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COLLAGEN HETEROGENEITY IN HUMAN CARTILAGE: IDENTIFICATION OF SEVERAL NEW COLLAGEN CHAINS

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Normal human hyaline cartilages contain five distinguishable collagenous proteins in addition to, and different from the α1(II) chain of Type II collagen. This report describes the characterization of three of these additional proteins. By the criteria of solubility, electrophoretic mobilities, ion-exchange and sieve chromatographic properties, amino acid compositions, and cyanogen bromide peptide profiles, at least two of these proteins, and possibly the third, are structurally distinct collagen α chains different from previously reported collagen chains. These findings imply further molecular heterogeneity of vertebrate collagens, and the existence of at least 9 different structural genes for collagen chains.

Introduction

Hyaline cartilage is primarily, or exclusively, affected in a relatively large number of human diseases, among which are osteoarthritis and the many human dwarfing disorders (chondrodysplasias). Previous studies have suggested that alterations of cartilage matrix collagen occur in osteoarthritis (1-3) and certain of the chondrodysplasias (4-5), but interpretation of these results depends upon complete understanding of normal hyaline cartilage collagen composition. Although numerous studies have documented that Type II collagen is the major collagenous component of hyaline cartilage (6-11), observations of several unidentified collagenous proteins in avian scleral cartilage (12) and embryonic limb cartilage (13) have suggested the existence of additional structural components of normal cartilage matrix.

We have examined normal human hyaline cartilage for the presence of collagen(s) different from Type II collagen and find 5 collagenous proteins in addition to the α1(II) chain of Type II collagen. This report describes the isolation and partial characterization of 3 of these additional collagen chains which together constitute approximately 10% of the total cartilage collagen.[2]

1. To whom to address correspondence.
2. Early fetal and, to a lesser extent, neonatal cartilages contain small amounts of 2 additional collagenous materials with electrophoretic and compositional properties similar to that of α1(IV) (15), and which may be derived from the fetal cartilage vascular system.

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Methods

Human costosternal and femoral head cartilages were obtained at autopsy over an age range from 24 weeks fetal gestation to 4 years postnatal; bovine costal cartilage was obtained at 18 months. Whole cartilage was washed with saline, the perichondrium removed by careful dissection, and the cartilage diced in ice-cold distilled water and briefly homogenized in a Polytron homogenizer (Brinkman Instruments) maintained between 0-4°C. The resultant slurry was centrifuged, and the recovered cartilage fragments were twice extracted with 4M guanidine, 50 mM TRIS-HCl[3], pH 7.4 at 4°C for 24 hours. Cartilage fragments were recovered, washed extensively with ice-cold distilled water, and suspended in cold 0.5 M CH_3COOH, 0.2 M NaCl containing pepsin (Worthington) at 1 mg/ml. This suspension was gently shaken in a 50 ml Teflon-glass homogenizer at 4°C, and after 10 hours of incubation, the suspension was homogenized at two-hour intervals for an additional 10 hours. The resultant solution was diluted with 2 volumes of cold 0.5 M CH_3COOH and gently stirred at 4°C for an additional 2-4 hours. Typically, a viscous cloudy suspension without discernable cartilage fragments was obtained. Dropwise addition of cold 4 M NaOH with constant stirring to adjust the pH to 8.6 resulted in a viscous water-clear solution. After centrifugation at 8.14×10^5 g-min, the supernatant solution contained 87% of the total tissue hydroxyproline, a minimum value uncorrected for unavoidable losses during preparation. Typically, a barely perceptible precipitate was observed after centrifugation, suggesting virtually complete solubilization of the cartilage matrix. The supernatant solution ("whole cartilage digest") was dialyzed against water, and the resultant precipitate was solubilized in 1M NaCl, 0.05 M TRIS-HCl pH 7.4 and dialyzed versus dilute CH_3COOH. The acid-insoluble fraction was collected by centrifugation, and the acid-soluble fraction (containing Type II collagen) recovered by dialysis and lyophilization.

Collagens Type I and III and αA and αB chains were prepared from human fetal membranes as previously described (14).

Methods for CMC[3] chromatography, discontinuous SDS-PAGE[3] using slab gels, preparation of CNBr peptides[3], and amino acid analysis have been previously noted (14).

Results and Discussion

The pepsin-resistant proteins in the whole cartilage digest were analyzed by SDS-5% PAGE; a characteristic electrophoretic profile is shown in Figure 1 (channels 1, 6 and 9), and compared to previously described collagen chains (channels 2-5). The major component of the whole cartilage digest has the mobility of the α1(II) chain, but between this position and that of the dimeric β components appear three additional materials. For descriptive purposes, these materials are labeled 1α, 2α and 3α in order of increasing electrophoretic mobility (Fig. 1, channels 6 and 7). It is apparent that 1α and 2α are easily distinguished, but that 3α is not clearly separated from the α1(II) chain. Side-by-side comparison of the relative mobilities of these materials to standard collagen chains reveals, for the most part, a lack of co-migration.

Fractionation of the whole cartilage digest on the basis of solubility in dilute acetic acid solutions yields two fractions, both of which contain native

3. Abbreviations: TRIS, tris (hydroxymethyl) amino methane; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; CMC, carboxymethyl-cellulose; CNBr peptides, peptides produced by digestion of purified material with CNBr.

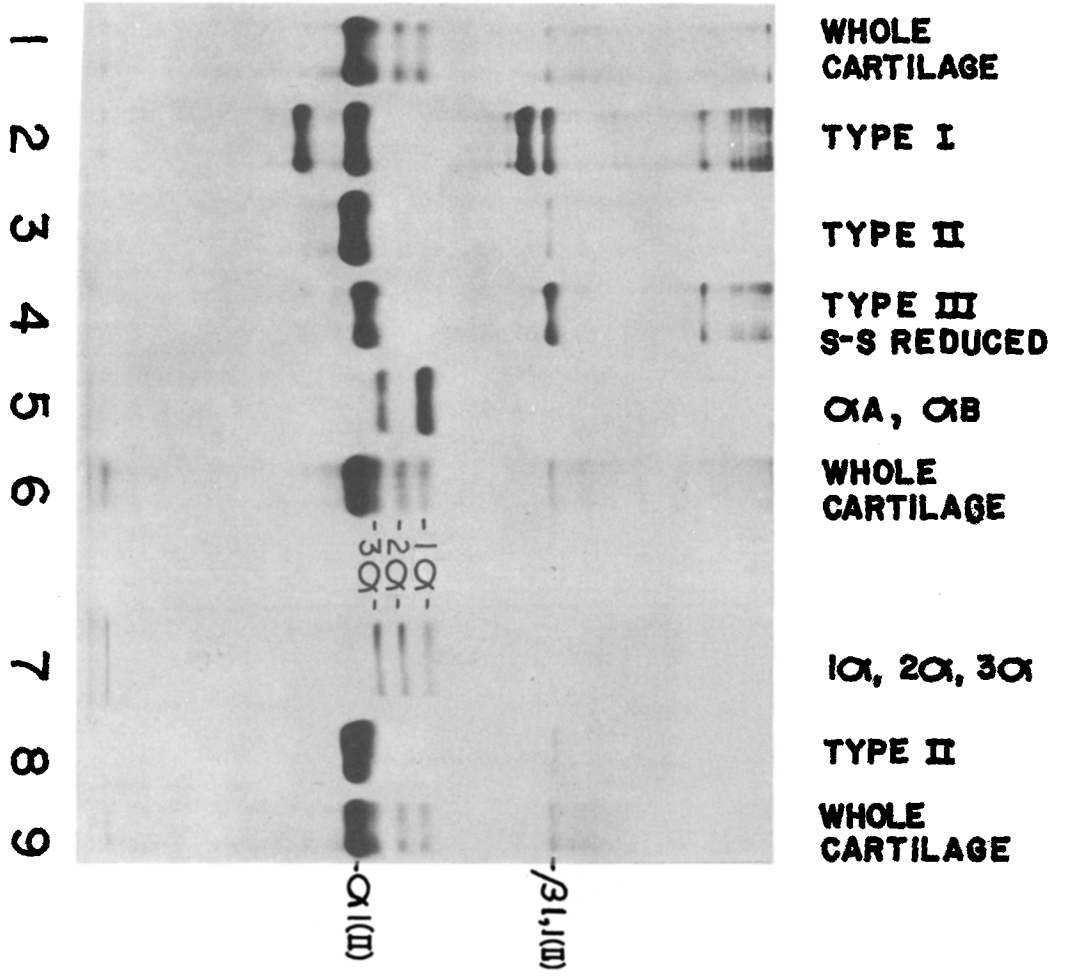

Figure 1. SDS-5% polyacrylamide gel electrophoretogram of standard collagen chains and various collagenous materials derived from cartilage. The content of each channel is indicated as are the electrophoretic positions of the α1(II) chain and β1,1(II), a dimer of two α1(II) chains. All depicted collagens are of human origin, and were prepared by pepsin extraction.

collagen molecules as indicated by resistance to repeated pepsin digestion at 4°C but complete digestion by nonspecific-protease-free bacterial collagenase (data not shown). The acid-soluble fraction contains α1(II) chains of Type II collagen (Fig. 1, channel 8), and the acid-insoluble fraction contains 1α, 2α and 3α chains (Fig. 1, channel 7). The component collagen chains of the acid-insoluble fraction may be resolved by CMC chromatography. Figure 2 depicts a typical CMC chromatogram and the results of SDS-5% PAGE analysis of individual column fractions. The 3α chain elutes in the same position as the α1(II) chain,

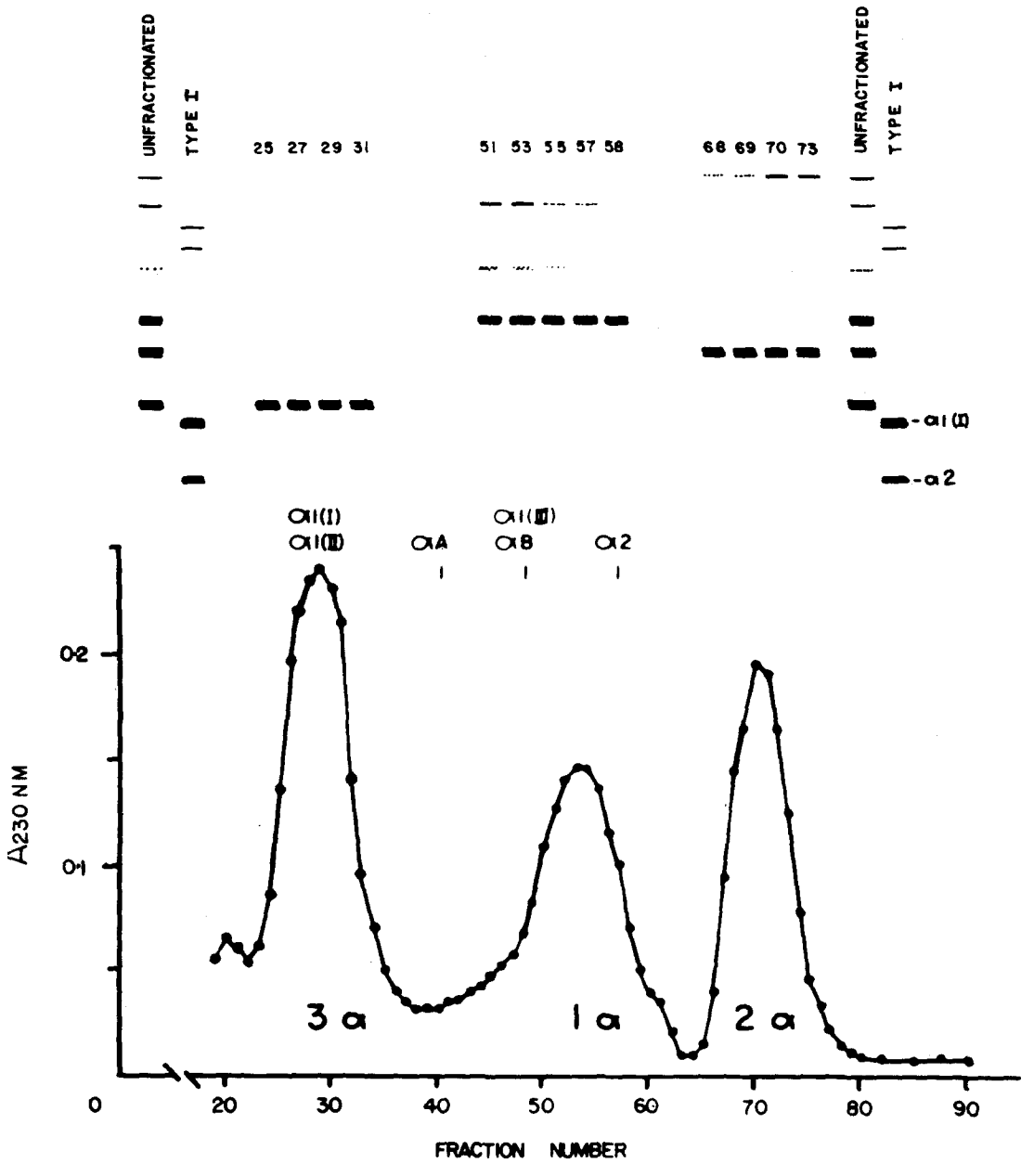

Figure 2. CMC elution profile of purified cartilage collagen(s) containing only 1α, 2α and 3α chains following heat denaturation. The column was equilibrated with 6 M urea, 0.04 M sodium acetate, pH 4.8 and developed with a superimposed linear gradient of NaCl from 0 - 0.09 M at 42° C. The elution position of standard collagen chains is indicated. SDS-5% PAGE of selected column fractions is represented above the corresponding elution positions and compared with the electrophoretic profiles of the original material applied ("unfractionated") and Type I collagen.

Table 1. Partial Amino Acid Compositions of Human Collagen Chains†

(Residues/1000)

	1α*	2α*	3α*	α1(II)*	αA**	αB**
4-Hypro	95	91	99	99	109	109
Thr	17	24	21	20	26	19
Pro	109	118	122	121	97	118
Gly	341	344	345	333	330	322
Ala	54	49	97	100	52	46
Val	24	25	17	18	27	18
Hylys	37	40	21	14	24	35
Lys	18	15	15	22	18	20
His	6	11	3	2	11	8
Arg	42	47	50	51	50	45

† The indicated collagen chains were isolated by pepsin digestion, hydrolyzed and chromatographed under identical conditions. The data are uncorrected for hydrolytic losses of threonine; no cysteine was present in any of the indicated collagen chains.

* Neonatal cartilage-derived collagen chains. Data are averages of triplicate analyses of three independent preparations.

** Human fetal membrane-derived collagen chains, composition from Burgeson, et al. (14).

whereas the 1α and 2α chains appear before and after the elution position of the α2(I) chain respectively.

Isolated 1α, 2α and 3α chains were separately co-chromatographed with human fibroblast ^{3}H-proline-labeled α1(I) on Bio-Gel A-5m; under conditions sufficient to widely separate α and β components, neither 1α, 2α or 3α resolved by more than a single fraction from the labeled α1(I) chain (data not shown). This result suggests that these chains have molecular weights similar to that of the α1(I) chain.

Partial amino acid compositions for 1α, 2α and 3α are recorded in Table 1, and compared to similar analyses of α1(II), αA and αB (14). The 1α and 2α chains are easily distinguished from 3α and α1(II) by a markedly lower content of alanine and larger contents of lysine plus hydroxylysine, and these same residues distinguish these chains from those of Type I-IV collagens (see Ref. 14 for collected amino acid compositions of human collagen chains). Comparison of 1α and 2α with the αA and αB chains reveals substantial compositional similarities, but 1α and 2α differ from αA in content of basic amino acid residues, and from αB in content of total imino acids and valine. In contrast, the composition of 3α is closely similar to that of the α1(II) chain except for increased hydroxylation of lysine.

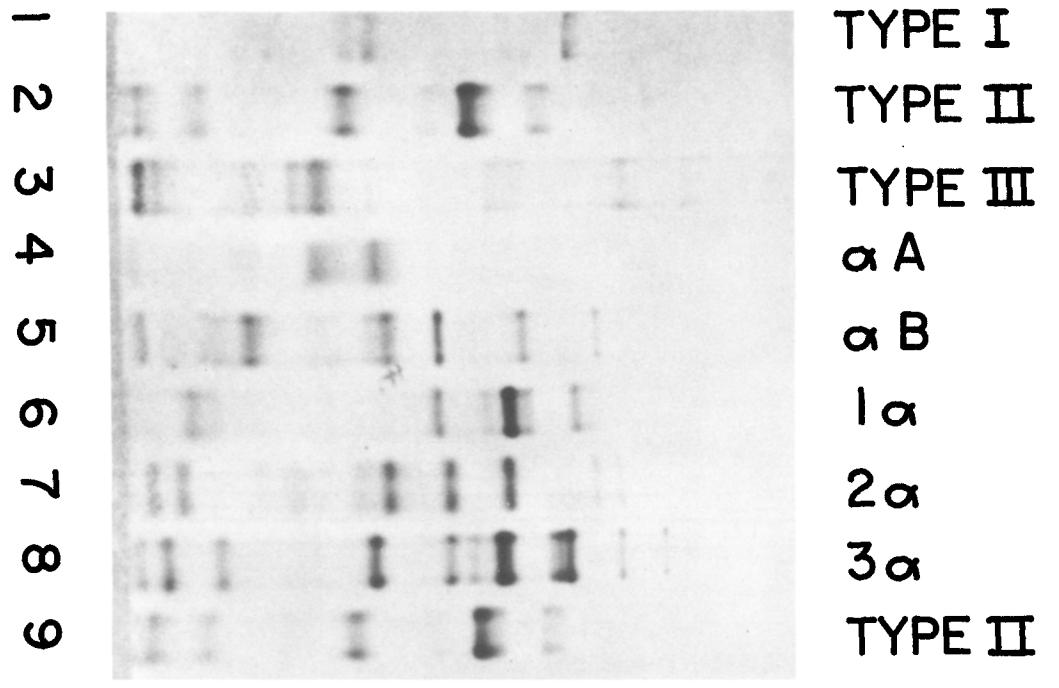

Figure 3. SDS-10% polyacrylamide gel electrophoretogram of CNBr peptides derived from standard collagens and purified collagen chains from human cartilage. The contents of each channel are indicated.

Electrophoretic comparisons of the cyanogen bromide (CNBr) peptides derived from purified 1α, 2α and 3α and from standard collagen chains are shown in Fig. 3. The CNBr peptide pattern of 1α (channel 6) and 2α (channel 7) are different from each other, from 3α, and from the profiles generated by standard collagen chains. The CNBr pattern of 3α is strikingly similar to that observed for α1(II) except that each constituent peptide has a somewhat lower electrophoretic mobility (compare channels 8 and 9).

These data indicate the presence of 2, and possibly 3, new collagen chains in human cartilage. By the combined criteria of solubility, electrophoretic mobility, CMC and Bio-Gel A-5m elution profiles, amino acid composition and CNBr peptide profile, both 1α and 2α are structurally distinct collagen chains whose molecular weights approximate that of previously described collagen chains. The 3α chain shares many features with the α1(II) chain, yet consistently exhibits altered solubility and slight differences in electrophoretic mobility, hydroxylation of lysine, and CNBr peptide profile. The present evidence does not permit distinction between the possibilities that the 3α chain is a structurally distinct polypeptide chain with substantial homology to α1(II), or

that it is an α1(II) chain modified at multiple sites by increased hydroxylation of lysine and addition of carbohydrate and/or other substituents. Butler, et al. (16) present evidence for sequence heterogeneity at three sites in the CNBr peptide, α1(II)-CB11, from bovine nasal cartilage, and propose the existence of at least two types of closely related α1(II) chains (designated α1(II) Major and α1(II) Minor) which are probably products of separate genes. The relationship, if any, between the 3α chain and α1(II) Minor is presently unknown.

Attempts to subfractionate 1α, 2α and 3α as native molecules have been unsuccessful, and a consistent apparent stoichiometric relationship between these chains has not been observed, and therefore, no conclusions may be presently drawn regarding the chain composition of these native molecules, or the number of distinct molecular species of collagen containing these chains. It seems apparent, however, that these materials are authentic cartilage matrix constituents since they have been observed in constant amounts relative to Type II collagen in early fetal through 4-year postnatal human cartilage, and have also been observed in 18-month bovine costal cartilage. Microanalysis of 1 mm slices of newborn human femoral head cartilage from articular surface to growth plate also revealed constant relative amounts of these chains, suggesting that these materials do not occur in spatially restricted areas of cartilage. (Data not shown.)

At present, at least 7 structurally distinct collagen polypeptide chains have been described (see Ref. 14), and the present results demonstrate the existence of two, and possibly three, additional collagen polypeptides. Therefore, at least 9 distinct structural collagen genes must exist in Man to account for the observed structural diversity of human collagen chains.

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