D-Periodic Distribution of Collagen Type IX along Cartilage Fibrils

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Abstract. It has recently become apparent that collagen fibrils may be composed of more than one kind of macromolecule. To explore this possibility, we developed a procedure to purify fibril fragments from 17-d embryonic chicken sternal cartilage. The fibril population obtained shows, after negative staining, a uniformity in the banding pattern and diameter similar to the fibrils in situ. Pepsin digestion of this fibril preparation releases collagen types II, IX, and XI in the proportion of 8:1:1. Rotary shadowing of the fibrils reveals a d-periodic distribution of 35–40-nm long projections, each capped with a globular domain, which resemble in form and dimensions the amino-terminal globular and collagenous domains, NC4 and COL3, of type IX collagen. The monoclonal antibody (4D6) specific for an epitope close to the amino terminal of the COL3 domain of type IX collagen bound to these projections, thus confirming their identity. Type IX collagen is therefore distributed in a regular d-periodic arrangement along cartilage fibrils, with the chondroitin sulfate chain of type IX collagen in intimate contact with the fibril.

A major question in cell biology is how individual macromolecules combine to form the often large and complex supramolecular structures of the extracellular matrix. Approaches to this problem include the direct microscopic visualization of tissue sections aided by chemical stains and immunological tools, reconstitution and binding studies with purified components in solution followed by analysis of the resulting products, and, where possible, direct x-ray analysis of highly ordered arrays in situ.

This strategy is particularly well exemplified by the many detailed studies of collagen fibrils and fibrillogenesis. This work has resulted in an understanding of the interactions that govern lateral association of fibrillar collagens (for recent reviews see references 3, 7, and 19). The quarter stagger model that arose from these studies has been considerably refined since its introduction, but still remains as the cornerstone of current models as it explains how fibrils formed in vitro from purified collagen molecules give rise to the characteristic staining patterns observed in the electron microscope. Although the fibril staining patterns produced in vitro match those seen in vivo, the diameter regulation of collagen fibrils in vivo and their complexity, including association with other components, especially proteoglycans, are features not reproduced by mixing solutions of collagens in vitro. In summary, two critical questions remain: how is the morphogenesis of fibrils regulated in vivo and what role might other molecules play in these processes?

This problem is particularly well illustrated in cartilage. The morphology of fibrils reconstituted from purified type II collagen in solution is vastly different from that of cartilage fibrils in situ. Large tactoidal aggregates with d-periodic staining patterns are formed under appropriate reconstitution conditions (21). In contrast, fibrils from chicken embryo sternal cartilage (for example) are long cylindrical structures with a uniform diameter of ~20 nm. This discrepancy has stimulated the search for additional components regulating fibril assembly. Although a number of proteins and proteoglycans have been shown to interact with type II collagen in vitro (6, 25, 33) there is to date little proof that these are the regulatory components in vivo. On the other hand, that proteoglycans and collagen fibrils are intimately associated can be seen from ultrastructural studies of the cartilage matrix (13). In addition, it has been proposed that proteoglycans are distributed in a periodic manner along type I collagen fibrils in various tissues, including annulus fibrosus (27), rat tail tendon, and cornea (26).

Cartilage also contains collagen types IX and XI, each of which must be an integral part of the fibrillar network, but whose roles and exact ultrastructural distributions are unknown. It is quite conceivable that collagen types II, IX, and XI interact to form cartilage fibrils. Certainly, type IX collagen has been detected on fine nonstriated fibrils in the pericellular matrix of fetal calf cartilage (10) and has been reported localized to the intersections of type II collagen fibrils in chicken embryo sternum (24). Evidence has also been presented for the existence of mixed fibrils of collagen types I and III (16) and collagen types I and V (2, 22), with type V collagen reportedly able to regulate the diameter of type I collagen fibrils formed in vitro (1).

Here we have isolated collagenous fibrils from cartilage and have analyzed them by a combination of protein–chemical, immunological, and ultramicroscopical methods. This
work demonstrates that a preparation of fibril fragments from cartilage contains collagen types II, IX, and XI and that type IX collagen is distributed d-periodically along these fibrils. The results illustrate the complexity of the collagen fibril in situ and raise a number of questions as to how its synthesis might be controlled.

Materials and Methods

Extraction, Isolation, and Purification of the Fibrils

Sternal cartilages were dissected from 17-d-old chicken embryos and the adherent perichondria carefully removed. All subsequent steps were performed at 4°C. The cleaned cartilages were homogenized with a Polytron, followed by extraction for 20 h in a 1 M NaCl solution buffered to pH 7.4 with 0.05 M Tris-HCl containing a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 M 6-aminohexanoic acid, 10 mM N-ethylmaleimide, and 10 mM disodium EDTA). The resulting suspension was centrifuged at 27,000 g for 30 min and the clear supernatant recentrifuged at 115,000 g for 2 h. The final pellet obtained was resuspended by vigorous vortexing in either the 1 M NaCl buffer described above or in an 8 M urea solution containing 150 mM NaCl and buffered to pH 7.4 with 0.05 M Tris-HCl and then recentrifuged at 115,000 g for 2 h. This procedure was repeated twice to obtain the cartilage fibril fragments free of single collagen molecules. For enzyme digestions, the final fibril pellet was resuspended in an isotonic Tris buffer, pH 8.0.

Enzymatic Digestions

Trypsin. To the fibril suspension in a 0.05 M Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl, trypsin was added to give a final concentration of 0.1 mg/ml and the digest incubated at room temperature with continuous stirring for 4 h. Afterwards the digest was centrifuged at 115,000 g for 2 h at 4°C. The pellet contains the trypsinized fibrils.

Pepsin. The fibril preparation, either directly or after trypsinization, was resuspended in a buffer of 0.5 M acetic acid, 0.2 M NaCl titrated to pH 2.0 with HCl, recentrifuged at 115,000 g for 2 h at 4°C, and resuspended in the above buffer. Pepsin was added to give a final concentration of 0.5 mg/ml and the digestion mixture incubated with continuous stirring at 4°C overnight. The digest was neutralized with crystals of Tris and centrifuged at 115,000 g for 2 h at 4°C. The supernatant contained the pepsin-resistant domains of the fibril collagens.

SDS-PAGE

Gradient gels of 4–10% wt/vol polyacrylamide were prepared and run using standard methods (20).

Scanning of the Gels

The Coomassie Blue-stained gels were scanned at λ = 560 nm on a Shimadzu Chromatogram Densitometer CD 50 (Desaga GmbH, Heidelberg, Federal Republic of Germany). In the collagen concentration range used, staining is a linear function of the amount of collagen applied to the gel. Estimations were based on scanning the α1(II) band for type II collagen, the high molecular weight (HMW) band for type IX collagen, and the α1(XI) band for type XI collagen, with appropriate corrections to obtain the respective collagen amount. The HMW bands of β and γ mobility, cross-linked collagen chains, were excluded from the estimations.

Amino Acid Analysis

Amino acid analysis of gas phase hydrolyzed samples was performed according to the dimethylaminobenzene sulfonyl chloride (DABS-CI) method (17). Separation of the derivatized amino acids was by high performance liquid chromatography using a Nucleosil 5-100-C18 reverse phase column (Macherey and Nagel, Oensingen, Switzerland).

Electron Microscopy

Negatively Stained Specimens. Aliquots of the purified cartilage fibril suspension were spotted onto sheets of Parafilm. Carbon-coated 400-mesh copper grids were floated on the drops for 1 min to absorb the fibrils, then
removed and excess suspension drained away with filter paper. The grids with the absorbed fibrils were then floated on a drop of a 2% uranyl acetate solution for 5 min, removed, drained of excess solution, and air-dried.

Rotary Shadowed Specimens. Purified fibrils were dialyzed against several changes of 0.2 M ammonium bicarbonate. Aliquots, either directly or after incubation with mAbs 4D6 or 2C2 for 1-2 h at 25°C, were mixed 1:1 vol/vol with glycerol and immediately sprayed onto freshly cleaved mica. After drying under a vacuum of approximately 10⁻⁷ torr for 3-5 h, the fibrils were shadowed at 9° with platinum/carbon and then at 90° with a supporting film of carbon. This film was then floated onto water and picked up on 400-mesh copper grids. Transmission electron micrographs were prepared. The contour lengths of structures in the micrographs were measured using a graphics tablet (Kontron Elektronik GmbH, Zürich).

Monoclonal Antibodies. The 2C2 and 4D6 mAbs were prepared and characterized as previously described (14).

Results

The low speed centrifugation of a 1-M NaCl extract of chicken embryo sternum effectively removed tissue debris. Centrifugation of the clear supernatant at high speed produced a noticeable pellet, which by repeated washing in 8 M urea and centrifugation was freed from individual collagen molecules as judged by SDS-PAGE (see Fig. 2, lane 1). The final pellet was then resuspended in an isotonic Tris buffer, pH 8.0, and examined in the electron microscope after negative staining (Fig. 1). Fragments of fibrils, with a periodic staining pattern of 65 nm and a highly uniform diameter of 16.7, SD 1.1 nm (n = 62) were observed. Very rarely, thicker fibrils were also present. Crude fibril preparations, examined before extensive washing were similar (not shown). The uniform appearance and dimensions of the thin fibrils closely resembled the in situ stained fibrils of 17-d embryonic chicken sternal cartilage (24).

The composition of these fibrils was examined on SDS-PAGE after proteolytic digestion with pepsin. Fibrils were either treated with pepsin directly or after incubation with trypsin to remove contaminating proteins and proteoglycans that might complicate the analysis by scanning of the stained gels. Under our conditions, trypsin treatment neither affects triple helical domains and nor does it change the appearance of the negatively stained specimens in the electron microscope. The washed (Fig. 2, lane 1) and also the trypsinized fibrils (lane 2) show a Coomassie Blue-stained material that does not enter the stacking gel, as would be expected for covalently cross-linked collagenous fibrils. Pepsin treatment caused this material to disappear with the concurrent appearance of bands with mobilities corresponding to the peptic fragments of collagens type II, type IX (HMW and LMW), and type XI (Fig. 2, lane 3). Scanning of the gel followed by a correction for the differing molecular weights of the collagens types II, IX, and XI gave a molar ratio of 8:1:1, respectively. The fibrils resist solubilization not only in 1 M NaCl or 8 M urea but also in 0.5 M acetic acid, the latter normally used to dissolve collagens, indicating the presence of covalent cross-links stabilizing the fibrils. Indeed, to release >90% of the collagens from the fibrils, relatively high amounts of pepsin were necessary (see Fig. 2, lane 3). The efficiency of pepsinization was confirmed by amino acid analysis, which otherwise relatively rigid collagenous portion, are notable features also visible in the molecules on the fibril surface. Where partial dissociation of the fibril has occurred (*), the individual molecules of type IX collagen appear to be aligned along the fibril. Bar, 100 nm.

Figure 3. Rotary-shadowed cartilage fibril. The fibril in this electron micrograph appears to be sheathed in type IX collagen (inset). The globular amino-terminal domain (1) and a short noncollagenous domain (NC3), which gives rise to a prominent kink (2) in the
Figure 4. Model showing the major features of type IX collagen. Three collagenous regions (COL1-3) and four noncollagenous domains (NC1-4) combine to form a molecule (28) that measures \( \sim \)190 nm in rotary-shadowed micrographs (15). The glycosaminoglycan chain is attached to the NC3 polypeptide (11, 18) at the NC3 domain (12, 15, 23). The sites of the epitopes for the 4D6 (Δ) and 2C2 (▲) mAbs are indicated.

revealed that 95% of the fibril hydroxyproline was solubilized by this procedure.

The fibrils were also examined in the electron microscope after rotary shadowing with platinum/carbon (Fig. 3). Especially noteworthy in these micrographs were the regularly spaced projections, each capped with what appears to be a globular domain. The dimensions and appearance of these projections closely resemble the amino-terminal globular (NC4) and collagenous (COL3) domains of type IX collagen (see Fig. 3, inset, and Fig. 4). Where partial dissociation of the fibril has occurred, the individual molecule appears to be aligned along the fibril.

To test whether these projections are indeed the amino terminal domains of type IX collagen, we used two monoclonal antibodies, 2C2 and 4D6, specific for type IX collagen (14). Both these antibodies were raised against the collagenous domains COL3 and COL2 (together comprising HMW) generated through pepsin cleavage of type IX collagen. The mAb 2C2 bound close to the carboxyl-terminal of COL2 and mAb 4D6 bound to an epitope near the amino-terminal end of COL3 (Fig. 4). Because of the proximity of the epitope of 4D6 to the amino-terminal globular domain (NC4) of type IX collagen, the antibody could be partly obscured by the amino-terminal domain. For this reason, a control panel of purified type IX collagen molecules, rotary shadowed after incubation with the 4D6 mAb, is presented for comparison in Fig. 5.

Incubation of the fibrils with the 4D6 mAb followed by rotary shadowing led to images such as those shown in Fig. 6. Here the 4D6 mAbs can be clearly seen binding close to the globular domains of the projections. The frequency of binding was \( \sim \)25%, consistent with the frequency observed with the 4D6 mAb on purified type IX collagen. This somewhat low frequency most likely reflects a partial steric hindrance by the amino-terminal domain of antibody binding to the epitope. Conversely, after incubation of the fibrils with the 2C2 mAb, only irregular binding could be observed, although the same antibody bound at a high frequency to individual type IX collagen molecules. Thus it would appear that the 2C2 mAb epitope is not readily available in the fibril, but, as expected, in regions of partial fibril dissociation increased binding of the 2C2 mAb was detected (not shown).

In an attempt to quantitate the relative binding of mAbs 2C2 and 4D6 to the fibrils, we measured in micrographs of rotary-shadowed specimens the orthogonal distance, relative to the fibril axis, of all antibodies from the outside edge of the fibrils. These results are presented in the histograms shown in Fig. 7. Whereas the 2C2 mAbs are randomly distributed, the 4D6 mAbs show a clear peak at 30–40 nm from the fibril. This corresponds to the length of the projections, measured from the middle of the amino-terminal globular domain to the edge of the fibril of 35–40 nm (Fig. 8).

The distribution of type IX collagen along the fibrils was analyzed (Fig. 9). Histograms were prepared of the distance between adjacent and clearly distinct projections of type IX collagen. Poorly resolved projections were not included in the data, a factor that would naturally bias the sample analyzed towards type IX collagen molecules projecting laterally from the fibril edge. This distribution centers on a broad peak at \( \sim \)65 nm, with smaller broad peaks at 130 and 195 nm. This periodicity corresponds to the “d-stagger” distance in fibrils of type II collagen of 67 nm (8, 21). Two additional observations are also noteworthy. Two type IX collagen projections frequently appeared to emanate from either the same point on one side of the collagen fibril (see, e.g., Fig. 5) or directly opposite on the other side of the fibril. On the other hand, projections from intermediate points within the d-period were relatively infrequent.

Discussion

The direct analysis presented here of isolated collagen fibril fragments from cartilage gives new insights into fibril architecture and structure. The uniformity in dimensions and

Figure 5. Rotary-shadowed micrographs of type IX collagen molecules after incubation with the 4D6 mAb. The 4D6 mAb ranges in appearance from triangular to globular and can be seen binding close to the amino-terminal globular domain, which is occasionally obscured. Bar, 100 nm.
appearance of 17-d chicken embryo sternal cartilage fibrils (24) facilitated both the ultrastructural analyses and the purification of fibril fragments. Biochemical evidence establishing the presence of collagen types II, IX, and XI in the isolated fibril population is forthcoming from peptic digestion of the fibrils. Quantification of the Coomassie Blue-

stained peptic fragments showed, as expected, that type II collagen makes up some 80% of the total collagen in the fibrils. Collagen types IX and XI contributed the remainder in approximately equal amounts. This does not provide proof that all three collagens coexist in the one fibril. This can only be established from ultrastructural analyses, shown here for type IX collagen and in a subsequent manuscript, to be published elsewhere, for type XI collagen.
The new approach developed here of rotary shadowing on isolated fibril fragments has enabled us to directly establish the ultrastructural distribution of type IX collagen. The identification of collagen type IX in the fibril is based on appearance, dimensions, and immunological identity utilizing a specific monoclonal antibody. The projections from the cartilage fibrils with their relatively rigid rodlike section capped by a terminal globular domain are closely comparable in form and dimensions with the amino terminal collagenous (COL3) and noncollagenous (NC4) domains of type IX collagen. This assignment is confirmed immunologically by the specific binding of the mAb 4D6, which binds both at the amino-terminal end of the COL3 domain of type IX collagen and at the corresponding position in the fibril projections. The evidence that the long arm of type IX collagen, including collagenous domains 1 and 2 (COL1 and COL2) lies along the surface rather than being buried deeply within the fibril is as yet only tentative, being based on observations of the partially dissociated fibril. The poor binding of the 2C2 mAb to the fibril indicates, however, that, at least, access to this epitope on the long arm is in some way hindered.

Type IX collagen represents ~10% of the fibrillar collagen, a proportion consistent with a distribution restricted to the surface of the collagen fibril. Indeed, it is difficult to envisage how type IX collagen with its interrupted triple helical structure and glycosaminoglycan chain (4, 28, 31) attached to the NC3 domain (12, 15, 23) could fit within the relatively tight packing of a fibril core. Possibly the best reference model for the packing within these fibrils to date is that proposed from the x-ray diffraction data for 17-nm collagenous fibrils from lamprey notochord sheath (3). These fibrils are composed of collagen types II and XI and have similar dimensions to the chicken embryo cartilage fibrils isolated here. Based on these data one would expect ~80 collagen molecules per fibril cross-section. If only eight of these are type IX collagen, then there can clearly not be a continuous sheath of type IX around the fibril surface. Therefore either type II and/or type XI collagen must also be exposed on the fibril surface.

The distribution of type IX collagen in the longitudinal axis of the isolated fibrils with a spacing of one d-period is clearly shown in the rotary-shadowed micrographs. That this periodicity is so readily observed is most likely also a function of a discontinuous sheath of type IX collagen on the fibril surface. The preparative procedure for rotary shadowing would certainly also play a role. Projections from the side of the fibrils would be encouraged to spread out on contact with the mica surface, whereas those lying along the top of the fibrils would tend to collapse onto the fibril and be lost to view. Because this methodology visualizes surface relief only poorly, the relative position of type IX collagen with respect to other collagens cannot be determined directly. However, if the previous localization of glycosaminoglycans to the d-band of type I collagen fibrils in tissue sections is mirrored in cartilage by the chondroitin sulphate chains of type IX collagen, then this would imply that the NC3 or "hinge" region is positioned next to this gap region in the fibril and in juxtaposition to the amino-telopeptide of type II collagen. Firm evidence in support of this has recently been obtained (9; van der Rest, M., and R. Mayne, manuscript submitted for publication). A tryptic peptide was isolated from a peptic digest of chicken cartilage and shown on the basis of its fluorescence spectrum to contain a trivalent hydroxyproidinium cross-link. Based on its amino acid double sequence, one peptide could be shown to be derived from the amino-terminal end of the COL2 fragment of the e2(IX) collagen chain, i.e., near the hinge, the other from the amino-telopeptide region of type II collagen. The corresponding peptide has also been isolated from bovine cartilage and sequenced (9) along with an additional peptide containing a trivalent cross-link between type IX and type II collagens. Although the exact organization of type IX collagen within the cartilage fibrils cannot yet be ascertained, the surface properties brought about by the projections of type IX collagen begin to emerge. The periodically arranged chondroitin sulfate chains may either extend away from the fibril to potentially interact with other component(s) of the cartilage matrix or may primarily interact with collagens or other molecules in the fibrils themselves. Another aspect to be considered is the orientation of the amino-terminal domain of type IX collagen in the fibrils. No function has yet been ascribed to this globular portion of type IX collagen, although it is reported to have a pI of 9.8 (30), making it clearly a candidate to interact with the polyanionic proteoglycans abundant in cartilage. In this context, the function of the COL3 domain may be to provide a spacer arm to project the NC4 domain away from the fibril to facilitate interactions with the proteoglycans. The COL1 and COL2 domains of type IX collagen (see Fig. 4) are presumably necessary for the interactions with collagen type II (and possibly type XI) in the fibril. The contact with the aggregating proteoglycans in the interfibrillar space as well as anchorage to cells may additionally be mediated by one or several of the cartilage glycoproteins described recently (5, 25, 29, 32, 33), some of which appear able to bind to collagen.

The concept of the collagen fibers as not only structural elements but as vehicles for the presentation in the extracellular milieu of varied macromolecular signals is attractive. The direct use of isolated fibrils in functional assays may provide insights into extracellular matrix interactions not possible with individual components. How the cell regulates the synthesis of these fibrils, in particular at the level of the gene with the coordinate regulation of six gene products for the three collagens necessary, is a challenging question.
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