Type III Collagen, a Fibril Network Modifier in Articular Cartilage*

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The collagen framework of hyaline cartilages, including articular cartilage, consists largely of type II collagen that matures from a cross-linked heteropolymeric fibril template of types II, IX, and XI collagens. In the articular cartilages of adult joints, type III collagen makes an appearance in varying amounts superimposed on the original collagen fibril network. In a study to understand better the structural role of type III collagen in cartilage, we find that type III collagen molecules with unprocessed N-propeptides are present in the extracellular matrix of adult human and bovine articular cartilages as covalently cross-linked polymers extensively cross-linked to type II collagen. Cross-link analyses revealed that telopeptides from both N and C termini of type III collagen were linked in the tissue to helical cross-linking sites in type II collagen. Reciprocally, telopeptides from type II collagen were recovered cross-linked to helical sites in type III collagen. Cross-linked peptides were also identified in type II collagen from a cross-link between three different collagen molecules, an alpha 1(III) telopeptide to the alpha 1(III) helix. This can only have arisen from a cross-link between three different collagen molecules, types II and III in register staggered by 4D from another type III molecule. Type III collagen is known to be prominent at sites of healing and repair in skin and other tissues. The present findings emphasize the role of type III collagen, which is synthesized in mature articular cartilage, as a covalent modifier that may add cohesion to a weakened, existing collagen type II fibril network as part of a chondrocyte healing response to matrix damage.

Fibrillar collagens are the most abundant vertebrate proteins. They provide the extracellular framework and mechanical strength of most animal tissues. There are seven collagens in the fibrillar collagen family, types I, II, III, V, XI, XXIV, and XXVII, encoded by 11 distinct genes (for review see Ref. 1). Based on phylogenetic analysis, fibrillar collagen genes can be subdivided into three distinct groups or clades (1–5). A-clade comprises a1(I), a1(II), a1(III), a2(I), and a2(V); B-clade is a1(V), a3(V), a1(XI), and a2(XI); and C-clade is a1(XXIV) and a1(XXVII). All fibrillar collagens are synthesized as procollagen molecules consisting of a long uninterrupted triple-helical domain (each alpha chain contains about 1000 amino acid residues) with globular extensions at both N and C termini and a minor triple-helical domain in the removable N-propeptide (1, 6).

Collagen types I, II, and III are the main fibril-forming molecules in vertebrates. Type I collagen is widely expressed and prominent in skin, tendon, bone and ligaments, and many other tissues but not in hyaline cartilages. The type I molecule is a heterotrimer of two alpha 1(I) chains and one alpha 2(I) chain (6). Type II collagen is restricted to cartilages, vitreous and intervertebral disc, and is a homotrimer of alpha 1(II) chains (7–9). Type III collagen is also a homotrimer of alpha 1(III) and appears to function as a copolymer with type I collagen in many tissues, including skin, tendon, ligament, vascular walls, periodontal ligament, and synovial membranes and is most prominent in highly compliant connective tissues (10–17). As with types I and II collagens, the strength of polymeric type III collagen depends on covalent cross-links formed by the lysyl oxidase mechanism (18–20). In addition to cross-links between the type III collagen molecules themselves, intertype cross-links also form to type I collagen, for example, in aorta which is rich in both collagens I and III (21). A small but significant amount of type III collagen becomes deposited in articular cartilage of mature joints, where it can be detected by immunofluorescence concentrated in the matrix surrounding chondrocytes throughout the depth of the tissue and particularly prominent in human osteoarthritic joints (22–24).

The collagen framework of hyaline cartilage is a highly cross-linked unique heteropolymer. In essence, the bulk type II collagen is polymerized on a template of type XI collagen, and type IX collagen covalently decorates the surface type II molecules of the nascent fibrillar networks most prominently in young tissue (25–28). All three collagen types, II, IX, and XI, are heavily cross-linked in the same fibril through the lysyl oxidase-mediated mechanism (29–32). In a study to understand better the structural role of type III collagen in cartilage, we have revealed that pN-type III collagen molecules are present in the extracellular matrix of adult human and bovine articular cartilages as covalently cross-linked polymers extensively cross-linked to the surface of type II collagen fibrils, suggesting a role in matrix reinforcement and a healing response to tissue damage.

EXPERIMENTAL PROCEDURES

Preparation of Collagens—Human knee joints were obtained from Northwest Tissue Services (Seattle, WA) from donors aged 18–75 with no obvious signs of osteoarthritis. Full thickness articular cartilage was sliced from the femoral and tibia condyles and from an equivalent site in a 4-year-old cow (bovine) knee. Minced tissue was extracted in 4 M guanidine HCl, 0.05 M Tris-HCl, pH 7.4, containing protease inhibitors (2 mM EDTA, 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 5 mM 1,10-phenanthroline) at 4 °C for 48 h to remove proteoglycans and other matrix proteins. The guanidine-insoluble tissue residue was then washed thoroughly with water and freeze-dried. Cross-linked collagens were solubilized by digest-

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In the natural text, the authors describe the isolation and purification of type III collagen from articular cartilage. The process involves several steps, including digestion with pepsin, bacterial collagenase, and trypsin, followed by various chromatography techniques to separate the collagen fractions.

1. **Pepsin Digestion**
   - The washed residue after 4 M guanidine HCl extraction was minced and digested with CNBr in 70% formic acid under N2 at room temperature for 24 h. The digests were diluted 15-fold with water and freeze-dried (34). Collagen CNBr-derived peptides were resolved by molecular sieve chromatography on an agarose A5m column (170 × 1.5 cm, 200–400 mesh, Bio-Rad), eluted with 2 M guanidine HCl, 0.05 M Tris-HCl, pH 7.5.

2. **Bacterial Collagenase Digestion**
   - The washed residues after 4 M guanidine HCl extraction were minced and digested with CNBr in 70% formic acid under N2 at room temperature for 24 h. The digests were diluted 15-fold with water and freeze-dried (34). Collagen CNBr-derived peptides were resolved by molecular sieve chromatography on an agarose A5m column (170 × 1.5 cm, 200–400 mesh, Bio-Rad), eluted with 2 M guanidine HCl, 0.05 M Tris-HCl, pH 7.5.

3. **CNBr Cleavage and Peptide Chromatography**
   - The washed residues after 4 M guanidine HCl extraction were minced and digested with CNBr in 70% formic acid under N2 at room temperature for 24 h. The digests were diluted 15-fold with water and freeze-dried (34). Collagen CNBr-derived peptides were resolved by molecular sieve chromatography on an agarose A5m column (170 × 1.5 cm, 200–400 mesh, Bio-Rad), eluted with 2 M guanidine HCl, 0.05 M Tris-HCl, pH 7.5.

4. **Antiserum and Western Blotting**
   - Two mouse monoclonal antibodies to human type III collagen were used. mAb 4G9 is specific to a conformational epitope in the globular N-propeptide domain (24). mAb 2C3 is specific to a proteolytic neoepitope at the C terminus of the α1(III) N-telopeptide sequence YDVKSGVAVGG, where K is a cross-linked lysine. Two mouse monoclonal antibodies to type II collagen were also used. mAb 10F2 recognizes a proteolytic neoepitope at the C terminus of a cleaved type II C-telopeptide sequence generated by pepsin (38), and mAb 1C10 is specific to a denatured epitope in the triple-helical domain of α1(II) near the C-terminal end (31).

5. **N-terminal Protein Sequence Analysis**
   - Purified cross-linked peptides were identified by ICAT (isotope-coded affinity tags) using reverse-phase liquid chromatography. The ICAT tagging reagent was used to derivatize the Cys residues of the peptides, allowing for their separation on a reversed-phase HPLC column. The peptides were then identified by mass spectrometry.

6. **Mass Spectrometry**
   - Individual protein bands after Coomassie Blue staining on SDS-PAGE were digested in-gel by trypsin (32, 39). The resulting peptides were subjected to microbore C8 column liquid chromatography (0.3 mm × 15 cm; Vydac) inter-
faced directly to a ThermoFinnigan LCQ Deca XP tandem mass spectrometer equipped with an electrospray ionization source. For protein identification, peptide fragments were compared with the NCBI nonredundant protein database using SEQUEST, an automated database search algorithm designed for use with tandem mass spectrometry (MS/MS) data. Cross-linked peptides were analyzed manually by calculating the possible MS/MS ions and matching these to the actual MS/MS ions (32).

RESULTS

Using interrupted SDS-PAGE with delayed reduction of disulfide bonds to resolve α1(III) from α1(II) chains, we were able to identify type III collagen in pepsin-solubilized material from all adult human articular cartilage samples examined (results from three representative donor joints are shown in Fig. 1). The chain identities, indicated by their migration on SDS-PAGE, were established beyond doubt by mass spectrometry and N-terminal protein sequence analysis. The type III collagen content of adult human articular cartilage varied between individuals in the range 0.5% to about 10% of total collagen, based on the recovered dry weights and relative intensity of Coomassie Brilliant Blue-stained bands on SDS-PAGE.

Fig. 2 shows a Western blot analysis using mAb 2C3 to probe the collagen chains extracted from adult human and bovine articular cartilage for a covalently attached type III collagen N-telopeptide. The 2C3 antibody is specific to human collagen III and does not cross-react with bovine collagen. Because collagen samples were not treated with DTT to reduce disulfide bonds prior to electrophoresis, the intramolecularly disulfide-bonded type III collagen chains ran near the top of the separating gel. From human cartilage three bands can be seen stained with 2C3, all of which were also stained by 1C10, the type II collagen-specific antibody. All three therefore were based on an α1(II) triple-helix but had an α1(III) N-telopeptide cross-linked to them. The gel shows that in addition to the signal from the main α and β chains of type II collagen seen by Coomassie Brilliant Blue staining in the pepsin digest, 2C3 also binds to a minor band running between them at 160 kDa not visible by Coomassie staining (Fig. 2). This band also reacted with mAb 1C10 and

FIGURE 1. Interrupted SDS-PAGE to detect α1(III) chains in pepsin extracts of human articular cartilage from three tissue donor knees. Lane 1, 18-year-old; lane 2, 60-year-old; lane 3, 73-year-old. For the reduced sample lanes (+DTT), DTT was added 15 min after starting the electrophoresis to resolve the α1(III) chain from α1(II) chain. The band immediately below α1(II) in lane 1 is a pepsin overcleavage product of α1(II).

FIGURE 2. SDS-PAGE/Western blot analysis to screen for type III collagen fragments covalently attached to type II collagen. Pepsin-solubilized type II collagens from mature human (H) and bovine (B) articular cartilages were resolved on SDS-PAGE without reducing disulfide bonds. Gels were either stained with Coomassie Brilliant Blue or electroblotted to polyvinylidene difluoride membrane and probed with a type III collagen N-telopeptide-specific antibody (2C3), type II collagen-specific antibody (1C10), or type III collagen N-propeptide-specific antibody (4G9). The type III N-propeptide/te-lopeptide is detected cross-linked to both human and bovine α1(III) chains. The arrow shows the position of the 160 kDa band that reacts with all three antibodies.

FIGURE 3. Molecular sieve chromatography of CNBr-digested human articular cartilage collagen. The CNBr digest of cartilage residue after 4 M guanidine HCl extraction was chromatographed on an agarose A1.5m (Bio-Rad) molecular sieve column (170 × 1.5 cm), eluted with a 0.05 M Tris-HCl buffer, pH 7.5, containing 2 M guanidine HCl, at a flow rate of 6 ml/h, collecting 3.0-ml fractions. Aliquots of collected fractions (4 μl) were assayed for mAb 4G9 immunoreactivity. The result shows that the N-propeptide domain of the type III collagen was retained in the cartilage matrix.

FIGURE 4. Interrupted SDS-PAGE/Western blot analysis of type III collagen from mature human articular cartilage. SDS-PAGE was run as in Fig. 1. mAb 10F2 was used to probe for the presence of a fragment of pepsin-cleaved type II collagen C-telopeptide linked to an α1(III) chain.
mAb 4G9, which shows the presence of the α1(II) chain and an α1(III) N-propeptide domain, respectively.

From these properties, this component appears to be an α1(II) chain cross-linked to an α1(III) N-telopeptide that still has a disulfide-bonded α1(III) N-propeptide trimer attached. Presumably this reflects a pepsin partial-cleavage product extracted from the cartilage matrix. The findings also imply that relatively large amounts of the N-propeptide domain of type III collagen are present in the extracellular matrix of adult cartilage. The presence of collagen type III N-propeptides in articular cartilage was confirmed by mAb 4G9 enzyme-linked immunosorbent assay across molecular sieved column fractions from a CNBr digest of the 4 M guanidine HCl-insoluble residue of adult cartilage (Fig. 3). The CNBr peptide components containing the N-propeptide domain eluted early in the chromatogram. Based on their elution positions on molecular sieve chromatogram and migration position on SDS-PAGE, the 4G9-reactive peptides in elution volume 85–130 ml have molecular masses of 100 kDa and above. The results indicate that the type III collagen N-propeptide domain is retained by most molecules of type III deposited and polymerized in cartilage matrix.

Fig. 4 shows that mAb 10F2 reacted with the α1(III) chain resolved on interrupted electrophoresis, indicating that α1(II) C-telopeptides were attached to some of the α1(III) chains. Such heterotypic cross-linking between type III collagen and type II collagen was also identified in extracts of adult bovine articular cartilage as follows. Because α1(III) chains are disulfide-bonded intramolecularly, they can be resolved from the bulk type II collagen and α2 chains in a pepsin digest by molecular sieve column chromatography (Fig. 5). Collagen recovered from the indicated pooled fractions enriched in type III collagen was digested with trypsin. Cross-linked peptides were further purified by IMAC and reverse-phase HPLC.

Using Cu²⁺ IMAC, several peptides containing histidine residues were selectively bound from the trypsin digest of the enriched type III collagen pool (Fig. 5). Four of these peptides were non-cross-linked linear peptides (Fig. 6a), but, in addition, divalent and tri-valent cross-linked collagen III peptides were also isolated. A prominent divalent cross-linked peptide was derived from the C-telopeptide of type II collagen (EKGPDPLQ) linked to the type II helical sequence that contained the residue 87 hydroxylysine cross-linking residue (GFP*GTP*GLP*GVK87GHR). Telopeptides from both N and C termini of type III collagen were also recovered linked covalently to the helical cross-linking sites in type III collagen (Fig. 6b). In addition, peptides from heterotypic cross-links between types II and III collagens were identified. One came from...
linkage of an N-telopeptide of type III collagen (DVXSGV-
AGGGIAGYP*GPAGPP*—) to the helical 930 site in type II
collagen (GLXGHR) (Fig. 6b). The structure of this heterotypic
cross-linked peptide was confirmed beyond doubt by MS/MS
(24). Another heterotypic cross-linked peptide was purified
from fraction 45 (Fig. 6a) and identified as a trivalent cross-
linked peptide linking the αI(II) C-telopeptide (EPL
X
GPDPQLD) to an αI(III) C-telopeptide (IAGIGGE
X
AGGFAGY) and the helical cross-linking site Lys87 in type III collagen (Fig. 7). In
addition to links to the helix of type III collagen, C-telopeptides
of collagens II and III were also found that were covalently
linked to the helix of type II collagen through a pyridinoline
residue. Thus, an αI(II) C-telopeptide (LGPRE
X
GPDPQLD) sequence and an αI(III) C-telopeptide sequence (IAGIGGEXA)
were found linked through pyridinoline to the type II collagen
helical cross-linking site at residue 87 in a peptide isolated from
a bacterial collagenase digest of adult human articular cartilage
Fig. 8).

Experiments designed to test whether the cartilage matrix
type III collagen was readily available for extraction as a poly-
mer associated with and cross-linked to type II collagen fibril
surfaces were carried out. Initial results indicated that of vari-
ous metalloproteinases tested, stromelysin-1 was the most effi-
cient under native conditions in extracting the type III collagen
pool without extracting significant amounts of type II collagen.
Fig. 9 compares the results of two serial 24-h extractions by
recombinant MMP3 at 37 °C (Fig. 9a) of minced articular car-
tilage from an osteoarthritic joint with pepsin extraction (Fig.
9b). The results of Western blotting using three different
monoclonal antibodies on replicate lanes show that MMP3
extracted very little type II collagen, which was detectable only
as intact $\alpha(II)$ chains (1C10 blot), but most of the type III collagen (detectable as cross-linked large fragments on 4G9 and 2C3 blots). Pepsin removed all of the type III collagen too, but cleaved mostly between the $\alpha(III)$ N-propeptide (disulfide-bonded trimer) and the main triple helix, whereas MMP3 did not cleave and release the free $\alpha(III)$ N-propeptide trimer, which was retained on the large fragments (Fig. 9a). Fig. 9c illustrates the various cleavage sites and molecular features of cross-linked pN-type III collagen.

**DISCUSSION**

The results confirm that significant amounts of type III collagen are present in adult human articular cartilage cross-linked covalently to other type III collagen molecules, suggesting their presence in the matrix as homotypic polymers of type III collagen presumably in the form of fine filaments of head-to-tail cross-linked molecules. Most of the cross-links formed between the type III collagen molecules are of the divalent variety, in contrast to type II collagen in which trivalent pyridinoline cross-links predominate (34). The results also indicate that in addition to type III-to-type III cross-links, the polymeric type III collagen is also heavily cross-linked to type II collagen. Telopeptides from type II collagen are linked to the helical cross-linking sites in type III collagen and, vice versa, telopeptides from type III collagen are linked to helical cross-linking sites in type II collagen.

The Western blot analyses of type III collagen extracted from adult human and bovine articular cartilages also revealed that a fraction of the molecules had been covalently linked to type II collagen (Fig. 2). A major site of linkage was between the C-helix (Lys$_{930}$) of $\alpha(II)$ and the $\alpha(III)$ N-telopeptide. This is the same site previously implicated for bovine articular cartilage (24). The results in Fig. 2 show that this cross-linkage in fact occurs between a longer form of the $\alpha(III)$ N-telopeptide in which the N-propeptide extension is retained and detected by

mAb 4G9. From the mass spectrometry results, all of the divalent cross-linked peptides identified in type III collagen of bovine cartilage, either from III to III or to II linkages, contained an additional 188-Da mass on the cross-linking residue. Such an adduct has been shown to be a maturation product of a pool of ketoamine cross-links in type II collagen of bovine cartilages that do not mature to pyridinolines. It results from ketoamine oxidation and arginine addition (40).

The results also reveal trivalent cross-linked peptides between mixed telopeptides of both types II and III collagens to helical residue 87 in either collagen II or collagen III (Figs. 6 and 7). This finding is important because it confirms that pyridinoline residues can link three different collagen molecules. Indeed, our original proposed mechanism of formation for pyridinoline cross-links was an aldol addition between two neighboring ketoamine cross-links within the microenvironment of the molecular packing arrangement of a fibril (41). The stoichiometry from C$_{14}$-lysine labeling of cartilage in vivo and in vitro (42–44) and the finding of a urinary peptide from bone resorption linking two $\alpha(II)$ N-telopeptides to a helical site (45) support this.

The CNBr-derived peptides in which N-propeptide domains of type III collagen were detected have estimated molecular masses $>100$ kDa (Fig. 3). This is larger than the monomeric processed N-propeptide trimer ($\sim 60$ kDa) and therefore cannot represent simply processed collagen III N-propeptides after synthesis, but the retention of N-propeptides on cross-linked pN-type III collagen molecules in cartilage matrix. Retained N-propeptides will prevent type III collagen from forming thick collagen fibrils (46–48), but will not impair divalent cross-linking internally in the polymer or trivalent bonds at the interface with type II collagen fibrils.

The most likely explanation for the polymeric form of type III collagen in cartilage matrix is a thin filamentous polymer of pN-type III molecules cross-linked head to tail at 4D-staggered sites but heavily cross-linked laterally to the surfaces of type II collagen fibrils wherever they interact. In effect, such a filamentous polymer might add cohesion to a swollen, and perhaps weakened, existing collagen II fibril network. It is notable that the earliest observed change in articular cartilage in experimental animal models of osteoarthritis is a swelling of the collagen fibril network (49) and that collagen III is expressed by chondrocytes of human osteoarthritic cartilage (50), in which its content has been reported to be enriched (51). Type III collagen is distinct from types I and II collagens in lacking 3-hydroxyproline in the triple helix, which we suspect may be related to an inability to form thick, homotypic fibrils (52). In skin and other tissues, immunogold electron micros-
The collagen type III polymer is susceptible to depolymerization and selective extraction by MMP3 cleavage in the triple helix and telopeptide domains as indicated by the scissors symbols.

The size of the fragments extracted by MMP3 with retained N-propeptides domain is consistent with depolymerization by cleavage in the main triple helix, most likely at the ¼ length collagenase-cleavage domain, which is especially susceptible in type III collagen to proteases other than collagenase including MMP3 (53, 54). Taken together, the results show that type III collagen molecules accumulate in mature human articular cartilage under native conditions, suggesting that it is accessible as a cross-linked polymer external to type II collagen fibrils. This concept is shown in Fig. 10.

The results in Fig. 10 and XI collagens decreases relative to type II collagen (55). The tilage cross-linked to the surface of type II collagen fibrils. The collagen molecules accumulate in mature human articular cartilage, suggesting that it is readily extracted by stromelysin (MMP3) digestion of collagen type III with retained N-propeptides has been detected present on the surface of type I collagen fibrils (13). Similarly, copy showed that collagen III with retained N-propeptides is present on the surface of type I collagen fibrils (13). Similarly, collagen type III with retained N-propeptides has been detected on the surface of type II collagen fibrils in human articular cartilage (23). The results in Fig. 9 show that type III collagen can be readily extracted by stromelysin (MMP3) digestion of human articular cartilage under native conditions, suggesting that it is accessible as a cross-linked polymer external to type II collagen fibrils. This concept is shown in Fig. 10.

FIGURE 10. Illustrated concept of collagen type III polymeric filaments interwoven with and cross-linked to a collagen type II fibrillar network.

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