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The Progeny of Rabbit Articular Chondrocytes Synthesize Collagen Types I and III and Type I Trimer, but Not Type II. Verifications by Cyanogen Bromide Peptide Analysis†

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ABSTRACT: The radioactive collagens synthesized by the fourth subculture progeny of rabbit articular chondrocytes were extracted and purified after limited pepsin digestion by neutral and acid salt precipitation. In order to identify the different types of collagen present, denatured collagen chains were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5% gels, electrophoretically eluted, and cleaved with cyanogen bromide, and the resultant peptides were fractionated by a new sodium dodecyl sulfate electrophoresis system (tris(hydroxymethyl)aminomethane-borate buffer, 15% gels). Comparison of these separate peptide profiles with those from α1(I) and α1(III) collagen chains permitted the unambiguous identification of these chains in the radioactive collagen synthesized by chondrocytes. Although cartilage slices predominantly synthesized α1(II) chains, only α1(I) chains were made by cells in fourth subculture. A large fraction of these α1(I) chains could not be accounted for by the presence of type I collagen. While in a native, triple-helical conformation, some of these extra α1(I) chains were completely separated from type I collagen by their solubility at pH 8.0 in 2.6 M NaCl and therefore identified as $[\alpha 1(I)]_3$, type I trimer. In addition to type I collagen and type I trimer, these chondrocyte progeny also synthesized type III collagen and two new collagen chains, X and Y. Each collagen type was further characterized by carboxymethylcellulose chromatography and its distribution between the medium and the cell layer. These findings support the idea that cultured chondrocytes assume a collagen phenotype similar to that of their undifferentiated mesenchymal cell precursors.

The chondrocyte phenotype has been shown to be unstable under conditions of in vitro growth. Variation in the culture medium, high plating densities, the presence of embryo extract, and the presence of fibroblasts caused loss of chondrocyte polygonal morphology, elimination of metachromatic matrix formation, and decreased incorporation of radioactive sulfate, while increasing cell mobility and proliferation (Coon, 1966; Abbott and Holtzer, 1968; Bryan, 1968; Marzullo and Lash, 1970). After cartilage collagen, $[\alpha 1(II)]_3$ or type II collagen, was identified as a specific gene product restricted to cartilagenous tissues (Miller and Matukas, 1969; Miller, 1971a, 1973; Miller et al., 1971; Strawich and Nimni, 1971), chondrocyte cultures were analyzed to determine their collagen phenotype. Layman, et al. (1972) demonstrated the synthesis of α2 chains by rabbit articular chondrocytes in monolayer culture indicating the presence of type I collagen, $[\alpha 1(I)]_2\alpha 2$. A similar observation was made by Schiltz et al. (1973) for chick chondrocytes cultured in the presence of embryo extract or 5-bromo-2′-deoxyuridine. Reexamination of the chick chondrocyte collagen phenotype, with the added sophistication of cyanogen bromide peptide analysis of isolated collagen α chains, provided the additional information that cloned senescent chondrocytes or those grown in 5-bromo-2′-deoxyuridine cease making type II cartilage collagen and synthesize both type I collagen and $[\alpha 1(I)]_3$, type I trimer (Mayne et al., 1975, 1976).

Recently we have presented data (Cheung et al., 1976) suggesting that rabbit articular chondrocytes in primary culture produce type III collagen and a new hydroxyproline-containing collagen chain, X, in addition to type II collagen. This analysis was performed by $NaDodSO_4$[1] polyacrylamide gel electrophoresis ($NaDodSO_4$ electrophoresis) on 5% gels (permitting the resolution of these additional collagens) rather than by conventional carboxymethyl (CM)-cellulose chromatography. Subsequent subcultures produced type I collagen, an elevated $\alpha 1{:}\alpha 2$ ratio, and an increased proportion of X and type III collagen.

In the present communication a new $NaDodSO_4$ electrophoresis system for analysis of collagen cyanogen bromide peptides is presented and standardized. This technique allowed the individual collagen peaks resolved by $NaDodSO_4$ electrophoresis on 5% gels to be unambiguously identified. Coupled with analysis by CM-cellulose chromatography and differential salt precipitation, these data demonstrated that the progeny of rabbit articular chondrocytes in their fourth subculture do not make type II collagen, but synthesize type I

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[1] Abbreviations used: $NaDodSO_4$ electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CM-cellulose, carboxymethylcellulose; DMEM, Dulbecco's modified Eagle medium; Tris, tris(hydroxymethyl)aminomethane.

trimer and type I collagen. Most important, however, is the fact that these cells also synthesize type III and X and Y collagen chains. Although X and Y chains are discussed in the context of changes in chondrocyte biosynthetic capacity, their detailed characterization will be communicated in a separate publication.

Materials and Methods

Dulbecco's modified Eagle medium (DMEM) with high glucose, fetal calf serum, and penicillin–streptomycin solution were obtained from Grand Island Biological Co. Hyaluronidase (HSE grade), trypsin (TRL grade), collagenase (CLS type II), and pepsin (PM grade) were obtained from Worthington Biochemicals. Aquasol and [2,3-^{3}H]proline (30–50 Ci/mmol) were purchased from New England Nuclear. Reagents for $NaDodSO_4$ electrophoresis were purchased from Bio-Rad Laboratories. All other reagents were analytical reagent grade, except urea which was recrystallized from absolute ethanol or deionized by ion-exchange chromatography. Carrier collagen, used for purifying labeled collagen, was rat-skin acid-soluble collagen, purified and stored without lyophilization essentially as described by Strawich and Nimni (1971).

Isolation and Culture of Chondrocytes. Chondrocytes were released by enzymatic digestion from slices of articular cartilage taken from 8-week-old male white rabbits (Cheung et al., 1976). All digestions were performed with stirring at room temperature in 20 mL of Gey's balanced salt solution under 10% CO_2–90% air. Cartilage slices were sequentially treated with hyaluronidase (0.05% w/v, 10 min), trypsin (0.2% w/v, 15 min), washed twice with 40 mL of Gey's balanced salt solution, and digested with collagenase (0.2% w/v, 30 min). The cells released from each of these digestions were discarded. The remaining slices were suspended in 10 mL of culture medium (DMEM/high glucose, 10% fetal calf serum, 1% penicillin–streptomycin) in a standard Petri dish. Two milliliters of the collagenase solution was added and the mixture was placed in a CO_2 incubator overnight. The cell pellet formed upon centrifugation was resuspended in culture medium, vortexed, and filtered through a Nitex filter to remove any aggregates or residual slices. This procedure released approximately 90% of the chondrocytes present in the original cartilage slices. The cells were plated at 1.5×10^6 cells per Falcon flask (75 cm^2). Cells were grown in a CO_2 environment (pH 7.2), fed twice per week, and transferred with trypsin (0.2%) at weekly intervals.

Collagen Labeling and Purification. Radioactive collagen was synthesized for 24 h by growing newly confluent chondrocytes in 7.5 mL of culture media containing tritiated proline (50 μCi/mL), β-aminopropionitrile (2.5 mM), and ascorbic acid (100 μg/mL). All subsequent purification steps were performed at 4 °C. The intact cell layer and the medium were treated in the flask for 24 h with pepsin (2 mg/mL in 1 M acetic acid, 7.5 mL) and then lyophilized. Subsequently, 3–5 mL of rat-skin acid-soluble collagen (200 μg/mL in 1 M NaCl–50 mM Tris-HCl, pH 7.5 at 4 °C) was added to each flask as carrier collagen. The suspension was titrated to neutrality with 2 M NaOH, shaken for 2 h and carefully transferred from the flask for overnight dialysis against 1 M NaCl–50 mM Tris-HCl, pH 7.5 at 4 °C. Any insoluble residue was removed by centrifugation at 10 000g for 30 min, and the radioactive collagen in the supernatant was precipitated by adding solid NaCl to 4 M. After 24 h the precipitated collagen was collected by cntrifugation, redissolved in 0.5 M acetic acid, and precipitated by adding solid NaCl to 1 M. The precipitated collagen was then dissolved and extensively dialyzed against 0.5 M acetic acid. This material was used for $NaDodSO_4$ electrophoresis, CM-cellulose chromatography, and differential salt-precipitation.

CM-Cellulose Chromatography. Collagen chains were fractionated by a modified version of the technique of Miller (1971a). The CM-cellulose column was pretreated before repeated use by saturation of its collagen binding sites with acid-soluble collagen (~150 mg in column buffer), incubation at 40 °C overnight, elution with 0.1 M NaCl in column buffer, and equilibration in column buffer. This eliminated the necessity of adding additional carrier collagen to the sample.

$NaDodSO_4$ Electrophoresis. Both 5 and 15% (w/v) acrylamide gels were cross-linked with 0.1% bisacrylamide and catalyzed by 0.05% ammonium persulfate and 0.03% (v/v) *N,N,N,N*-tetramethylethylenediamine. A continuous Tris–borate buffer (Sykes and Bailey, 1971) was used: 0.1 M Tris–0.075 M boric acid (pH 8.6)–0.1% $NaDodSO_4$. Samples containing 5–50 μg of collagen or 50–100 μg of peptides were dissolved in 20 mM Tris–borate (pH 8.6)–2 M urea–2% $NaDodSO_4$, at 55 °C for 1 h, and 30–50 μL was added to each gel. Gels (5 × 105 mm) were electrophoresed at 150 V for 3–3.5 h (5% gels) or at 240 V for 2–2.5 h (15% gels). Gels for visual observation were fixed in fresh 1% aqueous glutaraldehyde for 3 h prior to staining. Radioactive gels were cut into 1 mm slices, hydrolyzed in 0.2 mL of 0.4 M NaOH at 55 °C overnight and counted in acidified Aquasol (Cheung et al., 1976).

Electrophoretic Elution. Samples for CNBr peptide analysis were prefractionated on 5% gels, sliced, and held (<2 days) at 4 °C in a moist environment until the exact positions of the peaks were determined by counting a replicate gel. Slices from each peak were placed in an electroelution tube consisting of a glass tube (6 mm i.d. × 180 mm) filled with Tris–borate buffer, a porous disk 1.5 cm from the bottom of the tube, and a dialysis membrane bottom. Electroelution was performed at 300 V for 6 h with tap-water cooling. Eluted samples were removed from between the dialysis membrane and the porous disk, centrifuged to remove any gel particles, dialyzed overnight against 0.1% $NaDodSO_4$ at room temperature, and lyophilized. Recovery of eluted material was 90%.

Cyanogen Bromide Cleavage. Cyanogen bromide cleavage was performed under nitrogen for 4 h at 30 °C with a two- to four-fold (w/w) excess of CNBr (10 mg/mL in 70% formic acid) (Miller, 1971b). For electrophoretically eluted material, CNBr cleavage was performed after the addition of carrier acid-soluble collagen consistent with loading 50–100 μg of peptides per gel and more than 100 000 cpm of radioactive collagen peptides.

Results

The progeny of rabbit articular chondrocytes from the fourth subculture was labeled with tritiated proline in the presence of β-aminoproprionitrile and ascorbate for 24 h so that the collagens produced by these cells could be characterized. The labeling conditions and the processing of the labeled collagen by pepsin treatment and neutral and acid salt precipitation are described in detail in Materials and Methods. The residue after pepsin treatment and each supernatant fraction generated during purification were analyzed for tritiated hydroxyproline and proline by ion-exchange chromatography, and the intact nature of the collagen chains was verified by $NaDodSO_4$ electrophoresis. These data (not shown) demonstrated that >90% of the labeled collagen was present in the final sample.

$NaDodSO_4$ Electrophoresis. Purified, labeled collagens

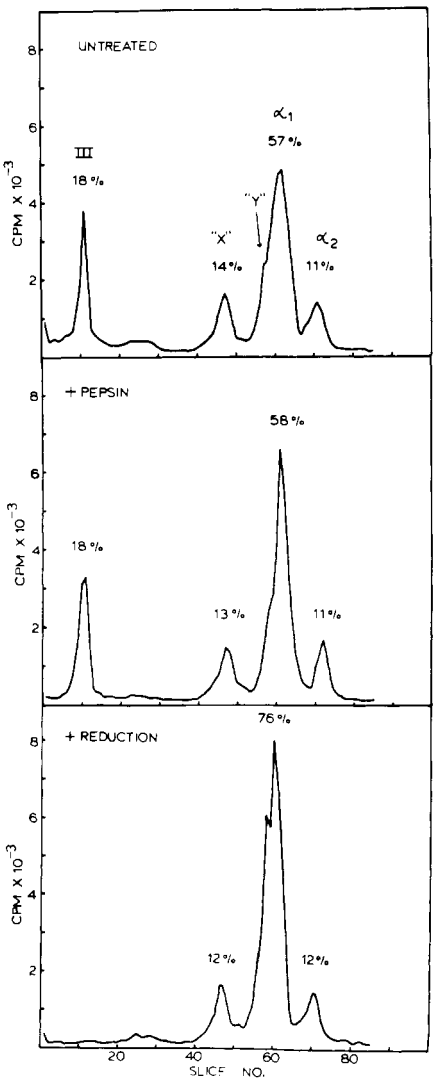

FIGURE 1: Sodium dodecyl sulfate electrophoresis of collagen from subcultured chondrocytes. The [^{3}H]proline-labeled collagen synthesized by the fourth subculture progeny of rabbit articular chondrocytes was treated with pepsin and purified as described. Denatured chains were fractionated by sodium dodecyl sulfate electrophoresis on 5% gels without additional treatment (untreated), after a second pepsin treatment for 24 h at 4 °C (+ pepsin), and after reduction with mercaptoethanol (+ reduction).

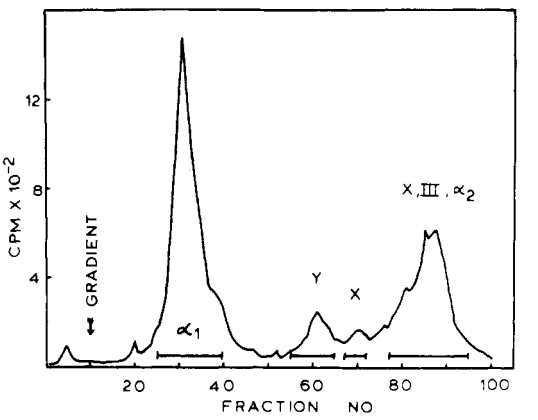

FIGURE 2: CM-cellulose chromatography of collagen from subcultured chondrocytes. Purified radioactive collagen was fractionated on a 1.6 × 9 cm column of CM-cellulose eluted with a 500-mL linear gradient (0.0–0.1 M NaCl in 1 M urea, 60 mM sodium acetate, pH 4.8). Each fraction was 3.2 mL and 100 μL was taken for scintillation counting.

were fractionated into several components ([α1(III)]$_3$, X, Y, α1(I), and α_2) by NaDodSO$_4$ electrophoresis on 5% gels using a continuous Tris-borate buffer system (Figure 1). In order to verify that the purified collagen was in a native triple-helical conformation, an aliquot was treated a second time with pepsin for 24 h at 4 °C. After lyophilization, this sample was electrophoresed concurrently with the starting material. The absence of changes in the amount of collagen or the proportion of the individual components (Figure 1) demonstrated helical conformation (Rubin et al., 1965; Müller et al., 1974; Mayne et al., 1975). At the same time, support for the identification of the trimeric material (slices 8–13) as type III collagen was obtained by electrophoresis after reduction with mercaptoethanol. This treatment resulted in depolymerization of trimer to material the size of α1 chains.

CM-Cellulose Chromatography. Radioactive collagen, similar to that fractionated by NaDodSO$_4$ electrophoresis in Figure 1, was submitted to CM-cellulose chromatography without additional carrier collagen (Figure 2). Material was pooled as indicated by the heavy bars and then electrophoresed on 5% gels after the addition of 15 μg of carrier collagen (gel profiles not shown). The data obtained identified the material as indicated above the bars in Figure 2. It is apparent that NaDodSO$_4$ electrophoresis (Figure 1) produced separate peaks for type III collagen, X, Y-α1, and α2 chains, while CM-cellulose chromatography did not completely resolve III, X, and α2 chains and placed Y in an elution position where β12 chains are expected. These results emphasize the potential for misinterpretation of CM-cellulose profiles. Also, quantitative analysis by CM-cellulose chromatography was difficult because only 60–85% recovery of radioactivity was possible whether or not extra carrier collagen was added. In contrast, the recovery of radioactivity from NaDodSO$_4$ electrophoresis was greater than 95% after quench correction. The major advantage of CM-cellulose chromatography was that homogeneous preparations of both Y and α1 chains could be produced.

CNBr Peptide Profiles Using NaDodSO$_4$ Electrophoresis. Positive identification of the collagens synthesized and fractionated by NaDodSO$_4$ electrophoresis was accomplished by comparing their CNBr peptide profiles with those of standard collagen chains. Lathyritic rat-skin collagen and α1 chains from collagen types I, II, and III were purified by conventional methods and cleaved at methionyl residues by treatment with CNBr. The peptides obtained were fractionated by NaDodSO$_4$ electrophoresis on 15% gels using the Tris-borate buffer system. A photograph of the stained gels is presented in Figure 3. Mixtures produced by combining peptides from two different chains in approximately a 1:1 ratio are also shown to emphasize the resolution of this method. The CNBr peptides from collagen types I and II were identified in NaDodSO$_4$ electrophoresis profiles by the coelectrophoresis of standard individual peptides obtained by CM-cellulose (Miller, 1971b) and subsequent agarose gel-filtration chromatography (Piez, 1968). The identification of the band labeled α1(III)-CB5+9 was made because its mobility (46–49 mm) was consistent with the molecular weight range (20 000–22 000) established for these peptides (Chung et al., 1974). Additional evidence for this identification was the presence of material with this mobility before reduction (presumably due to α1(III)-CB5) and the transfer of material from the upper portion of the gel (Figure 3, III) to this position upon treatment with mercaptoethanol (Figure 3, IIIR), presumably due to the depolymerization of disulfide-linked α1(III)-CB9 peptides (Chung et al., 1974).

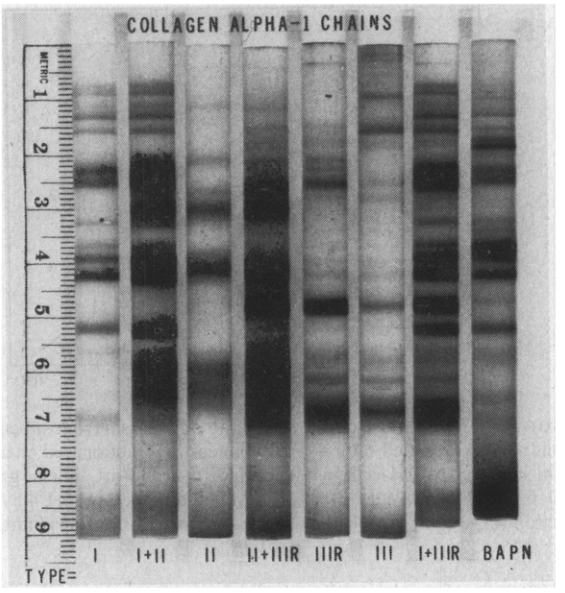

FIGURE 3: A photograph of Coomassie blue stained CNBr peptides from standard collagen α1 chains after fractionation by sodium dodecyl sulfate electrophoresis on 15% gels. Each gel is identified by a Roman numeral which indicates the type of α1 chain (I, II, III) cleaved by CNBr. The CNBr peptides from artificial mixtures of α1 chains (~1:1 by weight) and purified lathyritic rat skin collagen are also shown. "R" indicates the samples reduced with mercaptoethanol before electrophoresis. The CNBr peptides and their relative mobilities from α1(I) are: CB7, 38–40 mm (24 000); CB8, 41–43 mm (25 000); CB6, 51–53 mm (18 000); CB3, 55–57 mm (13 600); and CB4+5, 68–70 mm (4400 and 3900). Molecular weights in parentheses are from Butler et al. (1967) and Scott and Veis (1976a). Peptides from α1(II) are CB10,5, 28–31 mm (31 000); CB11, 40–42 mm (25 000); CB8, 58–61 mm (13 600); CB9,7+12, 61–68 mm (10 800 and 8000; respectively). Molecular weights are from Miller and Lunde (1973). The nomenclature α1(II)-CB10,5 is used throughout this paper to indicate both this peptide and bovine α1(II)-CB10 since they are indistinguishable by sodium dodecyl sulfate electrophoresis. Peptides from human fetal skin α1(III) are CB5+9, 47–50 mm (20 000 and 22 000, respectively). Molecular weights are from Chung et al. (1974).

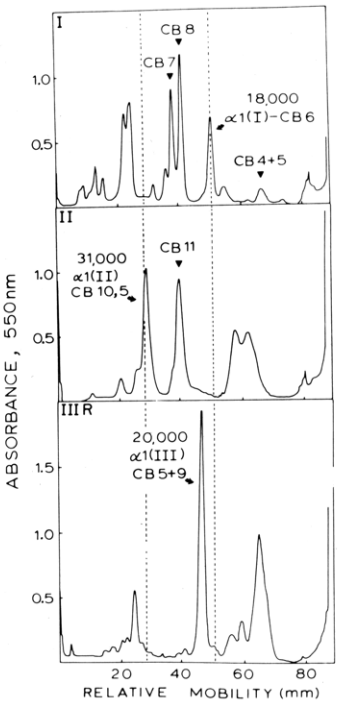

FIGURE 4: Spectrophotometric scans of the gels containing the CNBr peptides from α1(I), I; α1(II), II; and reduced α1(III) chains, IIIR. The gels are those photographed in Figure 3.

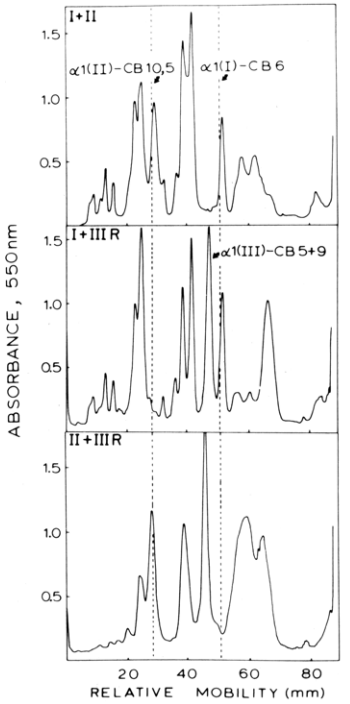

FIGURE 5: Spectrophotometric scans of gels containing CNBr peptides from the artificial pairs of α1 chains shown in Figure 3.

The major advantage of the Tris–borate, $NaDodSO_4$ electrophoresis system of fractionating CNBr peptides is that it resolves a single major band for each of the chains in the collagen types I, II, and III. The characteristic peptide peak derived from α1(I) chains was α1(I)-CB6 (51–53 mm); from α1(II), α1(II)-CB10,5 (28–32 mm); and from α1(III), the mixture of peptides α1(III)-CB5+9 (46–49 mm). This can most easily be seen in Figures 4 and 5 which present the spectrophotometric scans of the stained gels shown in Figure 3. This system provided separations not possible with the phosphate-buffered $NaDodSO_4$ electrophoresis system (Furthmayr and Timpl, 1971; Bradley et al., 1974) or its recent modifications (Scott et al., 1976; Scott and Veis, 1976b). Specifically, the present system separated α1(I)-CB7 from α1(I)-CB8, and α1(III)-CB5+9 from α1(I)-CB8. The latter separation is important as it permits accurate quantitation of the chains in the common I + III mixture by using an α1(III) peptide peak that represents approximately 40% of the material in the intact chain. Although the peak at 64–71 mm (Figure 4, IIIR) might also be used to quantitate the amount of type III chains, the fact that its region of highest mobility corresponded to that of α1(I)-CB4+5 (68–70 mm) indicates both a source of contamination and a heterogeneous population of peptides with molecular weights ranging from 10 000 (α1(III)-CB3) to 3600 (α1(III)-CB2). Scott and Veis (1976b) have identified a similar peak as α1(III)-CB8, although the mobility of this peak in their system was not consistent with the 12 000 molecular weight of this peptide (Chung et al., 1974).

If mixtures of collagen chains were quantitated on the basis of characteristic CNBr peptide peaks, the best results were obtained by continuing the electrophoresis until α1(I)-CB3 had been partially run off the bottom of the gel (manuscript in preparation). This approach permitted the peptide profiles to return to baseline adjacent to each of the characteristic peptide peaks. Additional benefit has been gained by analyzing

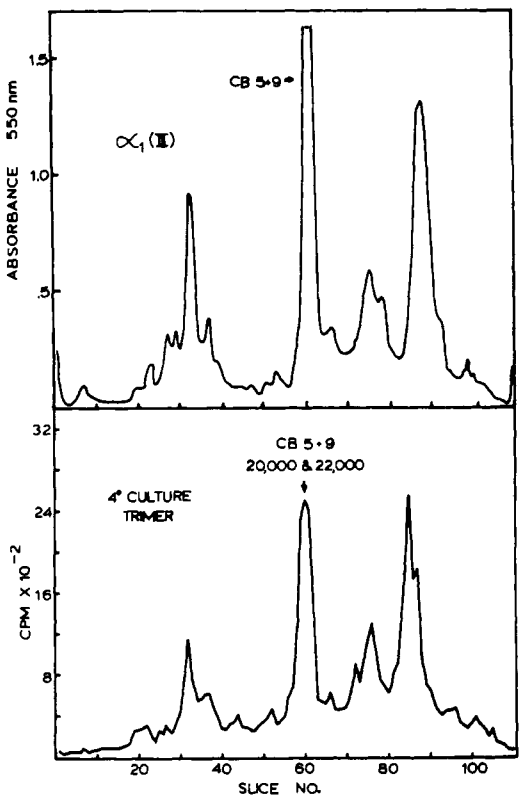

FIGURE 6: Comparison of CNBr peptide profiles of standard α1(III) chains (top) and the radioactive trimeric material synthesized by subcultured chondrocytes and electrophoretically eluted from the region corresponding to slices 8–13 in the top panel of Figure 1 (bottom).

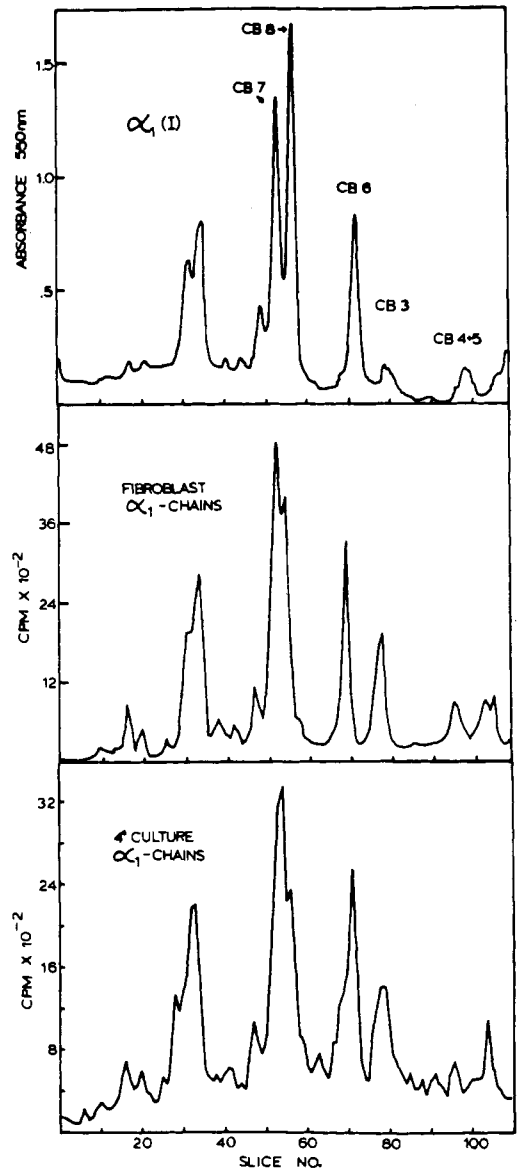

FIGURE 7: Comparison of CNBr peptide profiles of standard α1(I) chains (top), radioactive fibroblast α1 chains (middle), and α1 chains synthesized by subcultured chondrocytes and electrophoretically eluted from the region corresponding to slices 58–62 in Figure 1 (bottom).

radioactive collagens synthesized in the presence of β-aminopropionitrile (Figures 6–8). In this situation, radioactive peptides should not be lost or created by aldehyde-dependent cross-links and they can be quantitated directly by liquid-scintillation counting. We have used this approach to avoid errors due to differential staining and destaining, the loss of unfixed peptides, and the limitations of Beer's law.

Identification of Subcultured Chondrocyte Collagens. The radioactive collagens synthesized by fourth subculture chondrocytes were fractionated by $NaDodSO_4$ electrophoresis on 5% gels in order to obtain purified chains for CNBr peptide analysis. Approximately 200 000 cpm of labeled collagen was electrophoresed on each of ten analytical gels. The separation produced was identical with that shown in Figure 1. Slices from the trimer region (8–13) and the α1 chain region (58–62) were pooled separately and the radioactive collagen chains were electrophoretically eluted from the slices as described in Materials and Methods. These chains were then cleaved with CNBr and fractionated by $NaDodSO_4$ electrophoresis on 15% gels. This approach permitted each of the chains synthesized by a single 75-cm^2 flask of chondrocytes to be individually identified by CNBr peptide analysis. Only profiles from the trimeric material and α1 chains are presented.

Figure 6 compares the CNBr peptide profiles of standard type III collagen (absorbance at 550 nm) and the radioactive trimer synthesized by fourth subculture chondrocytes. Both the mobility and the proportion of the radioactive peptides demonstrate that the trimeric material was indeed type III collagen. By the same criterion, primary chondrocytes also produced type III collagen (data not shown).

Figure 7 presents the α1 chain CNBr peptide profiles. The top two panels compare the profiles of known α1(I) chains, purified by CM-cellulose chromatography. The spectrophotometric scan of stained peptides from lathrytic rat-skin α1(I) chains detected only a small amount of CB3, much less than would be expected on the basis of its molecular weight and the ratio of absorbance to molecular weight for CB6. A decreased affinity for stain rather than the actual absence of CB3 appeared to be responsible for this observation. In contrast, the proportion of CB3 approached the expected value when the radioactive peptides synthesized by cultured fetal rabbit fibroblasts were fractionated. This radioactive profile also suggests that no small peptides were completely lost during the fixing, staining, or destaining procedures. Another difference between the gel scan and the radioactive profile was the loss of resolution of CB7 and CB8 when radioactive slices were counted. This was mostly due to changing from a scanning slit width of 0.02 mm to gel slices approximately 1-mm thick. However, species differences in posttranslational modification of these peptides may also have been involved.

Figure 7 also provides a direct comparison of the α1 chains synthesized by fibroblasts and fourth subculture chondrocytes. In this comparison no species differences existed (both cell

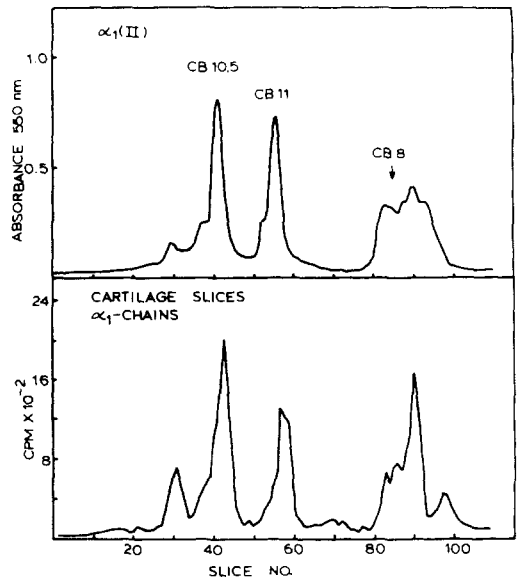

FIGURE 8: Comparison of CNBr peptide profiles of standard α1(II) chains (top) and radioactive α1 chains synthesized by freshly isolated cartilage slices and electrophoretically eluted from 5% gels after sodium dodecyl sulfate electrophoresis (bottom).

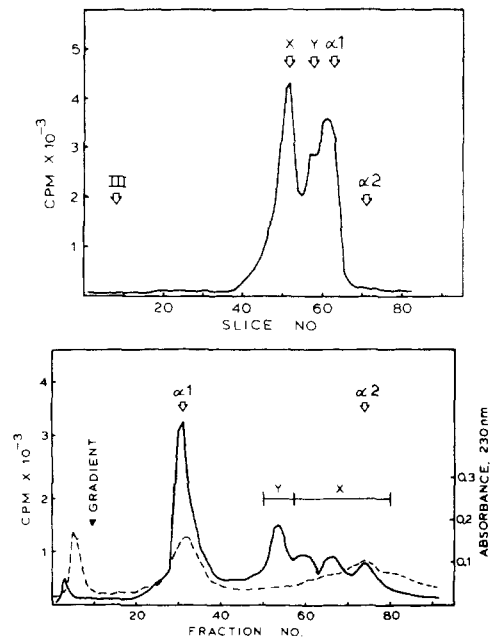

FIGURE 9: Radioactive collagens from the 2.6 M NaCl supernatant generated by differential salt-precipitation. Fractionation by sodium dodecyl sulfate electrophoresis on 5% gels (top). Fractionation by CM-cellulose chromatography (bottom). Lathyritic rat skin collagen (5 mg) was added to the sample to provide an internal standard for the elution position of α1 and α2 chains (dashed line).

cultures were derived from rabbit) and both profiles were based on counting radioactive peptides in gel slices. These two CNBr peptide profiles are identical. They also match the profile obtained from CM-cellulose purified α1 chains made by fourth subculture chondrocytes (not shown). Most important is the absence (between slices 35 and 45) of radioactive α1(II)-CB10 which is characteristic of cartilage collagen and represents approximately 30% of the intact α1(II) chain. Thus, the fourth subculture chondrocytes do not make type II collagen but instead synthesize only α1(I) chains. That the chondrocytes were making type II collagen before being released from cartilage is demonstrated by Figure 8. Some of the cartilage slices used to obtain the cells for culture were immediately placed in culture medium containing tritiated proline for 24 hr. The synthesized, radioactive α1 chains were electrophoretically eluted after $NaDodSO_4$ electrophoresis on 5% gels and their CNBr peptide profile compared with that of authentic α1(II) chains in Figure 8. The mobility and proportion of the CNBr peptides, and the absence of α1(I)-CB6 (slices 65–70), demonstrate that cartilage-specific collagen, type II, was the major α1 chain synthesized by the cartilage slices.

Salt Solubility at Neutral pH. Fourth subculture chondrocytes synthesized collagens with an α1:α2 ratio of 5.2:1 as determined by $NaDodSO_4$ electrophoresis in Figure 1. An erroneous ratio of 1.5:1 was obtained by CM-cellulose chromatography (Figure 2) because X and α1(III) chains eluted in the α2 region. Since only α1(I) chains were synthesized, the nature of the α1(I) chains present in excess of the 2:1 ratio expected for type I collagen was investigated. The fact that these extra α1(I) chains were resistant to pepsin digestion before and after purification and were precipitated by salt at both neutral and acid pH during purification demonstrates that these chains were in a native, helical conformation. Recently Mayne et al. (1975) have demonstrated that α1(I) chains in the conformation $[\alpha 1(I)]_3$, type I trimer, have greater solubility in salt solutions at neutral pH than α1(I) chains in the normal type I collagen conformation, $[\alpha 1(I)]_2\alpha 2$. Although we have surveyed pH from 7 to 8.5 and salt concentration from 2 to 2.6 M, we have been unable to obtain conditions that will separate all of the normal type I collagen from all of the type I trimer. However, precipitation by 2.6 M NaCl at pH 8 (50 mM Tris, 4 °C) left approximately 50% of the type I trimer in the supernatant completely separated from type I collagen. Analysis of this supernatant by $NaDodSO_4$ electrophoresis on 5% gels (Figure 9) did not detect α2 chains (type I collagen) or type III collagen; only X and Y-α1 chains were present. When the same supernatant was chromatographed on CM-cellulose (Figure 9), the α1 chains from type I trimer were completely separated from Y and X chains.

Solubility analysis also provided information about X and Y. Unlike type I trimer, few of these chains were found in the 2.6 M NaCl precipitate (data not shown). Their presence together in the supernatant suggests that they have similar solubilities or that they are components of the same helical structure. In addition, CM-cellulose chromatography of material devoid of III and α2 chains (Figure 9) verified the chromatographic heterogeneity of X indicated in Figure 2.

Extraction Characteristics of Subcultured Chondrocyte Collagens. Duplicate 75-cm^2 flasks of fourth subculture chondrocytes were labeled in order to determine the distribution of radioactive collagens in the medium and cell layer (acid extract). The labeled culture medium was removed and combined with a wash of the cell layer (37 °C serum-free DMEM). Flasks containing the attached cell layer were extracted at 4 °C for 24 h with 0.5 M acetic acid. Cell debris was removed from the acid extract by centrifugation. The medium, acid extract, and acid residue were then separately treated with pepsin and the labeled collagens were purified as described for whole cultures in Materials and Methods.

Less than 6% of the labeled collagen was found in the pepsin extract of the acid residue; this material was not further characterized. The purified collagens from the pepsin-treated medium and acid extract (cell layer) were fractionated by $NaDodSO_4$ electrophoresis on 5% gels. Data from duplicate flasks varied by less than 10% and were averaged. Calculations were used so that triple-stranded native collagen molecules

could be described rather than denatured, individual chains. These calculations were based on the following assumptions. (1) No type II collagen was synthesized by these cells (Figure 7). (2) The amount of α1(I) chains contributed by normal type I collagen can be calculated from the amount of α2 chains by using an α1:α2 ratio of 2:1. (3) X and Y form a single molecule, X_2Y, and the small amount of Y present in the Y-α1 peak can be calculated by assuming a X:Y ratio of 2:1 (unpublished data, manuscript in preparation). (4) Radioactive material present in the α1 region of $NaDodSO_4$ electrophoresis profiles which cannot be attributed to Y or α1(I) from the normal type I configuration is due to $[\alpha 1(I)]_3$, type I trimer. The quantity of each native collagen synthesized by subcultured chondrocytes and its distribution between the medium and the cell layer are shown in Figure 10. Although type I collagen and X_2Y represented the largest (36%) and the smallest (17%) fractions of the total synthesized collagen, each was incorporated into the cell layer to a similar extent (46% of the type I, 52% of X_2Y). In contrast, only 4% of the type III collagen was incorporated. Type I trimer represents a substantial fraction of the total collagen (27%). Since it differs from type I collagen by a single chain substitution, it is interesting that 30% of the type I trimer was incorporated into the cell layer. Although this is less than the incorporation of type I collagen, it still suggests biological function.

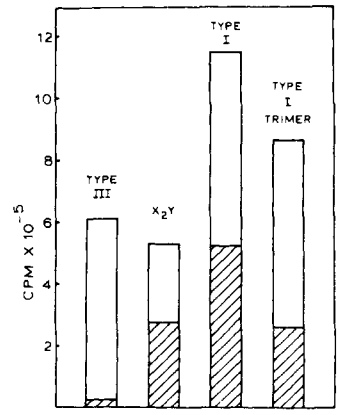

FIGURE 10: The distribution of native, radioactive collagens between the medium and the cell layer of subcultured chondrocytes. Experimental details are presented in the text. Open bars represent the medium; cross-hatched bars represent the cell layer.

Discussion

The present results demonstrate that the fourth subculture progeny of articular chondrocytes synthesize type I trimer, type I, and Type III collagen, but no longer synthesized the differentiated chondrocyte product, type II cartilage collagen. In addition to these previously known and characterized collagen chains, two new chains, X and Y, were also identified. The complete characterization of these chains by amino acid analysis, CNBr peptide analysis, and gel filtration chromatography has been completed and will be published separately. There is little question that all of these collagen chains are synthesized by the progeny of authentic chondrocytes. This conclusion is based on several lines of reasoning:

(1) Articular cartilage is a histologically homogeneous tissue (Hough and Sokoloff, 1975); in the present study only thin slices were taken from the center of the articulating surface.

(2) Cartilage slices were "washed" enzymatically to remove peripheral cells before >90% of the chondrocytes were released for primary culture.

(3) Contamination by underlying bone would not explain the results since osteoblasts have not been reported to synthesize type III or X_2Y collagen.

(4) Adult rabbit fibroblasts synthesize a different proportion of type III, X, and α2 chains than chondrocyte progeny in the present study or our previous report (Cheung et al., 1976).

(5) Most important, the amount of type III, type I, and X_2Y collagen synthesized *per cell* remains the same or declines slightly with serial subculture (manuscript in preparation). This observation is inconsistent with overgrowth by fibroblasts or any other cell type.

The collagen phenotype of subcultured chondrocytes is quite complex and sensitive to experimental conditions which can substantially alter the synthesis of one collagen type without altering the synthesis of the others. The methods presented in this paper were designed to evaluate such metabolic changes. Any intact chain that can be resolved on 5% gels can be quantitated, electrophoretically eluted, and subjected to CNBr peptide analysis. We have used this approach to identify α1(I) and α1(III) chains. Previously, CNBr peptide analysis by $NaDodSO_4$ electrophoresis in phosphate buffer (Furthmayr and Timpl, 1971) was used to identify purified type III collagen and procollagen in rat skin and human fibroblast cultures (Byers et al., 1974; Lichtenstein et al., 1975), to identify purified type II collagen in a rat chondrosarcoma (Smith et al., 1975), and to demonstrate the presence of type III collagen in insoluble bovine skin (Scott and Veis, 1976b) and the absence of type III collagen in the skin of patients with Ehlers-Danlos Syndrome type IV (Pope et al., 1975).

Since we have now verified by CNBr peptide analysis the synthesis of type III collagen by subcultured articular chondrocytes, these cells can be added to human aorta, skin, and uterine leiomyoma (Chung and Miller, 1974) and rat lung and human placenta (Epstein, 1974) as sources of type III collagen. In contrast, type III collagen was not detected in chick sternal chondrocyte cultures that had similarly lost their ability to synthesize type II collagen but synthesized type I collagen and type I trimer (Mayne et al., 1975, 1976). This may be due to the difference in specie, tissue, or technique, or to the unusual solubility characteristics of chick type III collagen.

In the present study, type III collagen synthesized by subcultured chondrocytes was found almost exclusively in the culture medium. This is not surprising since Lichtenstein et al. (1975) observed the same distribution in human fibroblast cultures. These authors demonstrated that most of this collagen was present as pro-α1(III) or the partially processed p-α1(III). Since type I collagen accumulated in the chondrocyte cell layer, it seems that the processing of type I procollagen can occur without the processing of type III procollagen. This suggests the absence or inactivity of a specific type III procollagen peptidase.

Type I trimer, originally isolated by Mayne et al. (1975, 1976), is a native, triple-stranded collagen with the conformation $[\alpha 1(I)]_3$. The extra α1(I) chains synthesized by subcultured chondrocytes in the present study are pepsin resistant and generally more soluble than type I collagen in salt solutions at pH 8.0. Thus they satisfy the criteria for type I trimer established by Mayne et al. (1975). Although X and Y were present with α1(I) chains in the 2.6 M NaCl supernatant, it is unlikely that they are involved in a helical structure with the α1(I) chains because serial subculture causes more synthesis of type I trimer but less synthesis of X and Y (manuscript in preparation). A significant proportion of the type I trimer accumulates in the cell layer indicating a functional capacity similar to the other collagens. The fact that proportionally less

type I trimer than type I collagen is found in the cell layer may reflect an influence of the substituted $\alpha 1$(I) chain on the activity of procollagen peptidase, matrix interactions, or fibril formation.

Two new collagen chains, X and Y, have been partially characterized in the present study. Both are pepsin resistant, insensitive to reduction by mercaptoethanol, and migrate between $\alpha 1$ and β chains when submitted to $NaDodSO_4$ electrophoresis on 5% gels. Y elutes from CM-cellulose as a homogeneous peak approximately where $\beta 12$ is expected. X, on the other hand, yields a complex elution pattern that is located where $\alpha 2$ and type III chains are expected. When in helical conformation, X and Y have identical solubility properties during differential salt precipitation at neutral pH. This and other accumulated data suggest that X and Y may be part of the same triple-stranded, helical structure with a stoichiometry of X_2Y. On the basis of the unambiguous distributon of X between the medium and the cell layer, the hypothetical molecule X_2Y was incorporated into the cell layer to a slightly greater degree than type I collagen, reflecting a possible physiological function.

Type III collagen is synthesized at a faster rate and is present in larger amounts during fetal development in both human skin (Epstein, 1974) and guinea-pig skin (Shuttleworth and Forrest, 1975). This collagen therefore appears to be associated with the more primitive cells of the mesenchymal cell lineage. In light of this trend, it is not particularly surprising that the synthesis of type III and type I collagen becomes evident when the progeny of chondrocytes lose their differentiated function, i.e., the ability to synthesize type II collagen. Such a mesenchymal cell collagen phenotype may merely reflect the embryonic heritage of the original chondrocytes (Abbott et al., 1974) and fits the term "dedifferentiated chrondrocyte" better than a phenotype of just type I collagen. Whether these cells can regain the collagen phenotype of differentiated chondrocytes has not been determined.

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