Collagen type IX from human cartilage: a structural profile of intermolecular cross-linking sites

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Type IX collagen, a quantitatively minor collagenous component of cartilage, is known to be associated with and covalently cross-linked to type II collagen fibrils in chick and bovine cartilage. Type IX collagen molecules have also been shown to form covalent cross-links with each other in bovine cartilage. In the present study we demonstrate by structural analysis and location of cross-linking sites that, in human cartilage, type IX collagen is covalently cross-linked to type II collagen and to other molecules of type IX collagen. We also present evidence that, if the proteoglycan form of type IX collagen is present in human cartilage, it can only be a minor component of the matrix, similar to findings with bovine cartilage.

INTRODUCTION

The major structural component of hyaline cartilage is type II collagen, which forms 80 % of the total collagen in fetal hyaline cartilage and 95 % in the adult. In addition, the tissue contains type IX (10 % of fetal, 1 % of adult collagen), type XI (10 % of fetal, 3 % of adult) and type X collagens, the latter being restricted to the hypertrophic and calcified zones of the growth plate and the deep calcified layer of mature articular cartilage. Cartilage also contains small amounts (< 1 %) of type VI collagen, which is found in most tissues [1–4].

The type IX collagen molecule is a heterotrimer of three genetically distinct chains, α1, α2 and α3 [4–8]. Each chain consists of four non-collagenous (NC) and three collagenous (COL) domains, termed NC1–NC4 and COL1–COL3 respectively [9]. The chains are bonded intramolecularly by disulphide bonds between cysteine residues located in the NC1 and NC3 domains [5,10]. As a result, pepsin digestion releases COL1, COL2 and COL3 triple-helical fragments in which COL1 and COL2 are disulphide-bonded trimers. COL1 and COL2 trimers were formerly termed LMW and HMW respectively [11]. Avian type IX collagen is a proteoglycan [12,13]. It possesses one chondroitin sulphate glycosaminoglycan covalently linked to a serine residue in the attachment site, Gly-Ser-Ala-Asp, located in the α2(IX)NC3 domain [14–17]. However, type IX collagen lacking an attached glycosaminoglycan has also been identified in bovine epiphyseal cartilage along with the proteoglycan form of the molecule [18].

Type IX collagen is one of the fibril-associated collagens with interrupted triple helices (known as FACITs) [19]. Studies in the chick using immunofluorescent antibodies directed against the COL2 domain have shown that type IX collagen molecules are co-distributed with type II collagen in cartilage, and appear to decorate the surface of the type II fibrils [20]. Rotary shadowing of collagen fibrils from embryonic chick sternal cartilage showed a D-periodic distribution of 35–40 nm projections capped by globular domains, which were identified as NC4(IX) domains by immunofluorescent staining using monoclonal antibodies directed against the N-terminal region of COL3(IX) [21]. Type IX collagen has been shown to be covalently cross-linked to type II collagen in bovine and chick articular cartilage [22–24]. Covalent bonds are formed between cross-linking sites at the N-terminus of COL2 in all three chains of the type IX collagen molecule and the N-telopeptide of type II collagen molecules, as well as from an interior site in the COL2 domain of α3(IX) to the C-telopeptide of type II collagen [where N- and C-telopeptides are short sequences that lack the $(GXY)_n$ repeat and form the N- and C-terminal ends of various collagen chains]. In addition, it has been demonstrated that bovine type IX collagen molecules are covalently cross-linked to other type IX molecules, by bonds between the COL2 domains of α1(IX) and α3(IX) and the NC1 domain of α3(IX) [24]. Most of the type IX collagen cross-linking residues in mature articular cartilage are tervalent pyridinolines [25], but in fetal cartilage bivalent, borohydride-reducible cross-links predominate [26].

In the present study we have examined the intermolecular cross-linking between collagen types IX and II, and between types IX and XI, in normal human articular cartilage. The results show a pattern of cross-linking that parallels what has been found in bovine tissue and define the cross-linking sites by sequence analysis.

MATERIALS AND METHODS

Preparation of type IX collagen

Human articular cartilage was dissected from the epiphyses of 100–120-day fetuses without evidence of connective tissue disease. The cartilage was washed extensively with 0.15 M NaCl/0.05 M Tris/HCl/protease inhibitors (2 mM PMSF, 2 mM EDTA, 5 mM benzamidine, 10 mM *N*-ethylmaleimide), pH 7.40, at 4 °C. Proteoglycans were extracted by washing three times with 4.0 M guanidine hydrochloride/0.05 M Tris/HCl/protease inhibitors, pH 7.40, for 24 h at 4 °C. The cartilage was then homogenized and digested with pepsin (1:10, dry weight) in 0.5 M acetic acid

Abbreviations used: NC, non-collagenous; COL, collagenous; DTT, dithiothreitol; TFA, trifluoroacetic acid; PVDF, polyvinylidene difluoride; N- and C-telopeptides, short sequences that lack the $(GXY)_n$ repeat and form the N- and C-terminal ends of various collagen chains.

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for 24 h at 4 °C. Serial precipitations in 0.9 M, 1.2 M and 2.2 M NaCl separated type II, XI and IX collagens respectively. Type IX collagen was desalted by dialysis against 0.1 M acetic acid and freeze-dried.

Reducible cross-linking residues were labelled with [^{3}H]$NaBH_4$ (400 mCi/mM) in 0.1 M sodium phosphate buffer, pH 7.40, at 25 °C [27]. Unreacted cross-links were further reduced with excess unlabelled $NaBH_4$, following which the reaction was terminated with acetic acid (5 %, v/v). The labelled collagen was dialysed against 0.1 M acetic acid and freeze-dried.

Column chromatography

COL1(IX) and COL2(IX) disulphide-bonded trimeric fragments generated by pepsin digestion were resolved by molecular sieve chromatography on an agarose column (Bio-Rad Laboratories A5m; 1.5 cm × 170 cm; 200–400 mesh), eluting with 2 M guanidine hydrochloride/0.05 M Tris/HCl, pH 7.50, at 7.2 ml/h and collecting 2.4 ml fractions. Individual COL2 domains were separated by reduction of cystine residues with 10 mM dithiothreitol (DTT) in 4.0 M guanidine hydrochloride/0.05 M Tris/HCl, pH 8.0, followed by carboxymethylation with 20 mM sodium iodoacetate [28]. The α1(IX) and α3(IX)COL2 domains were resolved from α2(IX)COL2 by reverse-phase HPLC on a C8 column (Brownlee Aquapore RP-300; 4.6 mm × 25 cm). The eluent was a linear gradient from 20 to 35 % solvent B in solvent A over 30 min at 1 ml/min, collecting 1 ml fractions, where solvent B was 0.085 % (v/v) trifluoroacetic acid (TFA) in acetonitrile/n-propanol (3:1, v/v) and solvent A was aqueous TFA (0.1 %, v/v) [24]. Telopeptides released from cross-linking by periodate cleavage were also resolved from individual COL2 domains by C8 reverse-phase HPLC.

Eluent was monitored for absorbance at 220 nm. Pyridinoline cross-links were detected by monitoring fluorescence using an HPLC fluorescence detector (Kratos 970; excitation 297 nm, emission > 380 nm) [29]. Borohydride-reducible cross-links and periodate-released telopeptides containing tritium label were detected by assaying an aliquot from each column fraction for tritium activity using a liquid scintillation counter (Beckman LS 1801).

Gel electrophoresis

SDS/PAGE was performed as described by Laemmli [30], and was used to confirm HPLC resolution of peptides and to separate proteins for electroblotting and sequence analysis. Unreduced proteins and reduced COL2 trimers were run on 7.5 % (w/w) polyacrylamide gels.

Electroblotting of proteins

For N-terminal sequencing, proteins were separated by SDS/7.5 %-PAGE and then transblotted on to a polyvinylidene difluoride (PVDF) membrane (Westran) using a Milliblot-SDE electroblotting apparatus (Millipore) [31]. After extensive washing of the membrane with ultrapure water (Millipore Milli-Q) to remove salt, it was stained with Coomassie Brilliant Blue to identify protein bands. The bands were then excised and subjected to N-terminal sequence analysis.

Periodate cleavage of borohydride-reduced cross-links

Purified ^{3}H-labelled COL2 domains were dissolved in 0.02 M acetic acid and reacted with an equal volume of 0.02 M $NaIO_4$ for 3 h in the dark at 25 °C to cleave reduced cross-links [27]. The reaction was terminated by adding 5 μl of ethylene glycol followed by excess unlabelled $NaBH_4$ in 0.1 M sodium phosphate buffer, pH 7.40. The telopeptides released in this manner were then brought up to 1 % TFA and resolved by reverse-phase HPLC fractionation on a C8 column (Brownlee Aquapore RP-300; 4.6 mm × 25 cm), following which they were identified by protein microsequencing.

Protein sequencing

Sequence analysis of phenylthiohydantoin N-terminal amino acids was performed on a gas-phase protein microsequencer (Porton 2090E) equipped with an on-line Hewlett-Packard narrow bore HPLC system. Samples were obtained directly from HPLC and from PVDF protein transfer and sequencing membrane.

Extraction of intact type IX collagen chains and treatment with chondroitinase ABC

Intact type IX collagen chains were extracted from slices of human fetal hyaline cartilage by washing either in 1.0 M NaCl/protease inhibitors, pH 7.4, or in 4.0 M guanidine hydrochloride/0.05 M Tris/HCl/protease inhibitors, pH 7.4, for 24 h at 4 °C. After centrifugation for 1 h at 40000 ***g*** (Sorvall RC5C) at 4 °C, supernatants were removed, dialysed against 0.1 M acetic acid and freeze-dried.

A 0.125 unit/ml solution was prepared of *Proteus vulgaris* chondroitinase ABC (ICN ImmunoBiologicals, Lisle, IL, U.S.A.) in 0.05 M Tris/0.06 M sodium acetate/protease inhibitors, pH 8.0. The freeze-dried cartilage extracts were added to the enzyme solution and gently stirred for 24 h at room temperature [32]. The reaction mixture was centrifuged for 1 h at 40000 ***g*** and 4 °C, following which the supernatant was removed for Western blot analysis.

Antibody preparation and Western blot analysis

A polyclonal antiserum was prepared by immunizing rabbits with type IX collagen extracted from bovine cartilage by mild pepsin digestion. The immunogen included structurally intact COL1, COL2, COL3, NC2 and NC3 domains. Rabbits were immunized by subcutaneous injection of 0.4 mg of the type IX collagen in 0.5 ml of PBS (0.14 M NaCl/3 mM KCl/0.01 M Na_2HPO_4/1.7 mM KH_2PO_4, pH 7.4) emulsified with an equal volume of Freund's complete adjuvant. Three subcutaneous booster injections of 0.4 mg of antigen emulsified with Freund's incomplete adjuvant were given at 3, 6 and 10 weeks after the initial injection.

Intact type IX collagen molecules were reduced with 0.05 M DTT to release individual type IX collagen chains, which were separated on SDS/7.5 %-PAGE and transblotted to a PVDF membrane for Western blot analysis. The PVDF membrane was first blocked with Tris-buffered saline (TBS; 0.5 M NaCl/0.02 M Tris, pH 7.5) containing 1 % BSA at 25 °C for 1 h. Transblotted proteins were reacted with the rabbit polyclonal serum diluted 1:100 in TTBS [TBS containing 0.05 % (v/v) Tween-20]/0.1 % BSA at 25 °C overnight. A secondary antibody, Biotin-SP-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories), was added at a dilution of 1:10000 in TTBS/0.1 % BSA at 25 °C for 1 h. The biotin was bound by an avidin–alkaline phosphatase conjugate (Extravidin; Sigma Chemical Co.) at 25 °C for 1 h. This was followed by the addition of the substrate, NitroBlue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase buffer (0.1 M NaCl/0.1 M Tris/5 mM

$MgCl_2$, pH 9.5), forming a blue–black precipitate [33]. The reaction was stopped by rinsing with PBS containing 0.02 M EDTA.

RESULTS

Column purification of COL2 domains

Following pepsin digestion of articular cartilage to release type IX collagen and [^{3}H]$NaBH_4$ labelling of reducible cross-linking amino acid residues, disulphide-bonded COL1 and COL2 trimeric fragments were resolved by molecular sieve chromatography (Figure 1). SDS/7.5%-PAGE of 100 μl aliquots from each fraction showed that the COL2 trimer eluted earlier than the COL1 trimer.

Cysteine residues of COL2 trimeric fragments were reduced with DTT and carboxymethylated with sodium iodoacetate to separate individual COL2 domains. Reverse-phase HPLC fractionation on a C8 column resolved these into two groups (Figure 2). SDS/7.5%-PAGE of 100 μl aliquots from each fraction showed that the early peak consisted of a 33 kDa protein fragment and the late peak contained both 33 kDa and 41 kDa protein fragments. Fractions corresponding to the early and late peaks were collected for further analysis (indicated by bars in Figure 2). Both peaks showed fluorescence and ^{3}H activity (results not shown), indicating that they contained reducible bivalent cross-linking residues as well as tervalent pyridinoline cross-linking residues. Based on direct N-terminal sequencing of electroblotted protein bands, and on previous studies on bovine type IX collagen [24], it was deduced that the 33 kDa protein fragment which eluted early represents α2(IX)COL2, whereas the one eluting late is α1(IX)COL2. The 41 kDa protein fragment represents α3(IX)COL2.

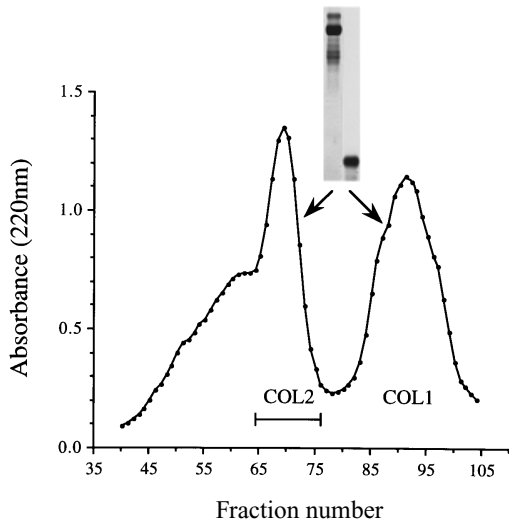

Figure 1 Molecular sieve chromatography of pepsin-extracted and [^{3}H]$NaBH_4$-labelled human fetal type IX collagen

The column (Bio-Rad Laboratories; agarose A5m column; 1.5 cm × 170 cm; 200–400 mesh) was eluted with 2 M guanidine hydrochloride/0.05 M Tris/HCl, pH 7.5 at 7.2 ml/h, collecting 2.4 ml fractions. SDS/7.5%-PAGE of 100 μl aliquots taken from each column fraction showed that COL2 trimers, eluting earlier, were resolved from COL1 trimers, which eluted later (inset). Fractions indicated by the bar were pooled for further analysis.

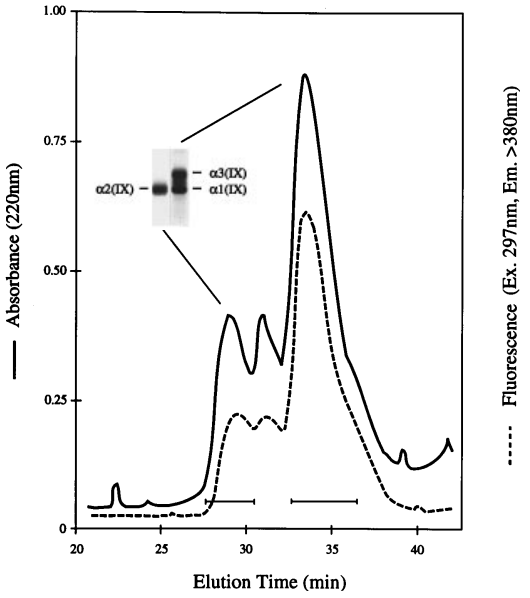

Figure 2 Reverse-phase HPLC fractionation of individual COL2 domains

The individual domains were prepared by reduction and carboxymethylation of cysteine residues in COL2 trimers. The C8 column (Brownlee Aquapore RP-300; 4.6 mm × 25 cm) was eluted with a linear gradient from 20 to 35% solvent B in solvent A over 30 min at 1 ml/min, collecting 1 ml fractions. Ex., excitation; Em., emission. SDS/7.5%-PAGE of 100 μl aliquots taken from each fraction showed that α2(IX)COL2 domains were resolved from α1 and α3(IX)COL2 domains (inset).

Periodate cleavage to release type IX and type II collagen telopeptides

Borohydride-reduced cross-links were reacted with $NaIO_4$ to release telopeptides from COL2 triple-helical cross-linking sites. The telopeptides were resolved from COL2 domains by reverse-phase HPLC on a C8 column (Figure 3). The eluent was monitored for absorbance at 220 nm. SDS/7.5%-PAGE of 100 μl aliquots collected from each fraction showed that the late peaks of 220 nm absorbance consisted of the COL2 domains of α2(IX) (Figure 3, upper panel) and of α1(IX) and α3(IX) (Figure 3, lower panel). Aliquots of 100 μl were collected from each fraction and assayed for ^{3}H. Fractions exhibiting ^{3}H activity were freeze-dried and subjected to N-terminal sequence analysis.

The α2(IX)COL2 preparation yielded two tritiated peptides: DEXAGGA and DEXAGGAQ (Figure 3, upper panel), where X represents the ^{3}H-labelled δ-hydroxynorvaline residue derived from the cross-link. Based on the published cDNA sequences of human type II collagen [34], these peptides represent variable pepsin cleavage products of the α1(II) N-telopeptide. The mixed α1(IX)/α3(IX)COL2 preparation yielded four tritiated peptides of distinct sequence (Figure 3, lower panel). One of these was the α1(II) N-telopeptide, DEXAGGAQ. Another, GGVGEXSGS, represents the α3(IX) C-telopeptide (i.e. NC1 domain) by identity with the bovine sequence [24]. Finally, summation of the sequences obtained from the two late peaks of ^{3}H activity, AGLGPREXGP and GPREXGPDPL, gives the sequence of the human α1(II) C-telopeptide, AGLGPREXGPDPL, which again had been variably cleaved by pepsin [34].

Protein sequencing of COL2 domains

Further aliquots of the COL2 domains partially resolved by reverse-phase HPLC (see Figure 2) were subjected to direct

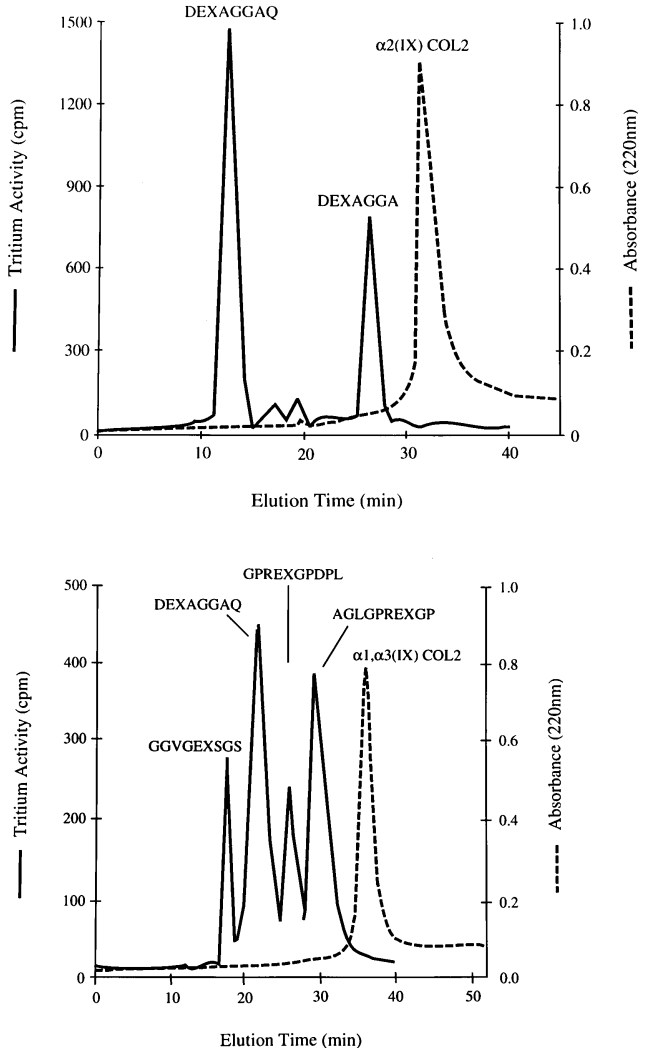

Figure 3 Reverse-phase HPLC fractionation of [^{3}H]$NaBH_4$-labelled cross-linking telopeptides

Telopeptides were released from α2(IX)COL2 (top panel) and from the mixture of α1(IX)COL2 and α3(IX)COL2 (bottom panel) by $NaIO_4$ oxidation. The C8 column (Brownlee Aquapore RP-300; 4.6 mm × 25 cm) was eluted with aqueous 0.1% (v/v) TFA at 1 ml/min for 5 min, followed by a linear gradient from 0 to 35% solvent B in solvent A over 35 min, collecting 1 ml fractions. The eluent was monitored for absorbance at 220 nm (broken line). Aliquots of 100 μl were collected from each fraction and assayed for ^{3}H activity (solid line). The N-terminal amino acid sequences, shown in single-letter code, were obtained by analysis of the indicated peak fractions. X represents the position of the ^{3}H-labelled δ-hydroxynorvaline residue that was derived from the cross-linking residue by periodate oxidation. The peptides could all be assigned in origin to type II or IX collagen telopeptide domains (see the text for details).

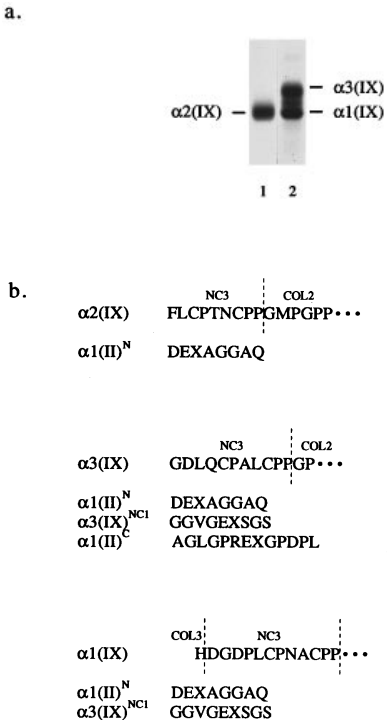

Figure 4 Direct N-terminal sequencing of electroblotted individual α1(IX), α2(IX) and α3(IX)COL2 domains

(**a**) SDS/PAGE resolved the individual COL2 chains (see the text for details). (**b**) α2(IX)COL2 yielded its own N-terminal sequence and the sequence of the α1(II) N-telopeptide to which it is cross-linked. Similarly, α3(IX)COL2 yielded the additional sequences of: (1) the α1(II) N-telopeptide, (2) α3(IX)NCl, and (3) the α1(II) C-telopeptide. α1(IX)COL2 yielded the additional sequences of the α1(II) N-telopeptide and α3(IX)NC1. X represents the cross-linked hydroxylysine residue (not detected on sequence analysis). The cross-linking sites in the COL2 helical domains themselves were not reached in these sequence analyses. The boundaries of the triple-helical domains are shown by vertical broken lines. The results indicate that in the tissue α2(IX)COL2 was linked only to the α1(II) N-telopeptide, α3(IX)COL2 was linked to an α1(II) N-telopeptide, α3(IX)NC1 or an α1(II) C-telopeptide, and α1(IX)COL2 was linked to an α1(II) N-telopeptide or α3(IX)NC1. Superscripts: N, N-telopeptide; C, C-telopeptide; NC1, C-terminal non-collagenous domain of the type IX collagen molecule.

sequence analysis after resolution on SDS/PAGE (Figure 4a). Thus column fractions containing the α2(IX)COL2 domain (lane 1) and the mixed α1(IX)/α3(IX)COL2 domains (lane 2) were pooled, freeze-dried and run on SDS/7.5%-PAGE. After electroblotting on to a PVDF membrane, direct N-terminal sequence analysis of each protein band was performed (Figure 4b). The band containing α2(IX)COL2 yielded both its N-terminal sequence and the sequence of the α1(II) N-telopeptide. The α3(IX)COL2 band yielded its N-terminal sequence, the α1(II) N-telopeptide sequence and a sequence corresponding to the C-telopeptide domain (NC1) of α3(IX). In addition, it yielded the telopeptide sequence of the α1(II) C-telopeptide. The α1(IX)COL2 band yielded its N-terminus and the sequences of the α1(II) N-telopeptide and α3(IX)NC1. The sequencing results obtained from the electroblotted proteins were confirmed by direct sequencing of freeze-dried column fractions.

These data, in combination with those obtained from C8 reverse-phase HPLC (see above), indicate that: (1) the α1(IX)COL2 helical region is cross-linked to an α1(II) N-telopeptide and α3(IX)NC1; (2) the α2(IX)COL2 helical region is cross-linked solely to the α1(II) N-telopeptide; and (3) the α3(IX)COL2 helical region, in addition to forming cross-links with the α1(II) N-telopeptide and α3(IX)NC1, is covalently cross-linked to the α1(II) C-telopeptide. The covalent interactions of type IX collagen with type II collagen and of type IX collagen with type IX collagen are illustrated schematically in Figure 5.

Western blot analysis of type IX chains

Western blot analysis of intact type IX collagen chains extracted by neutral 1.0 M NaCl or 4.0 M guanidine hydrochloride from whole human fetal hyaline cartilage was performed using a rabbit polyclonal serum raised against pepsin-digested bovine

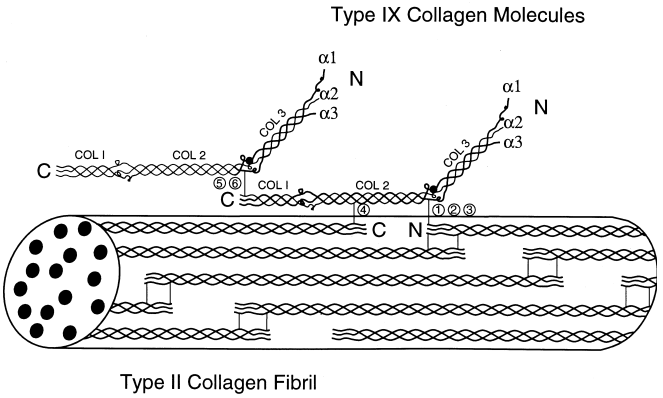

Figure 5 Schematic representation of the cross-links formed between type IX collagen molecules and between type IX and type II collagen molecules

1–3 indicate covalent bonds from the N-terminal helical region of the COL2 domain of α1(IX), α2(IX) or α3(IX) to the N-telopeptide of α1(II); 4 is the covalent bond from the mid region of α3(IX)COL2 to the α1(II) C-telopeptide; 5 and 6 are type IX to type IX covalent bonds, formed between the NC1 domain of α3(IX) and the N-terminal helical site in the COL2 domain of α1(IX) or α3(IX). Sites of cross-linking in the COL2 domains were based on previous findings with bovine cartilage [24].

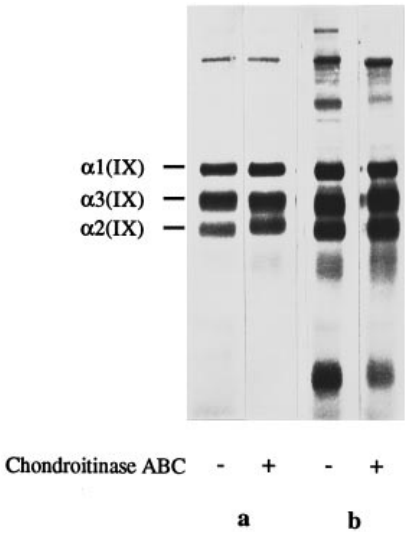

Figure 6 Western blot analysis of intact type IX collagen chains with (+) and without (−) chondroitinase ABC treatment

The intact chains were extracted from slices of fetal hyaline cartilage with neutral 1.0 M NaCl or 4.0 M guanidine hydrochloride. Following separation by SDS/7.5%-PAGE and electroblot transfer to PVDF membranes, the α chains were reacted with a rabbit polyclonal serum raised against pepsin-digested human type IX collagen. (**a**) Neutral 1.0 M NaCl extract before and after treatment with chondroitinase ABC. Whereas the α1(IX) and α3(IX) chains are unaffected, the α2(IX) chain following digestion migrates slightly more slowly and stains with a slightly greater intensity. (**b**) By contrast, chondroitinase ABC did not have a significant effect on α chains extracted with 4.0 M guanidine hydrochloride.

type IX collagen (Figure 6). The extracts were analysed with (+) and without (−) chondroitinase ABC digestion to degrade glycosaminoglycans. Treatment of the 1.0 M NaCl extract with chondroitinase ABC increased the apparent amount of the α2(IX) band and retarded it slightly (Figure 6a). Chondroitinase ABC did not have a significant effect on the type IX collagen chains in the 4.0 M guanidine hydrochloride extract (Figure 6b).

DISCUSSION

The results of the present study show that the cross-linking pattern of type IX collagen is similar in human and bovine hyaline cartilage [24]. Molecules of type IX collagen are cross-linked in the matrix to type II collagen by means of covalent bonds from the helical COL2 regions of α1(IX), α2(IX) and α3(IX) to the N-telopeptide of α1(II), and from another site in the helical region of α3(IX)COL2 to the C-telopeptide of α1(II). In addition, we have shown that type IX collagen molecules are cross-linked to one another, with covalent bonds forming between either α1(IX) or α3(IX)COL2 domains and the C-terminal NC1 domain of α3(IX).

Knowledge of how type IX collagen is cross-linked in the extracellular matrix of cartilage is relevant to an understanding of the pathogenesis of osteoarthritis. Degradation of articular cartilage by proteolytic enzymes, including stromelysin-1 (matrix metalloproteinase-3) released from chondrocytes in response to stimulation by cytokines, particularly interleukin-1, is a characteristic feature of joint destruction in osteoarthritis [35–37]. Immunohistochemical studies on human osteoarthritic cartilage have localized stromelysin-1 in superficial and transitional zone chondrocytes, with the intensity of stromelysin staining correlating directly with the histological/histochemical Mankin scores [38]. Stromelysin-1 is a well recognized proteoglycanase [39–41]. It has also been shown to cleave in the NC2 domain as well as to remove the large non-collagenous NC4 domain of type IX collagen [42]. An increase in the water content of articular cartilage with concomitant collagen network swelling has been noted as the earliest feature of the fibrillation associated with osteoarthritis [43]. This is due to the failure of a damaged collagen network in arthritic cartilage to oppose the swelling pressure of proteoglycans, and is seen in normal cartilage treated with bacterial collagenase [44]. In view of the covalent cross-linking of type IX collagen molecules with other type IX molecules and with type II collagen molecules, the action of stromelysin-1 may remove type IX collagen molecules from the surface of type II collagen fibrils, and thereby uncouple adjacent type II collagen fibrils. This may provide a specific mechanism for the reorganization of the collagen network in cartilage and, if uncontrolled in osteoarthritis, may lead to network failure.

Both a proteoglycan form having a chondroitin/dermatan sulphate side chain linked to the NC3 domain of α2(IX) and a non-proteoglycan form of type IX collagen have been identified in avian and mammalian cartilage [13–18]. We have performed Western blot analysis of intact type IX collagen chains from neutral 1.0 M NaCl and 4.0 M guanidine hydrochloride extracts of human fetal hyaline cartilage before and after chondroitinase ABC treatment. Our results suggest that the proteoglycan form is a minor component of the cartilage extracellular matrix. This is consistent with the observations of Ayad et al. [18] on bovine cartilage. Thus type IX collagen molecules with an attached glycosaminoglycan may represent a form of the molecule that has a special function, which may be unusually prominent in chick growth cartilage or be a transient intermediate. For instance, the sulphated polysaccharide may help to guide the type IX collagen molecule to the correct 'docking' site on the surface of a type II collagen fibril, after which it is cleaved to generate the final, non-proteoglycan, form of the molecule. However, this concept would require the presence of an as yet undescribed extracellular endoglycosidase.

In this context, it should also be noted that cartilage from a subject with diastrophic dysplasia showed an abnormal pattern of type IX collagen proteolysis fragments [45]. This recessively inherited condition has been linked to mutations in the gene

coding for a high-affinity sulphate transporter protein [46]. Conceivably, the consequences of the basic molecular defect may include selective undersulphation of the collagen α2(IX) glycosaminoglycan, with effects on the structure and function of the mature protein.

In conclusion, it is clear that the covalent association between type IX collagen molecules themselves as well as between type IX and type II collagen molecules is central to understanding the function of this protein in the extracellular matrix.

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