cell. Therefore type IX collagen may function as a regulator of fibre diameter in articular cartilage.

Type IX collagen; Cartilage; Fibrillogenesis; Immuno-localization

1. INTRODUCTION

Type IX collagen is one of several minor collagens found in articular cartilage. Considerable structural information has now been determined for both the avian and the mammalian molecules [1–5].

The molecule is composed of three distinct α-chains, two having molecular masses of 68 kDa and the third of 84 kDa [6]. There are three collagenous domains interrupted by short non-triple helical sequences and the α1-chain has a large globular N-terminal domain. One of the 68 kDa molecular mass chains, the α2-chain, has covalently linked glycosaminoglycans and migrates with a molecular mass around 115 kDa before treatment with chondroitinase ABC [7].

Immunohistochemical localisation studies have shown that in mature mammalian articular cartilage type IX is preferentially located in the pericellular region of the chondrocyte [8,9]. In contrast, studies on chick sternal cartilage have revealed a more even distribution throughout the tissue [10]. It has been suggested that the pericellular staining is an artefact of inadequate removal of proteoglycans by hyaluronidase treatment prior to antibody labelling and that the thickness of the section may contribute to this artefact. However, recent studies by Poole et al. [11] have shown that the chondrocyte is enclosed in a basket-like capsule together comprising a structure termed the chondron. We have isolated intact chondrons from both mature pig articular cartilage and rat chondrosarcoma and shown that these structures stain very intensely with antibodies to type IX collagen [12].

In this paper we present evidence for the function of type IX collagen from in vitro fibrillogenesis studies. These findings are consistent with our immunogold labelling experiments which show that type IX collagen is localised primarily to thin fibres which are more prevalent in close proximity to the chondrocyte.

Correspondence address: S.F. Wotton, AFRC Institute of Food Research, Bristol Laboratory, Langford, Bristol, Avon BS18 7DY, England

2. MATERIALS AND METHODS

2.1. *In vitro fibrillogenesis*

Type II and type IX collagen were prepared from rat chondrosarcoma as described [4]. These were dissolved in 0.005 M acetic acid at 1 mg/ml and the solutions clarified by centrifugation at 10000 × *g* for 30 min. Clarified solutions were incubated at 4°C and brought to pH 7.4 by the addition of 4 × PBS. The type II and type IX solutions were mixed in varying amounts

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

ranging from 0–15% type IX collagen. Fibre formation was carried out by incubating the mixtures at 37°C overnight. Fibres were viewed by phase contrast and electron microscopy after staining with phosphotungstic acid. Fibre diameters were measured from the micrographs using a Mitutoyo Profile Projector, type PJ250. Samples were analysed by SDS-PAGE using the Laemmli method [13] with a 4% stacking gel and 8% separating gel. Chondroitinase ABC treatment was carried out as described by Konomi et al. [14].

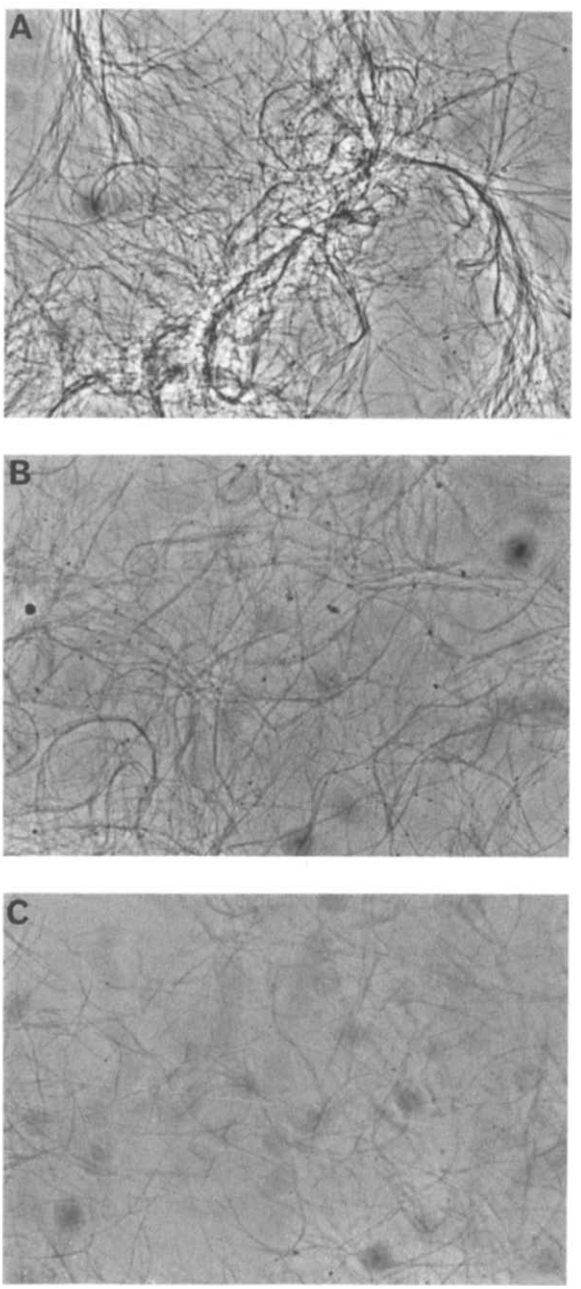

Fig.1. In vitro fibrillogenesis. Phase contrast microscopy of type II collagen fibres: (A) in the absence of type IX; (B) type II:type IX ratio 75:1; (C) type II:type IX ratio 7.5:1 (× 290).

Table 1

Fibre size during fibrillogenesis

	Small fibrils (nm)	Small fibres (nm)	Large aggregate (visual score 1–5)
Type II	4.6 ± 0.5	140–360	5
Type II + IX 75:1	6.3 ± 0.8	110–240	3
Type II + IX 7.5:1	5.2 ± 0.6	50–110	1

Diameters of small fibrils and small fibres measured from electron micrographs. Large aggregates scored visually from light micrographs (see fig.1). Type IX collagen affected the diameter of the small fibres and the large aggregates of type II in proportion to the amount of type IX present. No difference in the diameter of the small fibrils was apparent

2.2. *Immunogold-labelling of articular cartilage*

Adult pig articular cartilage was cut into 20 μm sections and digested with hyaluronidase (1 mg/ml PBS) for 1 h at 37°C prior to antibody labelling. Sections were incubated with a monoclonal antibody to type IX collagen for 2 h at room temperature or with non-immune serum as control. Sections were washed with PBS + 1% BSA before incubation with Protein A conjugated with 12–14 nm gold particles for 2 h at room temperature. After extensive washing in PBS + 1% BSA, sections were fixed with 3% glutaraldehyde in cacodylate buffer and post-fixed with osmium. After dehydration material was embedded in Spurr resin and thin sections cut for viewing in the electron microscope.

3. RESULTS

In vitro fibrillogenesis experiments using various ratios of type II:type IX collagen revealed that as the type IX ratio was increased there was a con-

Table 2

% alpha 1 (II) in fibrils

	Pellet	Supernatant
Rat type II	55	45
Rat type II + rat type IX 7.5:1	85	15

The presence of type IX collagen increased the percentage of type II collagen which formed fibres over that remaining soluble as measured by densitometry of SDS-polyacrylamide gels

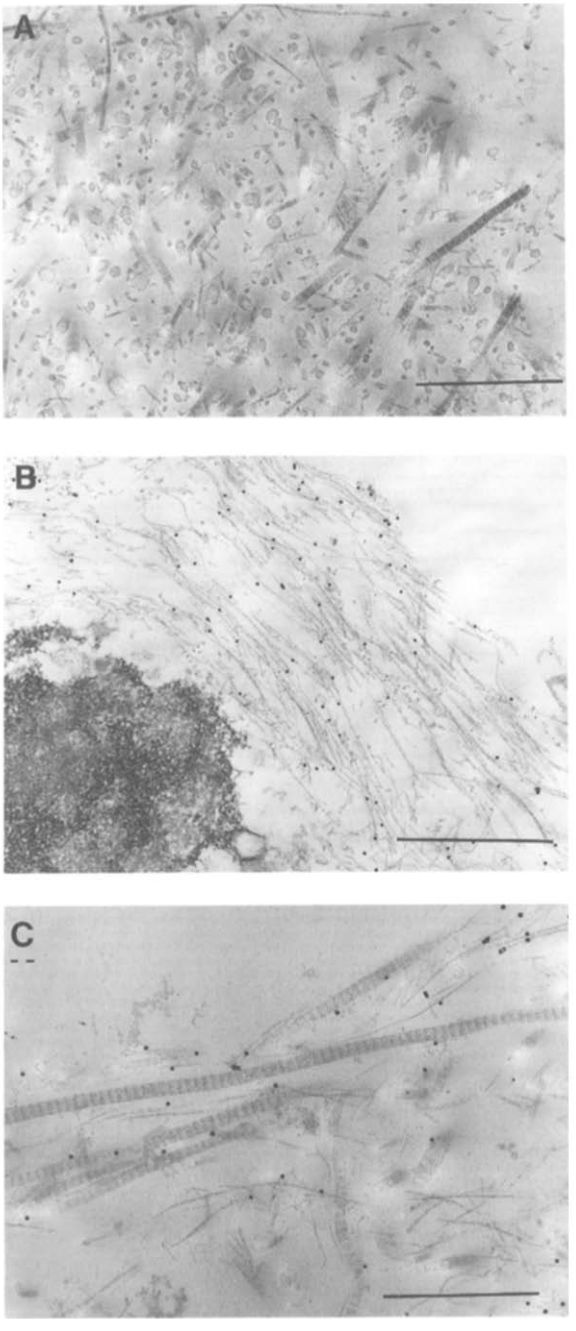

Fig.2. Immunogold localisation by electron microscopy of type IX collagen. (A) Control localisation with non-specific antibody; (B) high concentration of gold label pericellularly; localised with monoclonal antibody to type IX; (C) low concentration of gold label in inter-territorial matrix; localised with monoclonal antibody to type IX (× 28 000, bar = 1 μm).

comitant reduction in the fibre size as observed by phase contrast microscopy (fig.1). Type II collagen alone formed large aggregates which became progressively finer as the type IX ratio increased. The gross nature of the aggregates made fibre diameter measurement difficult and so they were scored on a visual basis on an arbitrary scale of 1 to 5 (table 1, column 3). A more precise measure of the fibre diameter was obtained after negative staining and electron microscopy. Two types of fibres could be identified: small thin fibrils and large, usually banded, fibres. The proportion of the finer fibrils increased with increasing type IX collagen although the size was unaltered (table 1, column 1). The larger banded fibres, however, were approximately 30% the diameter of type II collagen alone by the addition of 15% type IX (table 1, column 2).

To see if type IX was actually preventing fibrillogenesis, samples were centrifuged and the proportion of type II collagen in the pelleted fibres and supernatants determined by densitometric scanning of SDS-polyacrylamide gels. The results showed that type IX collagen actually enhanced the proportion of type II collagen which was incorporated into fibres (table 2). Chondroitinase ABC treatment of type IX collagen did not affect its ability to influence the fibrillogenesis of type II collagen.

The results of the immunogold localisation studies are shown in fig.2. Control sections revealed no significant background labelling. The antibody to type IX collagen can be seen to be preferentially distributed on the thin fibrillar material, with little labelling of the large banded fibres. The density of labelling is considerably higher in the pericellular region (fig.2B) in comparison with the inter-territorial matrix (fig.2C).

4. DISCUSSION

It is known from histological observations of articular cartilage that the pericellular region is composed of a fine meshwork of fibres whereas the inter-territorial matrix contains predominantly large fibre bundles [16]. Our previous studies have shown that type IX collagen in adult mammalian articular cartilage is preferentially located around the chondrocytes especially in the deeper zones [12,18]. The immuno-electron microscopy results

presented here confirm this distribution and reveal that the type IX collagen is associated almost exclusively with the small diameter unbanded fibres. Contrary to previous reports [15], our studies failed to show a localisation at fibre intersections.

Although these results appear to be at variance with other studies on embryonic chick sternal cartilage where the type IX collagen is reported to be more evenly distributed throughout the matrix [10,15], the proportion of type IX collagen is relatively high in this tissue and the whole of the matrix comprises small even-sized fibres. Indeed, in other tissues where a relatively high concentration of type IX collagen is present, foetal cartilage and vitreous humour, predominantly fine collagenous fibres are seen [17], which is consistent with our hypothesis.

That type IX collagen affects the fibre diameter of the collageneous matrix is supported by the in vitro fibrillogenesis experiments presented. These results show that in vitro, type IX collagen can interact with type II collagen to modulate fibril aggregation, although fibrillogenesis itself is not prevented. The precise nature of this interaction is at present unknown, but a recent paper by Eyre et al. [18] has demonstrated that type II and type IX collagen can be covalently linked through a pyridinoline cross-link. Type IX collagen is known to have a kink approximately 40 nm from the N-terminal end of the molecule coincident with a short non-collagenous domain which is the site of attachment of at least one of the chondroitin sulphate side chains [19,20]. In addition the N-terminus of the α1-chain has a large globular domain. From our results it would appear that the association of such a molecule with type II collagen fibrils prevents the close parallel alignment of these fibrils required for further aggregation. The fact that type IX collagen does not inhibit fibrillogenesis but only fibril aggregation suggests that it may only interact with the fibrillar form of type II rather than the individual molecules. Our results, however, suggest that the glycosaminoglycan side chains play no part in this interaction and subsequent inhibition of aggregation and their precise function remains to be elucidated.

We therefore conclude from these distribution and in vitro fibrillogenesis studies that one possible function of type IX collagen is the modulation of fibre formation of the major collagenous component of cartilage – type II collagen.

REFERENCES

[1] Shimokomaki, M., Duance, V.C. and Bailey, A.J. (1980) FEBS Lett. 121, 51–54.
[2] Shimokomaki, M., Duance, V.C. and Bailey, A.J. (1981) Biosci. Rep. 1, 561–570.
[3] Reece, C.A. and Mayne, R. (1981) Biochemistry 20, 5443–5448.
[4] Duance, V.C., Wotton, S.F., Voyle, C.A. and Bailey, A.J. (1984) Biochem. J. 221, 885–889.
[5] Bruckner, P., Mayne, R. and Tuderman, L. (1983) Eur. J. Biochem. 136, 333–339.
[6] Olsen, B.R., Ninomiya, Y., Lozano, G., Konomi, H., Gordon, M., Green, G., Parsons, J., Seyer, J., Thompson, H. and Vasios, G. (1985) Ann. NY Acad. Sci. 460, 141–153.
[7] Huber, S., Van der Rest, M., Bruckner, P., Rodriguez, F., Winterhalter, K. and Vaughan, L. (1986) J. Biol. Chem. 261, 5965–5968.
[8] Duance, V.C., Shimokomaki, M. and Bailey, A.J. (1982) Biosci. Rep. 2, 223–227.
[9] Hartmann, D., Magloire, H., Ricard-Blum, S., Joffre, A., Couble, M., Ville, G. and Herbage, D. (1983) Collagen Rel. Res. 3, 349–357.
[10] Irwin, M.H., Silvers, S.H. and Mayne, R. (1985) J. Cell Biol. 101, 814–823.
[11] Poole, C.A., Flint, M.H. and Beaumont, B. (1984) J. Anat. 138, 113–138.
[12] Poole, C.A., Wotton, S.F. and Duance, V.C. (1988) Histochem. J., in press.
[13] Laemmli, U.K. (1979) Nature 227, 686.
[14] Konomi, H., Seyer, J., Ninomiya, Y. and Olsen, B.R. (1986) J. Biol. Chem. 261, 6742–6746.
[15] Muller-Glauser, W., Humbel, B., Glatt, M., Strauli, P., Winterhalter, K. and Bruckner, P. (1986) J. Cell Biol. 102, 1931–1939.
[16] Meachim, G. and Stockwell, R.A. (1979) in: Adult Articular Cartilage (Freeman, M.A.R. ed.) pp.1–67, Pitman Medical, London.
[17] Ayad, S. and Weiss, J.B. (1984) Biochem. J. 218, 835–840.
[18] Eyre, D.R., Apon, S., Wu, J.J., Ericsson, L.H. and Walsh, K.A. (1987) FEBS Lett. 220, 337–341.
[19] Irwin, M.H. and Mayne, K. (1986) J. Biol. Chem. 261, 16281–16283.
[20] McCormick, D., Van der Rest, M., Goodship, J., Lozano, G., Ninomiya, Y. and Olsen, B.R. (1987) Biochemistry 84, 4044–4048.