REDUCIBLE CROSSLINKS OF TYPE I AND TYPE II COLLAGENS OF CHICKEN CARTILAGE

D. Brickley PARSONS and M. J. GLIMCHER

Department of Orthopaedic Surgery, Harvard Medical School and Children's Hospital Medical Center, Boston Mass. 02115, USA

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1. Introduction

Although type II collagen occurs principally in cartilage [1], it is not confined to that tissue [2–4] nor does it constitute the only genetically distinct type of collagen in cartilage. Type I collagen is present in human rib [5] chick, sternal [6,7], chick articular [8], bovine epiphyseal [9] and nasal cartilages [10,11], and is the predominant species in pig knee menisci and annulus fibrosus [12].

Although type I collagens of skin, tendon and bone (same species) have the same primary structure, posttranslational modifications distinguish them from one another [13], particularly in the profiles of reducible crosslinks [14]. The chemical nature and relative proportions of reducible crosslinks in type I and type II cartilage collagens were determined by extracting type I collagen from chick cartilage in denaturants, leaving type II collagen as the predominant species in the insoluble residue [8,9]. The two fractions were then reduced and analyzed separately.

It was found that type I collagen of cartilage has a reduced crosslink profile similar to that of type I collagen of bone. Hydroxylysinohydroxynorleucine (HylOHNle) is essentially the only reducible aldimine crosslink in type II cartilage collagen, which also differs from type I cartilage collagen in that it contains denaturant stable intra- and intermolecular bonds rendering it insoluble even after aldimine crosslinks are cleaved. The denaturant stable bonds in type II cartilage collagen are distributed differently than in insoluble tendon and skin collagen as evidenced by differences in swelling characteristics.

2. Materials and methods

Calvaria, mandible, skin, leg tendons, sternum and articular cartilage of the lower end of the femora and upper tibiae of freshly killed 10 week old white Leghorn chickens were used. Bone was freed of periosteum, and sternal cartilage of perichondrium. Articular cartilage was removed from bone by scalpel, leaving behind a visible layer of cartilage. Histological examination of the remaining ends of bone confirmed that the samples contained only cartilage.

Bone was cut into small pieces by hand, dried, pulverized at low temperature [15], decalcified in 0.5 M EDTA, pH 8.3, at 4°C, and washed free of EDTA in 0.01 M Tris-buffer, pH 7.4, and doubly distilled water. Cartilage was finely minced by hand, dried, and in some cases also pulverized for very short periods at low temperature. Skin and tendon were finely minced by hand.

2.1. *Extractions*

Aliquot samples of all of the tissues were sequentially extracted in 4 M $CaCl_2$, pH 7.4, 2°C every two days for 3 weeks (100 ml/g wet wt). Pooled extracts, clarified by centrifugation at 19 000 *g* for 2 h, were dialyzed exhaustively against water, concentrated and frozen. Insoluble residues were washed free of salt, dialyzed against 0.01 M Tris-buffer, pH 7.4, 2°C for several days, washed free of salt again and frozen, lyophilized or dried in a vacuum desiccator over P_2O_5.

2.2. *Reducible crosslinks*

10 mg aliquot samples of whole tissue, denaturant

extracts (as gels or lyophilized solids) and insoluble residues were reacted with $[^3H]NaBH_4$, the reduced samples hydrolyzed, chromatographed and counted in a liquid scintillation spectrometer [16]. Samples of insoluble residues were reacted with $[^3H]NaBH_4$ in 9 M KSCN at 25°C and the samples analyzed for reduced crosslinks as described. Other aliquots of denaturant insoluble collagens were extensively reacted with succinic anhydride [17] and reextracted in 4 M $CaCl_2$ for 1 week at 4°C. These insoluble residues were reduced with $[^3H]NaBH_4$ in both phosphate buffer and in 9 M KSCN. Swelling of the denaturant insoluble residues before and after reaction with succinic anhydride was measured by weighing the wet and dry samples equilibrated in 0.001 M Tris-buffer, pH 7.4 and in 0.3 M acetic acid before and after succinylation.

3. Results

Approx. 25–30% of the cartilage collagen is extracted as gelatin in $CaCl_2$. This gelatin has a lower hydroxylysine/hydroxyproline ratio than does the collagen of the whole tissue, while the insoluble collagen which remains is enriched in hydroxylysine (table 1) [8,9]. Amino acid analyses, and also SDS gel electrophoresis together with ion exchange chromatography of CNBr digested tissue [12] demonstrate that in the chick cartilage samples type I is the predominant (> 95%) species in the extracts, type II in the insoluble residue (> 95%). Reducible crosslink profiles (fig.1) of all extracts except cartilage are similar to those of native tissue (fig.1). Similar results have been previously demonstrated for partially renatured bone collagen extracted in denaturant salts [18]. There are striking differences between the reducible crosslink profiles of type I and type II cartilage collagens and between type II cartilage

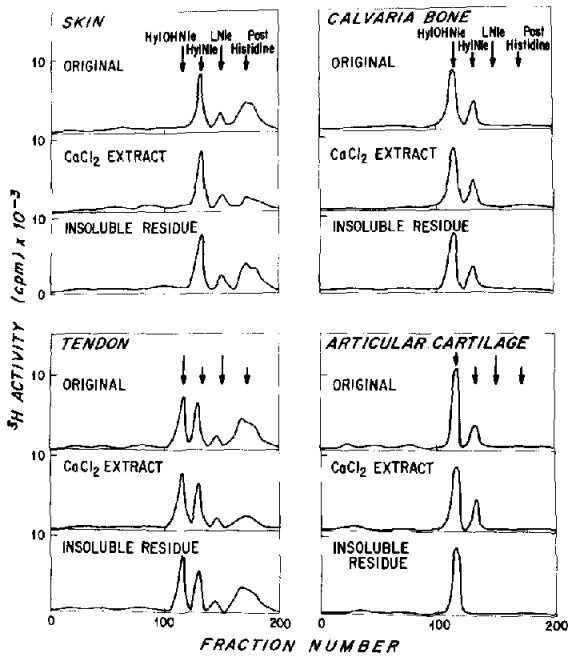

Fig.1. Ion exchange column chromatography of ^3H-labelled, reduced crosslink components of various whole tissues, extracts and insoluble residues. Profiles from sternal cartilage are similar to those of articular cartilage; those of mandibular bone are similar to calvarial bone.

Table 1
Hydroxyproline and hydroxylysine contents of connective tissues, extracts and residues of 10 week old postnatal chickens (residues/1000 amino acid residues)

	Whole		$CaCl_2$ extract		Insoluble residue	
	Hypro	Hylys	Hypro	Hylys	Hypro	Hylys
Sternum	91	16	95	9	93	21
Articular	93	14	93	9	96	20
Mandible	96	4.7	95	4.6	98	4.8
Calvaria	88	4.2	95	4.5	93	4.3
Skin	75	5.4	95	4.1	93	4.9
Tendon	93	9.1	94	8.1	95	9.7

collagen and the type I collagens of the other connective tissues. The crosslink profile of type I cartilage collagen is very similar to that of type I bone collagen and differs from other type I collagens, particularly skin. Type II cartilage collagen contains HylOHNle as virtually its only reduced aldimine (fig.1), with less than 2% hydroxylysinonorleucine (HylNle). Certain invertebrate collagens similarly contain only HylOHNle [16].

Reducible crosslinks are not detected in the $CaCl_2$-insoluble collagens when reduction is carried out in 9 M KSCN, or after reaction with succinic anhydride. Less than 3% additional collagen is solubilized in $CaCl_2$ after succinylation. The insoluble collagens of tendon and skin absorb 10–12 times as much fluid in acetic acid as they do at pH 7.4, while insoluble cartilage collagen imbibes only approx. 1.5 times as much under the same conditions. Succinylated skin and tendon collagen absorb about 10 times more fluid than do unreacted samples at the same pH; succinylated, insoluble collagens of cartilage absorb only approx. 1.5 times as much as unreacted samples.

4. Discussion

Appreciable amounts of type I collagen can be extracted from chicken cartilage by denaturants while type II collagen is insoluble in such solvents [8]. Since dehydro-HylOHNle, dehydro-HylNle and posthistidine (dehydrohistidinohydroxymerodesmosine) reform in partially renatured solid phase gelatins of skin, tendon and bone in proportions similar to native tissues, they are also likely to do so in cartilage. Therefore the reduced profiles obtained probably represents differences in the reducible components in the native cartilage collagens. This is consistent with experiments demonstrating only HylOHNle in native cartilage containing principally type II collagen, and HylOHNle and HylNle in cartilage containing predominantly type I (unpublished data). However, differences in the HylOHNle/HylNle ratio [19,20] may also depend on other factors such as the hydroxylysine content of the collagens [21–25] and on modifications of specific aldimines rendering them nonreducible, than on the proportions of type I and II present in the tissue. For example, both the HylOHNle/HylNle ratio [23,26,27] and the hydroxylysine content [22,23,25] of bone collagen decrease with age.

The denaturant stable [28] crosslinks in type II collagen prevent its solubilization even after cleavage of reducible aldimines and the generation of strong repulsive electrical forces along the polypeptide chains generated by succinylation of ϵ-NH_2 groups. Furthermore the denaturant stable crosslinks are so distributed that they prevent the separation of the molecules or chains [15,28,29]. The marked difference in swelling behavior indicates that the *distribution* of stable intra- and intermolecular bonds is different in type II collagen than it is in the insoluble type I collagens of skin and tendon. In this respect, type II collagen of cartilage resembles insoluble type I bone collagen [15,28,29].

Differences in reducible and non-reducible crosslinks of type I and type II cartilage collagen as reflected by differences in their interactions and in their reactions with other components of the cartilage matrix may play an important role in their biological function. Since type I collagens are more readily digested by tissue collagenases than is type II collagen [30], and since type I cartilage collagen is less crosslinked and more soluble than type II collagen, its turnover rate may be higher than type II. This would be consistent with studies demonstrating that cartilage proteoglycans interact more strongly with $\alpha 2$ chains (type I collagen) than with $\alpha 1$(II) chains [10,11], and have a much more rapid turnover rate than that of the total collagen [31]. Although the overall turnover of cartilage collagen is increased in osteoarthritis [32,33], differences in the *rates* of turnover of type I versus type II collagen could explain why the *content* of type I cartilage collagen is not increased in osteoarthritis [32,33], even though osteoarthritic cartilage has been reported to differ from normal cartilage by virtue of the fact that it synthesizes principally type I collagen when cultured in vitro [34].

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