A new look at vitreous-humour collagen

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The collagens of bovine vitreous-humour and nasal-septum cartilage have been extracted, fractionated and compared. Both tissues show the same heterogeneity of collagen types, consisting of type II, $1\alpha, 2\alpha, 3\alpha$ and C-PS collagens. The type II collagen of the vitreous humour was significantly more hydroxylated both in the lysine and proline residues than was that of cartilage. C-PS1 collagen, together with higher- M_r forms were present in the vitreous humour, but the higher- M_r forms were not seen in cartilage. Both C-PS1 and C-PS2 were present in vitreous humour and cartilage, but vitreous humour contained three times more of these collagens than did cartilage. Despite the difference in amount, the molar ratio C-PS1/C-PS2 was approx. 1 in both tissues, suggesting that they are components of a larger molecule. The $1\alpha, 2\alpha, 3\alpha$ collagens were present in the same concentration in both tissues. These three chains co-precipitated on dialysis against phosphate-buffered saline, pH 7.2, in a manner analogous to type V collagen.

Early biochemical and morphological studies established collagen as the major fibrous component of vitreous humour, and it was generally agreed that the collagen fibrils were fine, with diameters in the range 10–20 nm (Matoltsy *et al.*, 1951; Gross *et al.*, 1955; Olsen, 1965). However, the morphological studies were contradictory with regard to the axial periodicity of the fibrils, and it was suggested that the assembly of collagen molecules within the vitreous-humour fibrils was different from that of other tissues.

When it became apparent that collagen existed in more than one genetically distinct form, with type differences varying with tissue source (Miller, 1976; Bornstein & Sage, 1980), several investigations demonstrated that, like cartilage, the collagen of the vitreous humour was type II, containing three identical $\alpha 1(\text{II})$ chains (Swann *et al.*, 1975, 1976; Newsome *et al.*, 1976; Swann & Sotman, 1980). However, Swann and co-workers, in a comparison of unfractionated vitreous-humour and cartilage collagens, revealed differences in amino acid and carbohydrate composition and the presence in the vitreous humour of a major component and several minor components migrating more slowly than the $\alpha 1(\text{II})$ chains on gel electrophoresis (Swann *et al.*, 1976; Swann & Sotman, 1980). They therefore suggested that vitreous-

humour collagen was a specialized type II collagen in which certain 'terminal peptides' were retained after pepsin extraction and that these peptides altered the aggregation of the molecules, resulting in the unique fibrous form observed under the electron microscope (Swann *et al.*, 1976). The possible heterogeneity of vitreous collagen was excluded, since only one type of fibril was observed in the vitreous-humour preparations.

Recently, however, use of improved fractionation techniques for the study of cartilage has resulted in the isolation from that tissue of three minor collagen α -chains, $1\alpha 2\alpha 3\alpha$, first from human hyaline cartilage (Burgeson & Hollister, 1979) and subsequently from both the annulus and nucleus of the human intervertebral disc (Ayad *et al.*, 1981) and other mammalian (Shimokomaki *et al.*, 1980; Ayad *et al.*, 1981) and avian (Reese & Mayne, 1981; Von der Mark *et al.*, 1982) cartilages. The 1α and 2α chains are similar to the α -chains of type V collagen, but are genetically distinct (Burgeson & Hollister, 1979; Burgeson *et al.*, 1982), whereas the 3α chain is identical with the $\alpha 1(\text{II})$ chain, but is apparently more glycosylated.

Two further minor collagens have also been isolated from cartilage and the intervertebral disc, which have been called 'cartilage-phosphate-soluble' (C-PS) collagens because of their preferential solubility in phosphate buffers (Ayad *et al.*, 1981, 1982). These collagens are much shorter in length

Abbreviation used: SDS, sodium dodecyl sulphate.

than type II or the $1\alpha 2\alpha 3\alpha$ collagens. C-PS1 collagen has apparent M_r 110000 and consists of three similar chains of M_r 33000 linked by interchain disulphide bonds forming a short triple helix approximately one-third the length of the type II collagen molecule (Ayad *et al.*, 1981). C-PS2 collagen has apparent M_r 36000 and consists of three dissimilar chains of M_r 16000, 10000 and 8000 linked side-by-side by disulphide bonds in a short triple helix approximately one-seventh the length of the type II collagen molecule (Ayad *et al.*, 1982). The presence of these collagens was confirmed by Shimokomaki *et al.* (1980, 1981).

Since the vitreous humour has a gel-like structure similar to cartilage and disc (particularly nucleus pulposus) and contains type II collagen as the major collagen constituent, it seemed likely that the $1\alpha 2\alpha 3\alpha$ and C-PS collagens would also be present in the vitreous humour. The experiments described here were therefore carried out to test this hypothesis.

Experimental

Materials

Adult bovine nasal septa and eyes were obtained fresh from the local abattoir. Pepsin (twice-crystallized) and dithiothreitol were purchased from

Sigma. Acrylamide, *NN'*-methylenebisacrylamide, ammonium persulphate and *NNN'*-tetramethylethylene-diamine (which were specially purified for gel electrophoresis) and Bio-Gel A-5m were obtained from Bio-Rad Laboratories, Watford, Herts., U.K. CM-cellulose 52 (pre-swollen) was purchased from Whatman, Maidstone, Kent, U.K.

Isolation of collagen from vitreous humour and cartilage

Cartilage from the nasal septa was freed of adjacent perichondrium, diced and powdered in a stainless-steel bomb under liquid N_2 . The powdered tissue was incubated with 2M-MgCl₂/50mM-Tris/HCl buffer, pH 7.4, for 48 h at 4°C (10ml of buffer/g of wet tissue). The resulting suspension was centrifuged at 40000g for 1 h and the supernatant discarded. The residue was washed three times with distilled water, resuspended in 0.5M-acetic acid, pH 2.8, and incubated with pepsin (20mg of pepsin/g of wet tissue) for 48 h at 4°C. Vitreous humour was freed of adjacent tissue, centrifuged at 50000g for 3 h at 4°C and the resulting pellet incubated with pepsin as described for cartilage. The pepsin digests containing solubilized collagen were clarified by centrifugation at 40000g for 1 h at 4°C before fractionation.

Scheme 1. Fractionation of pepsin-solubilized collagen from bovine vitreous humour and cartilage. Bovine vitreous humour and cartilage were cleaned of adhering tissue and incubated with pepsin at 4°C for 48 h. The pepsin digests were then fractionated as shown.

Fractionation of pepsin-solubilized collagen

The total pepsin-solubilized collagen was fractionated as described previously (Ayad *et al.*, 1981, 1982), but with minor modifications as shown in Scheme 1. All collagen fractions were dissolved in 0.1 M-acetic acid, dialysed extensively against 0.1 M-acetic acid and either freeze-dried or stored at -30°C .

Quantitative determination of the different collagens

The relative amounts of the various collagens were assessed by analysing portions of the precipitates and supernatants obtained during the fractionation procedure outlined in Scheme 1 for hydroxyproline by the method of Woessner (1961). The sensitivity of this method was increased by replacing 2-methoxyethanol with 1-methylethanol and using a 10% (w/v) *p*-dimethylaminobenzaldehyde solution in 1-methylethanol. For precipitates containing mixtures of C-PS1 and C-PS2 collagens, hydroxyproline analyses were performed on portions of the individual collagens separated in the denatured state on agarose A-5m (Ayad *et al.*, 1982). The amount of each collagen was calculated by using the percentage hydroxyproline obtained by amino acid analysis and expressed as a percentage (w/w) of total pepsin-solubilized collagen.

Analytical procedures

The various collagens isolated (Scheme 1) were identified by sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis by the method of Laemmli (1970), but with 5% (w/v) acrylamide in the separating gel and no stacking gel. Samples were incubated at 100°C for 2 min in the sample preparation buffer with or without the addition of 50 mM-dithiothreitol (to reduce any disulphide bonds) before electrophoresis. The polypeptide bands were stained with Coomassie Brilliant Blue R and glycosylated peptides were located by the period acid/Schiff reagent (Glossmann & Neville, 1971). M_r values for various collagen chains and peptide derivatives were estimated by gel electrophoresis using α - and β -chains and CNBr-cleaved peptides of type I collagen as standard M_r markers.

Collagen samples were hydrolysed in constant-boiling HCl under N_2 for 24 h at 110°C and analysed for amino acids on a Jeol 6AH amino acid analyser.

Results

Fractionation and purification of vitreous-humour collagen

The fractionation and purification of vitreous-humour collagen (Scheme 1) was identical with that previously adopted for cartilage and disc (Ayad *et al.*, 1981, 1982) except that traces of type

II collagen contaminating the $1\alpha 2\alpha 3\alpha$ collagen were removed by selectively precipitating the $1\alpha 2\alpha 3\alpha$ collagen by dialysis against either phosphate-buffered saline (0.12 M-NaCl/10 mM- Na_2HPO_4 /3 mM- KH_2PO_4), pH 7.2, or 0.01 M-acetic acid at 4°C . Each collagen fraction was identified by comparison with the corresponding fraction isolated from bovine nasal cartilage under the same conditions.

Type II collagen

The major vitreous-humour collagen precipitated at 0.86 M-NaCl, pH 2.8, was virtually identical with type II collagen of cartilage, as shown by gel electrophoresis (Fig. 1, tracks 1 and 2). The vitreous-humour collagen, however, had a higher hydroxylysine content (Table 1a) and therefore an increased number of potential sites for glycosylation (Spiro, 1969). In this respect the $\alpha(\text{II})$ chains of vitreous-humour collagen were more intensely stained by the periodic acid/Schiff reagent than

Fig. 1. SDS/polyacrylamide-gel electrophoresis of bovine vitreous-humour and cartilage collagens

Samples of vitreous (V) and cartilage (C) collagens, isolated as described in Scheme 1, were analysed on SDS/5%-polyacrylamide gels in the absence of dithiothreitol and detected with Coomassie Brilliant Blue R. Samples in tracks are: 1 and 2, 0.86 M-NaCl, pH 2.8, precipitates (type II collagen) from C and V respectively; 3 and 4, phosphate-buffered saline precipitates ($1\alpha 2\alpha 3\alpha$ collagen) from C and V respectively; 5, 0.01 M-acetic acid-insoluble precipitate from V; 6 and 7, 4.5 M-NaCl precipitates from the phosphate supernatant (mixture of C-PS1 and C-PS2 collagens) from C and V respectively. The exact migration positions of the constituent chains of the cartilage collagens only are indicated.

Table 1. Amino acid compositions of bovine vitreous-humour (V) and cartilage (C) collagens

Amino acid	Composition (residues/1000 residues)								
	(a)		(b)		(c)			(d)	
	0.86M-NaCl, pH 2.8 precipitate (Type II)		phosphate-buffered saline, pH 7.2, precipitate (1 α 2 α 3 α)		0.02M-Na ₂ HPO ₄ supernatant, 5M-NaCl, pH 7.4, precipitate (C-PS1 and higher-M _r forms)			0.02M-Na ₂ HPO ₄ supernatant, 3.5M-NaCl, pH 7.4, precipitate (C-PS2)	
	V	C	V	C	V(1)*	V(2)*	C(1)*	V	C
Hyp	111	98	94	97	84	102	70	91	115
Asp	41	36	43	42	43	44	47	61	60
Thr	21	24	20	24	22	19	17	23	15
Ser	22	21	24	24	29	28	36	34	31
Glu	88	87	106	101	100	97	107	80	73
Pro	111	126	108	102	95	87	90	89	106
Gly	331	335	323	330	315	344	328	286	282
Ala	100	101	75	75	56	54	58	62	75
$\frac{1}{2}$ -Cys	—	—	—	—	5	4	1	15	13
Val	20	18	25	26	45	37	37	31	25
Met	9	9	9	9	8	—	7	6	11
Ile	12	10	17	18	25	23	26	28	24
Leu	30	26	38	38	54	59	47	59	48
Tyr	Trace	3	2	Trace	5	Trace	4	6	6
Phe	13	12	14	13	11	Trace	6	16	9
Hyl	26	19	36	32	33	30	32	28	14
Lys	17	19	15	16	18	17	31	23	21
His	2	4	6	4	6	10	11	15	14
Arg	46	52	45	49	46	45	46	47	57

* (1) and (2) are analyses of C-PS1 collagen and the combined components M_r 150000 and 132000 separated by chromatography on agarose A-5m.

those of cartilage. Amino acid analysis of the major vitreous-humour collagen showed that it was similar to, but not identical with, the type II collagen of cartilage, there being more hydroxylation of proline as well as lysine residues.

1 α 2 α 3 α Collagen

The gel-electrophoretic pattern of the phosphate-buffered-saline precipitate from vitreous humour is shown in Fig. 1 (track 4) and is similar to that from cartilage containing the 1 α 2 α 3 α chains (Fig. 1, track 3). The 2 α chain of vitreous-humour collagen, however, consistently migrated faster than that of cartilage. All three chains were unaffected by reduction and further pepsin digestion, and were periodate/Schiff-positive (particularly the 1 α and 2 α chains). The three chains of vitreous-humour collagen could also be selectively precipitated by dialysis against 0.01 M-acetic acid (Fig. 1, track 5) as first observed for the 1 α 2 α 3 α collagen of cartilage (Burgeson & Hollister, 1979). Their identity was further confirmed by amino acid analysis (Table 1b).

C-PS collagens

Fig. 1 (track 7) shows gel electrophoresis of the phosphate-soluble collagens from vitreous humour compared with the C-PS1 and C-PS2 collagens of cartilage (track 6). A detailed comparison of

vitreous-humour C-PS2 collagen with that of cartilage has been reported (Ayad *et al.*, 1982) and indicated the similarity between the two collagens, except for the extent of lysine hydroxylation, which is greater for the vitreous-humour collagen (Table 1d).

A relationship between C-PS1 collagen (M_r 110000) and the components of M_r 150000 and 132000 in vitreous humour has not been described. When C-PS1 and C-PS2 collagens were separated in the native state by differential salt precipitation at pH 7.4 (Scheme 1), the two components of M_r 132000 and 150000 consistently co-purified with C-PS1, precipitating preferentially at 4.5–5M-NaCl. The two components also co-eluted with C-PS1 on CM-cellulose under native conditions in a position similar to that of cartilage C-PS1 (Ayad *et al.*, 1981), but only at the leading edge of the elution profile, the remaining profile containing C-PS1 only (result not shown).

The higher- M_r components were, however, separated from C-PS1 collagen by chromatography on agarose A-5m in the denatured unreduced state. The major C-PS1 component was eluted in the position of α 1(II) chains as previously described (Ayad *et al.*, 1981), whereas the components of M_r 132000 and 150000 were eluted earlier (results not shown). The amino acid analyses of the

Fig. 2. SDS/polyacrylamide-gel electrophoresis of vitreous-humour C-PS1 and higher- M_r components after reduction of disulphide bonds

Samples of the 5M-NaCl, pH 7.4, precipitate from the Na_2HPO_4 supernatant (see Fig. 1) were analysed on SDS/7% polyacrylamide gels in the presence of 50 mM-dithiothreitol and stained with Coomassie Brilliant Blue R. C-PS1_r denotes the position of the reduced chains of cartilage C-PS1 collagen (M_r 33000). M_r values were determined by gel electrophoresis using the CNBr peptides and α/β -chains of type I collagen as standard M_r markers.

separated components (Table 1c) confirmed the identity of vitreous-humour C-PS1 collagen and indicated that the 132000- and 150000- M_r components were probably higher- M_r forms of C-PS1 collagen.

When the vitreous-humour C-PS1 collagen together with the higher- M_r components were reduced, several major polypeptides were observed with apparent M_r 33000, 46000, 76000 and 130000 (Fig. 2). This is in contrast with the major reduced polypeptides of M_r 33000 from cartilage C-PS1 collagen alone (Ayad *et al.*, 1981).

Quantitative analysis of the various collagen fractions showed that type II collagen accounted for only 77% of the total vitreous-humour collagen, in contrast with 86.5% of the total cartilage collagens. C-PS1 and C-PS2 accounted for 10 and 5% respectively of vitreous-humour collagen compared with only 3.5 and 1.5% for cartilage. The calculated molar ratio of C-PS1 to C-PS2 was approx. 0.7 for vitreous humour and 0.8 for cartilage. The amounts of $1\alpha 2\alpha 3\alpha$ collagen were the same for both tissues.

Discussion

Type II collagen was found to be the major vitreous-humour collagen, confirming previous findings (Swann *et al.*, 1975, 1976; Newsome *et al.*, 1976; Swann & Sotman, 1980), but did not possess the 'terminal peptides' proposed by Swann and co-workers (Swann *et al.*, 1976; Swann & Sotman, 1980). However, it did contain more hydroxylated lysine residues than did cartilage type II collagen, and these could be a potential site for glycosylation. An inverse relationship between carbohydrate content and fibril diameter is frequently observed (Spiro, 1969; Grant *et al.*, 1969), and this may account for the smaller diameter of vitreous-humour fibrils compared with those in cartilage (Matoltzky *et al.*, 1951; Gross *et al.*, 1955; Olsen, 1965).

A minor collagen fraction was completely separated from type II collagen by its preferential insolubility in both phosphate-buffered saline, pH 7.2, and 0.01 M-acetic acid and identified as the $1\alpha 2\alpha 3\alpha$ collagen. Chiang *et al.* (1980) first used dialysis against phosphate-buffered saline as a method of preparing type V collagen in a fibrillar form, and we have used this method to purify type V collagen (Abedin *et al.*, 1981, 1982). Our finding that phosphate-buffered saline also selectively precipitates $1\alpha 2\alpha 3\alpha$ chains, leaving type II collagen in solution, provides further evidence for the $1\alpha 2\alpha 3\alpha$ collagen chains being members of a 'type V family' of collagens (Reese & Mayne, 1981; Burgeson *et al.*, 1982). Moreover, although the 3α chain is different from the 1α and 2α chains, but similar to, if not identical with, the $\alpha 1(\text{II})$ chain (Burgeson & Hollister, 1979), it consistently co-precipitates with the 1α and 2α chains and it has been impossible to fractionate the $1\alpha 2\alpha 3\alpha$ collagen into more than one type of molecular species either by CM-cellulose or DEAE-cellulose chromatography in the native state (S. Ayad, unpublished work). This suggests that the 3α chain probably exists in the same triple helix as the 1α and/or 2α chains [i.e. $(1\alpha 2\alpha 3\alpha)$ or $(1\alpha)_2 3\alpha$ plus $(2\alpha)_2 3\alpha$] rather than as an independent molecular species $(3\alpha)_3$. It also argues against the major collagen of rabbit vitreous humour consisting of a mixture of $\alpha 1(\text{II})$ and 3α chains (Burke, 1980).

The presence of both C-PS1 and C-PS2 collagens in vitreous humour has been noted previously (Ayad *et al.*, 1982). Vitreous humour also contains two components of M_r 132000 and 150000, which from their physicochemical behaviour appear to represent higher- M_r forms of C-PS1 collagen (M_r 110000). In this respect the major disulphide-bonded collagen of chick cartilage exists predominantly in a high- M_r form ($M_r \sim 150000$) (Reese & Mayne, 1981; Von der

Mark *et al.*, 1982; Reese *et al.*, 1982), one of its three component chains being cleaved at a non-helical site during isolation (Reese *et al.*, 1982). Also, the electrophoretic profile of the reduced vitreous-humour disulphide-bonded collagens (Fig. 2) is qualitatively similar to that reported for the chick cartilage collagen. It is possible that the major disulphide-bonded collagen of vitreous humour consists of a mixture of molecules in which one, two and all three chains have been cleaved at a non-helical site to give rise to components of M_r 150000 and 132000, together with C-PS1 collagen (110000 M_r), respectively on denaturation before reduction. Interestingly these higher- M_r forms of vitreous-humour disulphide-bonded collagen could not be converted into C-PS1 *in vitro* by further pepsin digestion. However, other more specific proteinases may well have caused cleavage at the non-helical site during isolation. The major disulphide-bonded collagen of chick cartilage has been shown to be susceptible *in vitro* to several neutral proteinases (Liotta *et al.*, 1982).

An important observation was that although the total amounts of C-PS1 and C-PS2 collagen and their higher- M_r forms were three times greater in vitreous humour than in cartilage, the molar ratios of C-PS1 to C-PS2 were approximately the same in both tissues ($\sim 1:1$), which would strongly suggest that these collagens are components of the same larger molecule which exists in the tissue before extraction.

Swann & Sotman (1980) investigated vitreous-humour collagen and postulated that the type II collagen of vitreous humour, in contrast with that of cartilage, had 'terminal peptides', most of which were removed on reduction. It is now almost certain that what they were observing was in fact the C-PS1 collagen and its higher- M_r forms. These workers also observed some residual α -size components after reduction, which presumably corresponded to the $1\alpha 2\alpha 3\alpha$ collagen.

It is not yet known which cells synthesize the minor collagen of vitreous humour nor where these collagens are located. Different cell types synthesize the collagen of vitreous humour at different stages of development, the early vitreous-humour collagen being synthesized by the neural retina, whereas later in development the hyalocytes are more active (Newsome *et al.*, 1976). In the case of cartilage, C-PS1 and C-PS2 collagens are specifically located in the territorial region surrounding the chondrocytes, which indicates that they are in fact synthesized by these cells (Evans *et al.*, 1983). The difference in amounts of CP-S collagens between the two tissues may be related to the fact that adult vitreous humour requires a much greater active synthesis of collagen to maintain its gel state and normal eye function (Snowden *et al.*, 1982).

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