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IDENTIFICATION OF ALTERNATIVELY SPLICED VARIANTS OF TYPE II PROCOLLAGEN IN VITREOUS

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Adult and foetal bovine vitreous were analyzed for the presence of unprocessed and partially processed forms of type II procollagen. Type II procollagen can exist in two alternatively spliced forms: a short form which lacks a large part of the N-propeptide due to the splicing out of exon 2 and a long form in which exon 2 is expressed and which has a full-sized N-propeptide. Both splice variants were demonstrated and analyses of type II pN-collagen demonstrated that the long form predominated, the ratio of the long form to the short form being 5:1 in foetal vitreous and 1.5:1 in adult vitreous. In foetal vitreous 35% of the type II collagen extracted was in the pN form, but there was very little unprocessed type II procollagen or type II pC-collagen. In contrast, adult vitreous extracts contained relatively less type II pN-collagen, but contained larger amounts of type II pC-collagen and unprocessed type II procollagen.

Type II collagen is the major fibril-forming collagen of vitreous, cartilage and the nucleus pulposus of the intervertebral disc (1-3). Additionally, low levels of type II collagen are expressed transiently in a number of foetal tissues (4,5). Type II collagen (at least in vitreous and cartilage) is found in heterotypic collagen fibrils that also contain type V and/or type XI collagen gene products and are decorated on the surface by type IX collagen (6-10). In cartilage and vitreous, type II collagen has been estimated to account for between 69-87% of the total collagen (11-13).

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Type II collagen comprises three identical α1(II) chains that are encoded by a single gene containing 54 exons. It has recently been shown that the pre-mRNA of type II procollagen undergoes alternative splicing of exon 2 which results in the presence or absence of a globular domain near the N-terminal region of the N-propeptide (14). Cartilage expresses predominantly the short form (lacking exon 2), whereas a number of non-cartilaginous foetal tissues transiently express the long form (4,5,15). There are no previously published data to show which form is present in the vitreous and in this communication we show that both forms are present, and that the long form predominates in extracts from foetal and adult bovine vitreous.

MATERIALS AND METHODS

Materials: All chemicals were obtained from the Sigma Chemical Co. unless otherwise stated. Bacterial collagenase form III was obtained from Advance Biofactures Corporation, Lynbrook, New York. Procollagen type I/II N-proteinase purified from chick embryo tendons (16) was a generous gift from Dr. R. Watson (School of Biological Sciences, University of Manchester, U.K.).

Extraction procedures: Adult and foetal (1st to 3rd trimester) bovine eyes were obtained from a local abattoir and the vitreous removed and frozen (-30 °C) within three hours post-mortem. On thawing, proteinase inhibitors were added to the pooled adult or foetal vitreous to give a final concentration of 2 mM EDTA, 10 mM NEM, 2 mM PMSF and 5 mM benzamidine. The vitreous was then centrifuged at 30,000 g for two hours at 4 °C and the pellet discarded. NaCl was added to the supernatant to a final concentration of 4.5 M. After stirring overnight at 4 °C a precipitate was collected by centrifugation (as above). The precipitate was resuspended in 50 mM Tris-HCl, pH 7.4 containing 4.5 M NaCl. Following centrifugation (as above) the precipitate was collected. The precipitate was then redissolved in, and dialysed into, 50 mM Tris-HCl, pH 7.4 containing 0.15 M NaCl and the supernatant collected following centrifugation at 30,000 g for 30 minutes at 4 °C. This supernatant was dialysed extensively against distilled water and lyophilised.

Enzyme digestions: Aliquots of the lyophilised material were digested with N-proteinase, pepsin or bacterial collagenase. For N-proteinase digestion, samples were resuspended in 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 5 mM calcium chloride and 0.05% Brij 35 (200 µl of buffer per mg of sample) (17). N-proteinase was added (200 units/mg sample) and the samples were digested for 40 hours at 30 °C. For pepsin digestion aliquots were resuspended in 0.5 M acetic acid and incubated with pepsin (1:50 dry weight enzyme:substrate ratio) for 24 hours at 4 °C. Bacterial collagenase (20 units/100µg of lyophilised sample) was added to samples that had been resuspended in 50 mM Tris-HCl, pH 7.2 containing 5 mM calcium chloride, 1 mM PMSF and 5 mM NEM and the digestion was performed for 16 hours at 37 °C.

SDS/PAGE and Western blotting: Samples were analyzed in the presence (reduced) or in the absence (non-reduced) of 50 mM dithiothreitol by discontinuous 6.5% polyacrylamide gel electrophoresis in SDS (18). The resultant gels were stained with either Coomassie Brilliant Blue R or subjected to Western blotting. Coomassie Blue-stained gels were scanned using an LKB 2202 Ultroscan laser densitometer.

For Western blotting a previously characterized anti-human type II collagen serum (anti-II) was used (19). Transfer to nitrocellulose was by the method of Towbin et al. (20) with a modified buffer containing 48 mM Tris, 39 mM glycine, 20% (v/v) methanol and 0.0375% (w/v) SDS. Following transfer, the nitrocellulose sheet was incubated with anti-II (1:1000 dilution) followed by the secondary alkaline-phosphatase-conjugated antiserum (1:1000 dilution) and colour development was by the method of Blake et al. (21).

RESULTS AND DISCUSSION

Previous analyses of the soluble collagens from adult bovine vitreous demonstrated the presence of type II procollagen (13). We now present further data showing various partially processed forms of type II procollagen in both adult and foetal bovine vitreous. SDS /PAGE analysis under non-reducing conditions followed by Coomassie Blue staining (Fig. 1 A, lanes 1-4) and immunoblotting with anti-II (Fig. 1 B, lanes 1-4) showed that above the predominant (fully processed) α1(II) component there were two additional components (lanes 1 and 3). These two components were removed by prior treatment with N-proteinase (lanes 2 and 4), demonstrating that they represented two different forms of type II pN-collagen.

Type II procollagen possesses interchain disulphide bonds in the C-propeptide; Consequently, SDS/PAGE under reducing conditions followed by Coomassie Blue staining (Fig.

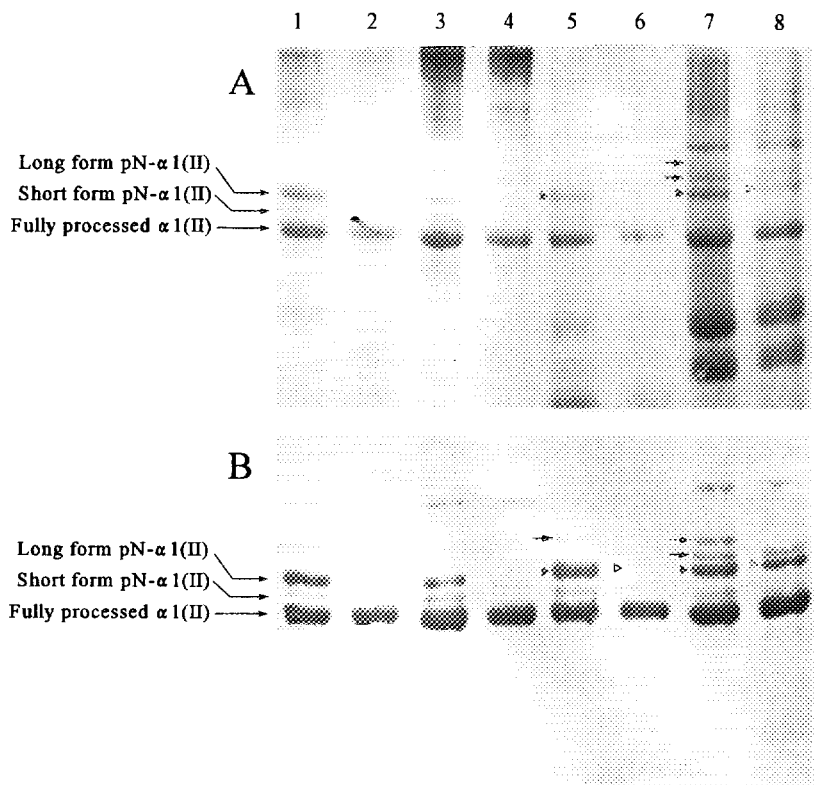

Fig. 1. Identification of partially processed forms of type II procollagen from foetal and adult vitreous.
Foetal (lanes 1-2, 5-6) and adult (lanes 3-4, 7-8) bovine vitreous extracts were analyzed by SDS/PAGE under non-reducing (lanes 1-4) and reducing (lanes 5-8) conditions. Samples in lanes 2,4,6 and 8 were digested with N-proteinase. (A) Shows a polyacrylamide gel stained with Coomassie Blue and (B) shows a Western blot with anti-II. Following reduction, the long form of pN-α1(II) and pC-α1(II) co-migrated (indicated by ▸). The unprocessed procollagen forms (long and short) are indicated by arrows (→). The pC-α1(II) component is indicated by an unfilled triangle (▹) and is barely visible in the foetal sample (lane 6).

1 A, lanes 5-8) and immunoblotting with anti-II (Fig. 1 B, lanes 5-8) additionally revealed unprocessed type II procollagen and type II pC-collagen. Furthermore, reduction resulted in the slightly slower migration of the higher M_r form of pN-α1(II) due to cleavage of intrachain disulphide bonds (see below) (Fig.1 A and B, lanes 1, 3, 5 and 7).

The relative proportion of pN-α1(II) (both forms) to fully processed α1(II) was estimated (by laser densitometric scanning of Coomassie Blue-stained polyacrylamide gels) to be 1:1.9 in foetal vitreous and 1:10 in adult vitreous extracts. Foetal samples contained very little unprocessed type II procollagen or type II pC-collagen (Fig. 1 A and B, lanes 5 and 6). By contrast, adult vitreous contained relatively larger amounts of unprocessed type II procollagen and type II pC-collagen (lanes 7 and 8). Following reduction the higher M_r form of pN-α1(II) co-migrated with pC-α1(II), a phenomenon that has been observed previously in SDS/PAGE analyses of the culture medium from human costal chondrocytes (15). Two distinct components were immunoblotted above the co-migrating pN-α1(II) and pC-α1(II) components (Fig. 1 B, lane 7); these probably correspond to the two alternatively spliced forms of unprocessed type II procollagen. Following N-proteinase digestion and reduction (lane 8) the pC-α1(II) component produced a clearly visible band. A weakly staining component is visible just above the main pC-α1(II) component (Lane 8) which possibly represents incomplete digestion by N-proteinase of the short form of unprocessed type II procollagen (although this is unlikely as the pN-α1(II) components were completely digested by N-proteinase, lane 4) or substitution of the C-propeptide by an N-glycan, producing a higher M_r form of pC-α1(II) (22).

Additional confirmation that the immunoblotted components were collagenous and derived from type II procollagen was obtained by digestion of samples with pepsin and bacterial collagenase prior to analysis by SDS/PAGE. Pepsin digestion (Fig.2, lanes 2 and 4) resulted in a single component that migrated slightly faster than the fully processed α1(II) chain; this represented pepsinised type II collagen. Bacterial collagenase digestion removed all the components immunoblotted in Fig. 1 B (data not shown).

Based upon analysis of cDNA clones of rat and human type II procollagen it was concluded that a large part of the N-propeptide was missing compared to the pro-α1 chains of collagen types I and III (23, 24). However, it has recently been shown that exon 2 of the type II procollagen gene (COL2A1) is subject to alternative splicing (14). This alternative splicing results in the presence or absence of a 69-amino acid cysteine-rich domain near the N-terminus of the N-propeptide of type II procollagen. The predominant procollagen mRNA expressed by mature chondrocytes was the short form lacking exon 2 (termed type IIB), whereas prechrondrocytes expressed predominantly the long form with exon 2 (termed type IIA) (15).

Vitreous, cartilage and intervertebral disc are the only mature tissues known to contain type II collagen. We demonstrate that foetal and adult vitreous contain both splice variants of type

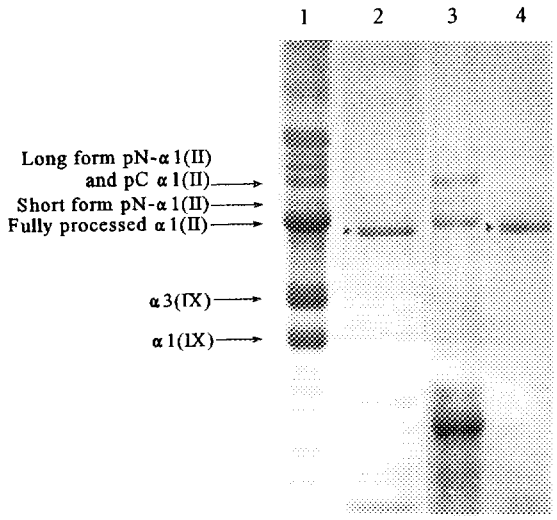

Fig. 2. The effect of pepsin digestion on vitreous extracts.
Adult (lanes 1-2) and foetal (lanes 3-4) bovine vitreous extracts were analyzed by SDS/PAGE under reducing conditions. The resultant gel was stained with Coomassie Blue. Samples were analysed with (lanes 2 and 4) and without (lanes 1 and 3) prior digestion by pepsin. The pepsinised α1(II) component is indicated by a triangle (‣).

II procollagen. The ratio of the long form to the short form (estimated by laser densitometric scanning of type II pN-collagen in Coomassie Blue-stained polyacrylamide gels) was 5:1 in foetal vitreous, decreasing to 1.5:1 in the adult tissue. This is the first demonstration of the long form of type II procollagen predominating in a mature fully differentiated tissue and contrasts with cartilage in which the short form predominates.

The removal of the N-propeptide is essential for normal fibrillogenesis (25). However, at least in the case of fibrils containing collagen types I and III, there is evidence to suggest that a proportion of the collagen molecules (probably those on the surface of the fibrils) retain the N-propeptide (26). As our analyses were of the soluble component following centrifugation of vitreous, any type II pN-collagen associated with the insoluble collagen fibrils would not have been analysed. However, previous extraction studies on the insoluble pellet following centrifugation (9) failed to extract significant quantities of partially processed type II procollagen. Furthermore, even if a significant proportion of total vitreous type II pN-collagen were associated with the fibrils, this would not affect the results for the ratios between the two forms in vitreous unless there was preferential incorporation of one splice variant into the fibrils.

In cartilage, the α3(XI) chain has been shown to be the same gene product as the α1(II) chain (27) and therefore, component(s) attributed to type II collagen could be derived from the

α3(XI) chain. Vitreous contains a collagen that is similar to cartilage type XI collagen (containing α1(XI) and α2(V) chains), but it is not known whether this (type V/XI) collagen contains an α3(XI) chain (10). However, it is unlikely that any of the components identified as being type II collagen in this study are due to an α3(XI) chain as pepsin digests of the vitreous (Fig. 2) failed to reveal the pepsinised forms of α1(XI) and α2(V). Moreover, we have shown previously that type V/XI collagen is virtually absent from the supernatant following centrifugation of bovine vitreous (13).

A number of functions have been attributed to the propeptides of fibrillar collagens including the feedback regulation of collagen synthesis (28, 29) and the regulation of fibril diameter (26, 30). Other authors question the idea that the retained N-propeptides on the surface of type I collagen fibrils regulate fibril diameter and suggest instead that they modulate interactions between the collagen fibril and other matrix components (25, 31). Clearly the functions of the N-propeptides of fibrillar collagens have yet to be fully elucidated, but the finding that vitreous and cartilage differ in their predominant splice variant of type II pN-collagen may, with further studies, provide new insights into their functions.

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