Biochemical properties and immunolocalization of minor collagens in foetal calf cartilage

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1. INTRODUCTION

Type II collagen is the major collagen of cartilage. However, new collagenous chains have been described in different cartilaginous tissues, recently. The 1α, 2α and 3α chains were extracted from human and bovine hyaline cartilage [1]. Other minor collagenous components were extracted from neonatal pig and human cartilage noted M, CF_1, CF_2 [2–4], from bovine nasal cartilage and human intervertebral disc noted CPS_1, CPS_2 [5,6] and from chicken sternal cartilage noted HMW, LMW [7,8] and M_1, M_2 [9].

Here, we report the partial characterization of 3 collagenous fractions obtained after limited pepsin treatment of foetal calf cartilage and isolated according to their solubility properties. The 1.2 M NaCl fraction contains the 1α, 2α and 3α chains. The 2.0 M NaCl and 3.0 M NaCl fractions contain at least 7 collagenous chains, which are related to the disulfide-bonded new chains enumerated above, but show some major differences, Antibodies against the 1.2 M and 2.0 M NaCl fractions were raised in rabbits and their specificity tested by radioimmunoprecipitation. The localization of the corresponding collagenous chains was achieved by indirect immunofluorescence in epiphyseal proper and growth cartilage from foetal calf cartilage.

2. MATERIALS AND METHODS

2.1. *Preparation and purification of collagens*

Epiphyseal cartilage was obtained from foetal calves (7–9 months). Thin tissue slices were homogenized under liquid nitrogen. Proteoglycans were extracted with 4 M guanidinium chloride, pepsin treatment and precipitation of the pepsin-soluble fraction were performed as in [1]. Salt fractionation of the collagenous components was achieved as in [2,3]. For further purification the different fractions (1.2 M, 2.0 M and 3.0 M NaCl) were passed down a DEAE-cellulose column under native conditions [2] to remove contaminating acidic glycoproteins.

2.2. *Biochemical characterization*

SDS–polyacrylamide gel electrophoresis was performed as in [10] with 6.75% acrylamide gels, with and without reduction with dithiothreitol (DTT). Apparent M_r values were calculated by comparison to β_{11} and α_1 components of type I collagen and cyanogen bromide peptides of the α_1 (I) chain. For molecular sieve chromatography, samples were chromatographed on an agarose (Biogel A-1.5 m; 200–400 mesh, Biorad) column (2.5 × 220 cm) eluted with 1 M $CaCl_2$–0.05 M Tris (pH 7.5) at 20 ml/h.

Amino acid analyses were carried out on a Jeol-5AH automatic analyzer after hydrolysis of samples with 6 N HCl for 24 h at 110°C.

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2.3. *Preparation and purification of antibodies*

New Zealand white adult rabbits were injected intradermally with 1 mg antigen in 0.5 M acetic acid emulsified with an equal volume of Freund's complete adjuvant. Two, 4 and 6 weeks after the initial injection, intradermal booster injection of 1 mg antigen in 0.5 M acetic acid emulsified with Freund's incomplete adjuvant was administered. The antibodies were purified by immunoadsorption on affinity columns composed of bovine types I and II collagens and of 1.2 M and 2.0 M NaCl fractions conjugated to glutaraldehyde-activated Ultrogel ACA 22 resin.

2.4. *Radioimmunoprecipitation*

Collagens were labelled with ^{125}I by the lactoperoxidase method [11] and radioimmunoprecipitation was performed as in [11]. The binding of ^{125}I-collagen to antibody was expressed as a percentage of the added collagen found in the precipitate.

2.5. *Immunofluorescence staining procedure*

Fresh frozen sections (6 μm) of epiphyseal proper and growth cartilage from foetal calf were treated with bovine testicular hyaluronidase for 2 h at 37°C. Indirect immunofluorescence technique was performed as follows: the sections were exposed to purified anticollagen antibodies for 20 min at room temperature, treated with FITC-conjugated goat antiserum against rabbit IgG (Institut Pasteur Production) and observed on a Leitz-Dialux fluorescence microscope.

3. RESULTS AND DISCUSSION

3.1. *Characterization of collagenous fractions*

3.1.1. 1.2 M NaCl fraction

This fraction, precipitated at 1.2 M NaCl in 0.5 M acetic acid contains 3 chains with electrophoretic (fig.1a) and compositional properties (not shown) similar to the 1α, 2α and 3α chains described in [1].

3.1.2. 2.0 M and 3.0 M NaCl fractions

The 2.0 M NaCl fraction reveals at least 7 bands (called X_1–X_7 chains) on SDS–polyacrylamide gel electrophoresis (fig.1b,c) whereas the 3.0 M NaCl fraction contains only the low M_r chains (X_5–X_7). The two fractions comprise ~5% of the total collagen in foetal calf and human cartilage but their amount decreases with ageing of tissue.

Chain X_3 was described in all preparations but the relative amounts of the X_1 and X_2 chains depended upon the conditions of pepsin treatment (fig.1b,c). The X_3 chain of 125 000 M_r was isolated by chromatography on Biogel-A 1.5 m (fig.2, peak 2 consistent with M_r 125 000). With reduction the X_3 chain gave rise to at least 3 major components (fig.1e,i) called X_3R_1, X_3R_2 and X_3R_3 of 40 000, 37 000 and 31 000 M_r. The electrophoretic behaviour and the amino acid composition (table 1) of the X_3 chain are similar to those reported for the collagenous components M [2,3], CPS_I [5,6], HMW [7,8] and M_I [9]. However, with reduction M and CPS_I both give rise to only one chain of 33 000 M_r. The X_3 chain can rather be compared to HMW [7,8] or M_I [9] giving rise with reduction, respectively, to 3 and 4 components but with different M_r-values.

Observed in addition to the X_3 chain, the X_1 and X_2 chains of 180 000 and 160 000 M_r were eluted as a single peak on Biogel A-1.5 m (fig.2, peak 1) consistent with 180 000 M_r. However, SDS–polyacrylamide gel electrophoresis of the material corresponding to this peak revealed that an important amount of the X_3 chain was eluted with the X_1 and X_2 chains. With reduction, in addition to the 3 components arising from the X_3 chain (fig.1i) two further products of 50 000 and 62 500 M_r were observed (fig.1h).

The X_4 chain (fig.1f,j) is a disulfide-bonded collagenous fragment, of 31 500 M_r by SDS–polyacrylamide gel electrophoresis and 33 000 M_r by molecular sieve chromatography, which can be compared to CF_2 [3], CPS_2 [6], LMW [7,8] and M_2 [9]. With reduction the X_4 chain gave rise to one component of 13 000 M_r. The amino acid composition of the X_4 chain (table 1) is different from that reported for CF_2 [3] and is rather similar to that of CPS_2 [6], LMW [7] and M_2 [9]. In particular the X_4 chain contains much less hydroxyproline and proline (110 and 116 residues/1000) than CF_2 (198.5 and 146.8 residues/1000).

The X_5, X_6 and X_7 chains (fig.1g,k,l) obtained in higher yields in the 3.0 M NaCl fraction are non disulfide-bonded components of 19 500, 15 000 and 13 000 M_r. They were eluted as a single peak on Biogel A-1.5 m (fig.2, peak 4) consistent with

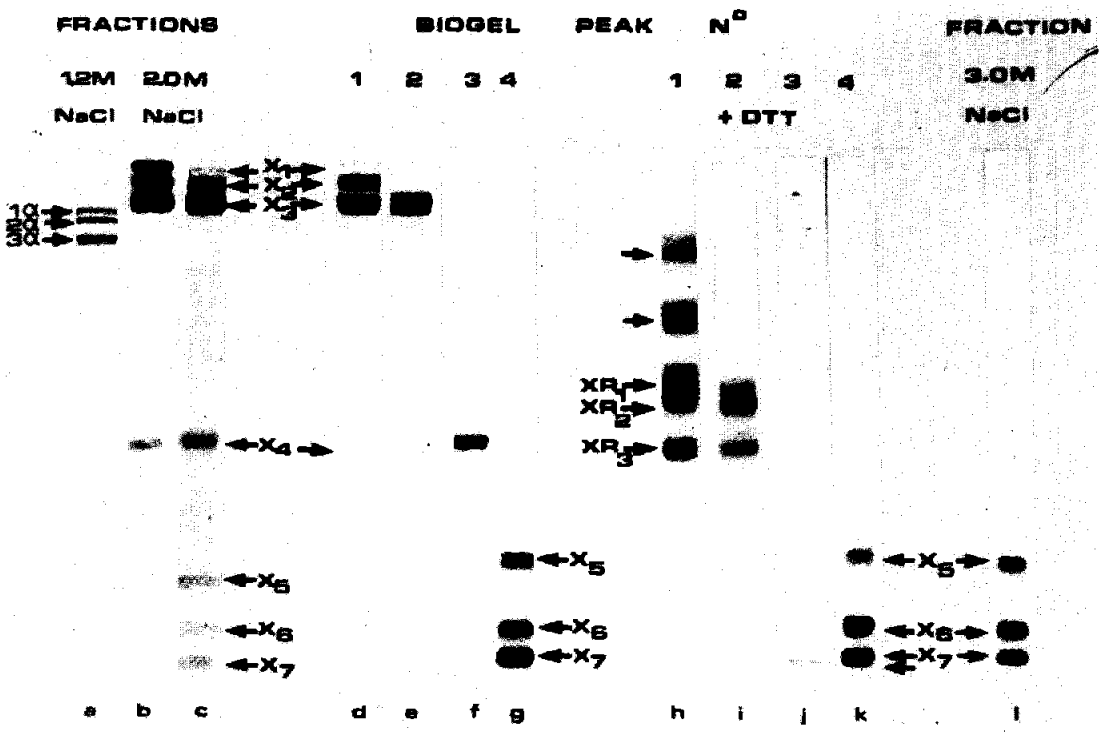

Fig.1. SDS–polyacrylamide gel (6.75%) electrophoresis, without and with (+ DTT) reduction: (a) of the 1.2 M NaCl fraction; (b,c) of two different preparations of the 2.0 M NaCl fraction; (d–k) of the material recovered from agarose gel filtration and corresponding to the 4 peaks of the chromatogram presented in fig. 2; (e) of the 3.0 M NaCl fraction.

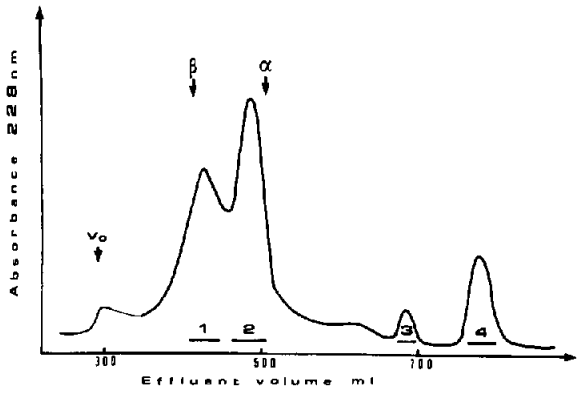

Fig.2. Agarose (Biogel A-1.5 m) molecular sieve elution pattern of the 2.0 M NaCl fraction. The column (2.5 × 200 cm) was eluted with 1 M $CaCl_2$ in 50 mM Tris–HCl (pH 7.5) at 20 ml/h. Arrows show the elution position of β and α components of type I collagen and the bars indicate the 4 fractions which were pooled for further analysis.

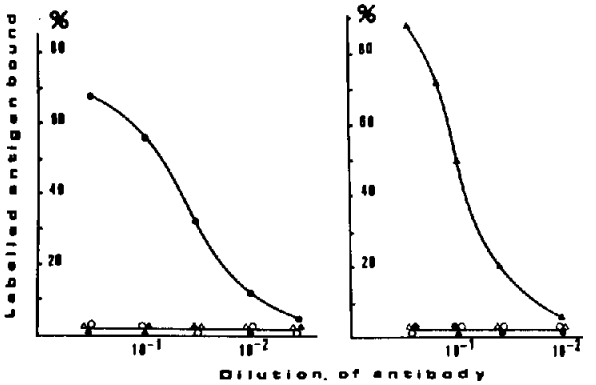

Fig.3. Reactions of rabbit antisera to; (a) 1.2 M NaCl and (b) 2.0 M NaCl fractions with labelled collagens. The ^{125}I-labelled antigens used were: the (•) 1.2 M NaCl fraction, (▲) 2.0 M NaCl fraction and (○) type I and (△) type II bovine collagens.

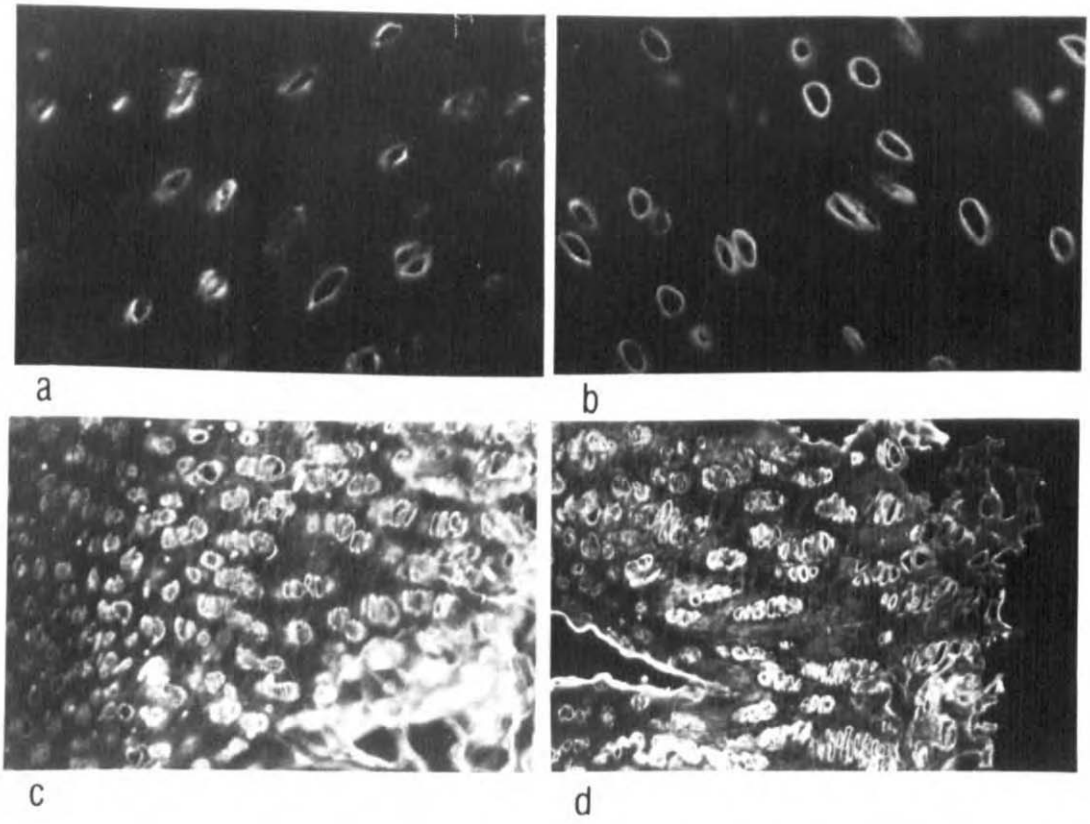

Fig.4. Epiphyseal proper (a,b) and growth (c,d) cartilage from foetal calf after hyaluronidase treatment, stained with anti-1.2 M NaCl fraction (1α, 2α, 3α chains) antibodies (a,c) and with anti-2.0 M NaCl fraction (X_1–X_7 chains) antibodies (b,d). Specific staining with both antibodies can be detected on the pericellular surface of chondrocytes from the two different zones of the epiphyseal cartilage.

17 000 M_r. The amino acid composition of these chains (table 1) shows a very high content of proline and hydroxyproline (159 and 160 residues/1000), similar to that reported for CF_1 and CF_2 [3] and for C_4 [8].

It is now important to determine if these 7 chains (or fragments) are parts of a single molecule of collagen. The presence of higher yields of X_5, X_6 and X_7 chains in the more soluble fraction is in favour of the existence of at least 2 different molecular species as already suggested for M, CF_1, CF_2 [2,3] and CPS_1, CPS_2 [4,5]. These molecules may belong to a unique large molecule analogous to type IV collagen, whose 'four domain structure' [12] includes triple helical and non-collagenous (pepsin-sensitive) segments cross-linked or not by disulfide bridges or other covalent bonds.

3.2. *Characterization of antibodies and immunofluorescent localization*

The two collagenous fractions were immunogens in rabbits and induced antisera with good binding capacity after 3 injections. Following purification the antibodies showed typical binding profiles with ^{125}I-labelled antigens (fig.3). High concentration of antiserum bound 70–90% of the labelled antigen, indicating that iodination had not destroyed antigenicity. Both antisera showed a strong reaction for the immunizing antigen and a weak one with the other collagenous fraction and with bovine types I and II collagens.

Anti 1.2 M NaCl and 2.0 M NaCl fractions gave an intense staining located around the chondrocyte lacunae from both the epiphyseal proper and growth cartilage (fig.4). The labelling of the anti 2.0 M NaCl fraction seemed to be more restricted

Table 1

Amino acid composition (residues/1000) of the components recovered from agarose gel filtration (fig.2)

Peak :	1	2	3	4
Chains :	X_1,X_2,X_3	X_3	X_4	X_5,X_6,X_7
4 Hyp	102	85	110	165
Asp	41	45	50	34
Thr	17	18	20	20
Ser	33	35	42	23
Glu	105	109	89	57
Pro	100	101	116	160
Gly	325	330	301	331
Ala	49	49	54	33
Cys	7	nd	nd	nd
Val	37	38	31	28
Met	9	12	nd	nd
Ile	23	22	25	27
Leu	43	46	47	47
Tyr	4	3	1	0
Phe	6	5	7	5
Hyl	25	28	32	9
Lys	15	14	30	35
His	6	7	9	1
Arg	53	53	36	25

around the chondrocytes than the anti 1.2 M NaCl fraction which gave a more diffuse fluorescent ring. Anti 1.2 M NaCl fraction (1α, 2α, 3α chains) did not stain the extracellular matrix. If the structure of the 3α chain is similar to that of the α_1 (II) chain as postulated [13] the fraction of the antibodies directed against 3α chain may have been eliminated during the immunoadsorption step on type II collagen or this 3α chain is far less immunogen than 1α and 2α chains.

A similar immunolocalization was obtained with related collagenous fractions [2,4] and with type V collagen [14]. Nevertheless we failed to show the presence of type V collagen by classical biochemical methods. The labelling observed in [14] may be due to a cross-reactivity of anti-type V antibodies with the 1α, 2α chains and/or with the 2.0 M NaCl fraction. Indeed biochemical properties of 1α, 2α chains are similar to type V collagen chains [1,6]. The protease susceptibility of the 1α, 2α chains and HMW fraction was similar to that of type V collagen in [13,15].

The chondrocytes appear to be surrounded by an exocytoskeleton including one or several new collagens whose molecular composition and organization remain to be established.

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