Specific glycanforms of type IX collagen accumulate in embryonic chick sterna after 17 days of development

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Type IX collagen is a key component of the extracellular matrix of cartilage where it occurs at the surfaces of type II collagen fibrils as a glycanated molecule. The function of the glycosaminoglycan (GAG) side chain of the molecule is, however, unknown. We have shown that type IX collagen in chicken sternal cartilage is synthesized with a unimodal distribution of GAG chain size, but at post 17 days of development three predominant glycanforms of type IX collagen accumulate. Such accumulation did not occur in sterna from day 15 embryos. In day 17 embryos predominant glycanforms were found in the caudal region of the sternum. By day 19 of development the three predominant glycanforms are widespread throughout the caudal and cephalic regions. The results indicate that developmental and anatomical changes occur to type IX collagen that depend on the size of the GAG chain attached to the α2(IX) chain of the molecule.

Key words: collagen/cartilage/glycosaminoglycan/development/proteoglycan

Introduction

The ability of cartilage to withstand repeated cycles of compression and relaxation can be attributed to the extracellular matrix, which comprises very long collagen fibrils surrounded by glycoproteins, proteoglycans and hyaluronan. Of special importance glycanated type IX collagen molecules occupy the surfaces of the fibrils (Eyre et al., 1987; van der Rest et al., 1988; Bruckner et al., 1988; Vaughan et al., 1988; Shimokomaki et al., 1990; Yada et al., 1990), with type IX and II collagens oriented in an anti-parallel relationship (Wu et al., 1992) and the glycosaminoglycan side chains protruding into the interfibrillar space. The role of the GAG side chain of type IX collagen in the development and structure of cartilage is unclear.

Type IX collagen belongs to the family of fibril associated proteoglycans and hyaluronan. Of special importance glycanated type IX collagen molecules in chicken cartilage, bovine cartilage and bovine vitreous contain GAG chains of 15–60 kDa (based on chondroitin sulfate and protein molecular weight standards; Yada et al., 1990; Bishop et al., 1992), whereas the type IX collagen in chicken vitreous contains GAG chains of approximately 350 kDa (based on hyaluronic acid standards; Yada et al., 1990).

Here we have found that the α2(IX) chain of chicken type IX collagen is synthesized with a GAG chain having a unimodal size distribution. In young embryos, the distribution of size remains constant 24 h after synthesis. However, at later stages of development, selective disappearance of certain type IX collagen molecules occurred resulting in the accumulation of three predominant populations of type IX collagen molecules having small, medium, and large GAG chains. The accumulation of these specific glycanforms occurs immediately prior to hatching of the chick embryo, indicating a possible structural role in stabilizing the cartilage extracellular matrix.

Results

Separation of type IX collagen glycanforms

The majority of radiolabeled proteins extracted from the sternal matrix into buffered 1 M NaCl did not bind to a column of DEAE cellulose under the conditions used here (Figure 1, peak A). Bound proteins were eluted on a linear gradient of NaCl in four peaks (Figure 1, peaks B–E). The contents of each peak were examined using SDS–PAGE and fluorography. The identity of type IX collagen in the eluted fractions was confirmed by Western blot analysis using a polyclonal antibody which recognised the NC4 domain of chicken type IX collagen (Douglas et al., 1998). Type II collagen and the small amounts of type IX collagen that lacked a GAG chain (COLIX) were present in the nonadsorbed fractions (data not shown). Glycanforms of type IX collagen (COLIX+) eluted in peaks C, D and E (Figures 1 and 2). To confirm that the type IX collagen glycanforms differed only in respect of their GAG chain, samples of each glycanform were treated with chondroitinase ABC lyase and the products separated by SDS–PAGE. Removal of the GAG chain caused the α2(IX) chain of all three glycanforms to migrate to the same position in SDS-gels (Figure 2).

In order to determine the relative amounts of type IX collagen in peaks C, D, and E, the chains of type IX collagen were separated by SDS–PAGE (under reducing conditions) and the α3(IX) chains quantitated using a phosphoimager. Relative intensities of α3(IX) (dotted line in Figure 1) corresponded precisely with the radioactivity profile of the DEAE chromatogram (solid line in Figure 1) and confirmed that the amount of radioactivity in peaks C, D, and E in the DEAE chromatogram was a measure of the relative abundance of the three glycanforms. The results showed that there was an unequal ratio of the three
Characterization of the disaccharide composition of type IX collagen glycanforms

We considered the possibility that the differences in migration of \( \alpha_2(IX)^* \) chains in SDS-gels arose from differences in sulfation levels of the chondroitin sulfate disaccharides, which comprised the GAG chain of the molecule. The disaccharide compositions of type IX collagen GAG chains from peaks D and E were determined. The relative percentages of chondroitin-4-sulfate and chondroitin-6-sulfate were 85% and 15% respectively, for both peaks D and E (data not shown). Nonsulfated and multisulfated forms of chondroitin were not detected. The results showed that the composition of the GAG chains of type IX collagen from peaks D and E were indistinguishable.

The type IX collagen glycanforms differed in the hydrodynamic volumes of the GAG chain

Type IX collagen molecules from peaks C, D, and E were treated with bacterial collagenase and the released GAGs examined by gel filtration chromatography (Figure 3). GAG chains from type IX collagen in peaks C, D, and E eluted from a Superdex 200 FPLC column in 14 ml, 13 ml, and 12 ml, respectively, of equilibration buffer. For descriptive purposes, estimates of GAG molecular weights (compared with globular protein standards) showed that those in peaks C, D, and E were 79 kDa, 145 kDa, and 308 kDa, respectively.

Selective degradation of some glycanforms

To examine the biosynthesis of the predominant glycanforms, pulse-chase experiments were performed as described in Materials and methods and radiolabeled type IX collagen molecules were extracted from the sterna and chromatographed on columns of DEAE cellulose. At the end of the pulse (0 h), the 6 h chase and the 12 h chase, type IX collagen eluted from columns as a single peak (Figure 4). Type IX collagen from the 24 and 48 h chase incubations eluted as three separate peaks (Figure 4, lower panels). Quantitation of radiolabeled protein showed that frank loss of protein had not occurred at 24 h post labeling. That is, the peak heights of type IX collagen glycanforms were approximately 2000 c.p.m./200 \( \mu l \) at 0 h, 12 h, and 24 h (Figure 4). At 48 h, the peak heights had fallen to 1000 c.p.m. indicative of overall degradation. Thus, the appearance of the separate peaks of type IX collagen was consistent with selective disappearance of certain glycanforms of type IX collagen.

Accumulation of specific glycanforms of type IX collagen is developmentally regulated

To learn more about the biological relevance of the predominant glycanforms, we determined their occurrence during development of the sterna. Chick sternal cartilage was dissected from 12 dozen embryos at days 15, 17, and 19 days of development and cultured for 30 h in the presence of \( \beta \)APN and a mixture of U-L-\(^{14}\)C amino acids. The mass of the sterna at 15, 17, and 19 days of development were 7.2 \pm 2.2, 14.8 \pm 1.6, and 19.2 \pm 4.8 mg, respectively. The length of the sterna at 15, 17, and 19 days of development were 10.5 \pm 1.0, 12 \pm 0.8, and 14 \pm 0.8 mm, respectively. Alizarin red staining of the sterna showed that mineralization of the extracellular matrix was beginning in the cephalic region of sterna from 17 day embryos and was more advanced by the end of the 30 h culture duration of the experiment (equivalent to a total developmental age of 18.25 days). In sterna harvested from 19 day embryos, mineralization was readily

glycanforms: peak C accounted for 11% and peaks D and E accounted for 44% and 45%, respectively, of the glycanated type IX collagen extracted from the sterna.
Fig. 3. Gel filtration chromatography of free GAG chains. The $^{35}$S-labeled GAGs were released from the three predominant glycanforms by bacterial collagenase, mixed with globular protein standards and applied to a column of Superdex 200 for FPLC gel filtration chromatography. Disaccharides and GAGs eluting from the column were detected by liquid scintillation counting. Bold lines, without treatment with chondroitinase ABC lyase; dotted lines, after treatment with chondroitinase ABC lyase. The peak eluting late in the profile corresponded to digested collagenous peptides (bold lines) and disaccharides (dotted lines). The peak appearing in the void volume of the column (Vo) corresponded to material resistant to collagenase and chondroitinase ABC lyase. Vt, Total included volume of the column; C, position at which GAGs from peak C (see Figure 1) eluted; D, position at which GAGs from peak D (see Figure 1) eluted; E, position at which GAGs from peak E (see Figure 1) eluted. The positions at which molecular weight (kDa) standard proteins eluted are shown at the top of the diagram. The standards were alcohol dehydrogenase, bovine serum albumin, cytochrome C, and vitamin B12.

Fig. 4. DEAE-cellulose chromatography of biosynthetically labeled matrix type IX collagen glycanforms. Pulse-chase experiments were performed on sterna from 17 day chick embryos as described in Materials and methods using U-L-$^{14}$C-amino acids and Na$_2^{35}$SO$_4$ for 4 h and chased for up to 48 h. Proteins extracted from the extracellular matrix of sterna chased at 0 h, 6 h, 12 h, 24 h, and 48 h post label were applied to columns of DEAE-cellulose and nonbound proteins eluted. Bound proteins were eluted on a linear gradient of 0–400 mM NaCl and fractions of 10 ml collected. Radioactivity in each fraction containing type IX collagen was assayed by liquid scintillation counting (bold lines). The relative radioactivity in α3(IX) chains of type IX collagen was determined by phosphoimaging of SDS-gels (dotted lines). Bins in the 24 h and 48 h samples indicate the predominant glycanforms.

apparent in the caudal region at the start of the experiment. The sternae were bisected at the midline and the proteins in the cephalic and caudal regions extracted separately into buffered 1 M NaCl. Type IX collagen in the two half sternae were chromatographed separately on columns of DEAE cellulose. Type IX collagen from the cephalic and caudal regions of day 15 sterna (and incubated for a further 30 h in culture, data not shown) and from the cephalic region of day 17 sterna (incubated for a further 30 h; Figure 5)
eluted from the columns in a symmetric peak of protein, indicating a unimodal distribution of GAG chain size (data not shown). We noted that the type IX collagen from the caudal region of day 17 sterna contained the two most abundant predominant populations of glycanforms that were seen in earlier experiments (Figure 5). At day 19 of development (plus 30 h of culture), both the cephalic and caudal regions of the sterna contained the 3 predominant populations of glycanforms (Figure 6).

**Discussion**

The present study made three observations concerning the size of the GAG chain on type IX collagen molecules in embryonic chick sternae. First, the type IX collagen was synthesized as a broad range of glycanforms that differed in the size of their GAG chain. This shows that the biosynthetic apparatus and the secretory pathway in embryonic chondrocytes is relatively insensitive to the size (length) of the GAG chain of the molecule. Second, at stages post 17 days of development, the population of glycanforms in the cartilage underwent major changes in that some glycanforms predominated while others disappeared, possibly by degradation. Third, the accumulation of the three specific glycanforms begins in the caudal region of the cartilage at day 17 and spreads throughout the sternum prior to hatching.

A possible explanation for the accumulation of the three glycanforms was that the unimodal distribution of GAG size changed to the trimodal distribution by selective, rather than indiscriminate, loss of type IX collagen glycanforms. This was shown in pulse-chase experiments in which the peak of $^{14}$C/$^{35}$S-protein remained unchanged but troughs appeared in the protein distribution, indicative of selective loss of protein. Nuclear magnetic resonance spectroscopy, computer simulations, and rotary shadowing electron microscopy suggest that GAGs have regular secondary structures and can undergo self-interactions to form duplexes (Scott et al., 1992). The three specific glycanforms might, therefore, accumulate in the sternal cartilage because they form stable interactions either with proteoglycans or other type IX collagen molecules. It is noteworthy that type IX collagen molecules have been observed at sites where collagen fibrils intersect (Mullerglauser et al., 1986).
At day 18 of gestation the chick sternum undergoes a developmental change when the cephalic region begins to mineralize (Lu Valle et al., 1992). The appearance of predominant glycanforms in whole sterna at day 17 suggested to us that their appearance might coincide with onset of mineralization. However, we showed that the predominant glycanforms first appeared in the nonmineralized caudal region of the day 17 sterna following 30 h in culture. The predominant glycanforms were only found in the cephalic region in day 19 sterna, which is approximately 48 h after the start of cephalic mineralization. Therefore, although the sternum is undergoing an important developmental change at a time when predominant glycanforms are detected, the two events appear to be unrelated. These observations are consistent with there being a distinct population of nonhypertrophic cartilage cells in the caudal region of 17 day embryonic sterna, and later throughout the sterna, which are responsible for the accumulation of the 3 glycanforms of type IX collagen.

The embryonic chick sternum is a rapidly developing structure, especially during late embryogenesis. The sternum, together with other thoracic structures in late embryogenesis, must be able to withstand the attachment of flight muscles and in turn the stresses they exert when the chick begins to move its wings. The predominant glycanforms might therefore have a role in fine-tuning the biomechanical properties of the sternum, in binding growth-factors involved in chondrocyte differentiation, or in the adhesion of chondrocytes to collagen fibrils (for review, see Hardingham and Fosang, 1992). Another possible function of specific glycanforms might be to regulate interfibrillar distance, in which hydrated glycancated type IX collagen molecules generate a “cylinder of hydration” around the fibril.

Materials and methods

Materials

Uniformly labeled \(^{14}\text{C}\)-l-aminos, Na\(^{2}\text{35SO}_{4}\), \(^{3}\text{H}\)-glucosamine, and \(^{14}\text{C}\)-formaldehyds were purchased from NEN Research Products, Dulbecco’s modified Eagles medium from Seralab, DEAЕ cellulose pre-swollen microgranular anion exchanger DE52 from Whatman Biosystems Ltd., extra fine P-2 Gel from Bio-Rad, and chondroitinase ABC lyase and pre-stained protein molecular weight standards from Sigma. All other chemicals were of reagent grade (Sigma and BDH).

Organ culture of chicken embryo sternum

Typically, sterna of 50 dozen embryos were dissected free of perichondria and washed in modified Kreb’s solution (111 mM NaCl, 5.4 mM KCl, 1.3 mM KH\(_{2}\)PO\(_{4}\), 1.3 mM MgCl\(_{2}\)-6H\(_{2}\)O, 1.8 mM CaCl\(_{2}\)-2H\(_{2}\)O, 4.0 mM NaHCO\(_{3}\), 12.5 mM Na\(_{2}\)HPO\(_{4}\), 3.1 mM NaH\(_{2}\)PO\(_{4}\), 0.8 mM MgSO\(_{4}\), and 13 mM glucose). The sterna were incubated at 37 °C in 200 ml of DMEM supplemented with 1 μCi/ml of U-L-\(^{14}\text{C}\)-amino acids, 10 μCi/ml of Na\(^{2}\text{35SO}_{4}\), 64 μg/ml BAPN, 50 μg/ml ascorbic acid, 100 μg/ml penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine. After 16 h the culture medium was decanted and a further 200 ml of fresh supplemented DMEM was added containing the radiolabeled amino acids and Na\(^{2}\text{35SO}_{4}\). The sterna were incubated for a further 7 h. The culture medium was decanted and the sterna incubated with 100 ml of supplemented DMEM for 16 h, and the sterna and culture medium were stored separately at -80°C. In earlier experiments we examined the use of tritiated amino acids (both mixtures of all amino acids and high specific activities of proline alone) but noticed that the type IX collagen produced under these conditions was more difficult to monitor during subsequently purification of the protein. The mixture of U-L-\(^{14}\text{C}\)-amino acids resulted in the biosynthetic labeling of type IX collagen that was readily examined by SDS-PAGE and autoradiography.

Isolation of type IX collagen from the sternal matrix

All procedures were carried out at 4 °C and the methods were based on previous published work (Bruckner et al., 1985). Approximately 50 dozen metabolically radiolabeled 17 day chick embryo sterna were powdered under liquid nitrogen in a stainless-steel mill and extracted for 4 × 24 h with 4 × 100 ml of a 50 mM Tris–HCl (pH 7.4 at 20°C) buffer containing 1 M NaCl and a protease inhibitor cocktail (25 mM EDTA, 0.02% sodium azide, 1 mM p-amino benzoamide, 10 mM N-ethylmaleimide, and 1 mM phenylmethylsulfonyl fluoride). Proteins were recovered from the extraction buffer by precipitation with ammonium sulfate (176 mg/ml) and centrifugation at 17,000 × g for 90 min. The pellet was redissolved in 50 ml of a 50 mM Tris–HCl (pH 7.4 at 20°C) buffer containing 400 mM NaCl, 0.2% Triton X-100, and 0.02% sodium azide (buffer A), and the insoluble debris removed by centrifugation at 27,000 × g for 30 min. The resulting supernatant was dialyzed against 2 × 50 volumes of a 50 mM Tris–HCl (pH 7.4 at 20°C) buffer containing 0.2% Triton X-100, 2 M urea, and 0.02% sodium azide (buffer B) for a total of 24 h.

Chondroitinase ABC lyase digestion

Samples were dialyzed at 4°C against 2 × 1000 volumes of a 0.1 M Tris–HCl (pH 8.0 at 20°C) buffer containing 0.04 M sodium acetate, 25 mM EDTA, 0.02% sodium azide, and 10 mM N-ethylmaleimide. Digestions were carried out at 37°C for 4 h with 0.2 U/ml of chondroitinase ABC lyase.

SDS-PAGE, fluorography and phosphoimaging

Proteins were separated by discontinuous SDS–PAGE (7% separating gel and 3.5% stacking gel) and the fluorography performed by exposing dried gels to preflashed Kodak XAR film at -70°C. To determine the relative amounts of the glycanforms of type IX collagen eluted from the ion-exchanger, proteins were separated by discontinuous SDS–PAGE and the dried gels exposed to a Fuji phosphoimaging plate and processed using a Fuji Bas 2000 image analyzer. The intensities of bands were measured using Millipore whole band analyzer software.

Quantitation of type IX collagen glycanforms

The relative amounts of the glycanforms of type IX collagen eluted from the DEAЕ-cellulose were estimated from the intensity of the α3(IX) chain after SDS–PAGE and phosphoimaging of the individual chains of the molecule. The α3(IX) chains were selected for quantitation for three reasons. First, the intensity of the α2(IX)β chain was an unreliable measure of the quantity of type IX collagen since the intensity was made up of contributions from \(^{14}\text{C}\) and \(^{35}\text{S}\). Second, measurement of α1(IX) chains was
not always possible because the α2(IX)* chain migrated too close to the α1(IX) chain to allow reliable peak separation. In additional experiments, not described here, we showed that measurement of the α1(IX) chain was a valid approach for quantifying the type IX collagen as long as the α2(IX) chain did not migrate close to or along with the α1(IX) chain. Third, in all the glycanforms examined the α2(IX) chains migrated slower than the α3(IX) chain.

Estimation of GAG chain size by gel filtration chromatography

Glycanforms corresponding to peaks C, D and E (see Figure 1) were concentrated by ultrafiltration on an Amicon YM30 membrane. One ml samples were dialyzed at 4°C against 2 x 1000 volumes of a 100 mM Tris–HCl (pH 7.4 at 20°C) buffer containing 2 mM CaCl₂, 20 mM N-ethylmaleimide and 0.2% Triton X-100. The samples were incubated at 37°C for 4 h with 70 U/ml of bacterial collagenase. Following digestion, the samples were clarified by centrifugation at 15,000 × g for 10 min. One hundred-fifty microfilters of the supernatant (containing the GAG chains) was added to a mixture of unlabeled protein molecular weight standards (50 µl of a mixture of alcohol dehydrogenase (150 kDa, 2.5 mg/ml), bovine serum albumin (66 kDa, 5 mg/ml), cytochrome C (12.4 kDa, 1 mg/ml), and vitamin B₁₂ (1355 Da, 0.1 mg/ml)). A chondroitinase ABC lyase digestion (described above) was performed on the remaining supernatant (collagenase digested). The chondroitinase ABC digested samples were clarified by centrifugation at 15,000 × g for 10 min. One hundred-fifty microfilters of the supernatant (containing the disaccharide units) was added to a mixture of unlabeled protein molecular weight standards (50 µl). The collagenase digested and collagenase + chondroitinase ABC lyase digested samples containing the protein standards were applied at a flow rate of 250 µl/min to a column of Superdex 200 gel filtration resin (Pharmacia FPLC) preequilibrated with 50 mM Tris–HCl (pH 7.4 at 20°C) buffer containing 400 mM NaCl and 0.02% sodium azide, and 500 µl fractions were collected. Proteins eluting from the column were detected by absorbance at 280 nm by an in-line UV monitor. Radioactivity in fractions was determined using a liquid scintillation counter. The reproducibility of the column was confirmed when protein standards were eluted by the same volume of buffer in seven consecutive runs. A calibration plot was generated for each run by plotting log (protein molecular weight) against eluted volume (ml). The Kav values were calculated for each GAG chain using the equation $K_{av} = (V_e - V_o)/(V_t - V_o)$, where $V_o$ = void volume of the column (8 ml), $V_e$ = elution volume of the GAG, and $V_t$ = the geometric bed volume (24 ml). An estimation of the MW of the GAG chains were determined by comparing the Kav values with a previously published standardization of Kav values against MW using Sephadex 200 gel filtration (Wasteson, 1971).

Disaccharide analysis

Sterna from 600, 17 day old chicken embryos were dissected free of perichondria and washed immediately in modified Kreb's buffer (described above) supplemented with 64 µg/ml βAPN, 50 µg/ml ascorbic acid, 100 µg/ml penicillin G, and 100 µg/ml streptomycin. The sterna were incubated at 37°C for 4 h in 50 ml of modified Kreb's buffer (without 0.8 mM MgSO₄) supplemented with 10 µCi/ml of a U-L-¹⁴C-amino acids, 124 µCi/ml of Na₂⁻¹⁵SO₄, 64 µg/ml βAPN, 50 µg/ml ascorbic acid, 100 µg/ml penicillin G, and 100 µg/ml streptomycin. The sterna were separated from the buffer and 5 dozen sterna immediately frozen in liquid nitrogen. This sample was the t = 0 h sample in subsequent experiments. The remaining 20 dozen sterna were sorted into four groups of 5 dozen and each group was incubated separately at 37°C in 20 ml of DMEM supplemented with 64 µg/ml βAPN, 50 µg/ml ascorbic acid, 20 mM l-proline, 100 µU/ml penicillin G, 100 µg/ml streptomycin, and 2 mM l-glutamine, for 6, 12, 24, and 48 h, respectively. At each time point the sterna were separated from the culture medium and immediately frozen in liquid nitrogen. Proteins were extracted from the sternal matrix and the radiolabeled proteins separated by DEAE cellulose chromatography as described above.

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Abbreviations

GAG, glycosaminoglycan; β-APN, 3-aminopropionitrile fumarate; DEAE, diethylaminoethyl; PAGE, polyacrylamide gel electrophoresis; COLIX and COLIX, type IX collagen with and without a GAG chain, respectively; α2(IX)* and α2(IX), the alpha two type IX collagen chain, with and without a GAG chain, respectively.
References


