THE COLLAGEN OF OSTEOGENIC CARTILAGE IN THE EMBRYONIC CHICK*

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Received April 16,1973

SUMMARY

The diaphyseal region of tibiae, and vertebral bodies from 8-day chick embryos were cultured in the presence of tritiated proline and the radioactive proteins were extracted and co-purified with carrier collagen. Chromatography on carboxymethylcellulose indicated that the radioactively labelled proteins eluted as a single peak which coincided with the carrier $\alpha 1$ chains. On DEAE-cellulose, the radioactively labelled $\alpha 1$ chains eluted with authentic cartilage $\alpha 1$ (II) carrier. The transitory chondrogenic regions of the embryo thus produce a collagen molecule similar, if not identical, to the principal collagen molecule found in cartilaginous structures in the adult.

Chondrogenesis in the embryonic limb and vertebral body has been extensively used as a model for the study of differentiation. The generally accepted criteria for differentiated cartilage are characteristic cell and tissue morphology, synthesis of chondroitin sulfate, and the appearance of a metachromatically staining extracellular matrix. A potentially diagnostic criterion has come from recent studies that the collagen of adult cartilaginous tissues such as epiphysis, articular cartilage and sternum is a different gene product from that found in skin, tendon and bone (1-4).

The collagen molecule isolated from most vertebrate tissues is composed of three polypeptide chains of molecular weight 95,000 daltons. These chains consist

*This is publication #599 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities. This investigation was supported by the National Institutes of Health (training grant HD 00362 and research grant AM 3564) and the National Foundation-March of Dimes (CRBS 229).
**Recipient Faculty Research Award, American Cancer Society (PRA 107).

of two distinct types, $\alpha 1$ type I and $\alpha 2$, differing in amino acid composition and occurring in a $\alpha 1{:}\alpha 2$ ratio of 2 to 1, designated $[\alpha 1\ (I)]_2\alpha 2$. Cartilage collagen, designated $[\alpha 1\ (II)]_3$, is composed of three α chains ($\alpha 1$ type II) identical in composition, but differing from both $\alpha 1$ type I and $\alpha 2$ (1).

The discovery of this cartilage-specific collagen molecule in adult cartilages has raised the question of the timing and control of its appearance in the chondrogenic regions of developing embryos. Also during embryogenesis of the long bones and vertebrae a temporal transition from "undifferentiated" mesenchyme, through cartilage, to bone matrix occurs. As an initial step in elucidating the control mechanisms in skeletal morphogenesis, we asked whether these observed tissue changes also involve changes in collagen type.

In this report we demonstrate that the transient cartilage of the embryonic tibial diaphysis and of the vertebral bodies, at 8 days of development, are producing collagen of a type which is similar, if not identical, to that which has been isolated from adult cartilages.

MATERIALS AND METHODS

Tibial diaphyses and vertebral bodies were dissected from 8-day White Leghorn chick embryos, previously incubated at 38°C, and cleaned of all adhering tissues. Each culture consisted of 18 diaphyses or about 6 vertebral columns. The tissues were cultured overnight at 37°C in 2 ml of Dulbecco-modified Eagle's medium supplemented with 10% fetal calf serum, 50 ug/ml β-aminopropionitrile, 100 ug/ml ascorbic acid, and 50 μCi/ml ^{3}H proline.

The cultures were extracted at 4° with 0.15 M sodium phosphate pH 7.6 for 24 hours, after which 10-20 mg of previously purified lathyritic chick skin collagen was added as carrier. The extract was clarified by centrifugation and the collagen was precipitated by dialysis against several changes of cold 0.01 M Na_2HPO_4. The

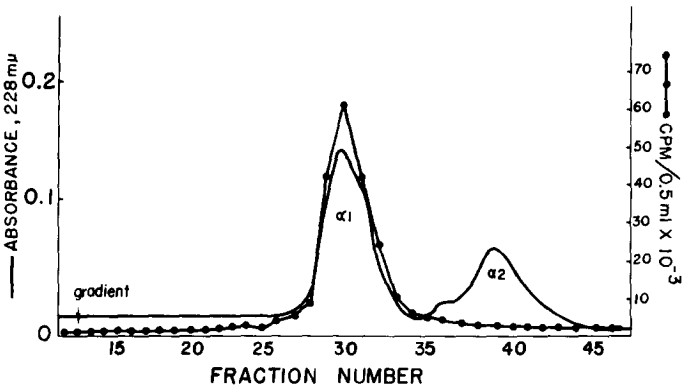

Figure 1: Chromatogram from a CM-cellulose column of the radioactive material purified from the diaphyseal region of the 8-day chick embryo tibia and unlabelled 3-week old lathyritic chick skin collagen carrier. Absorbance, solid line; radioactivity, dotted line.

precipitate was dissolved in 0.5 M HAc, the collagen reprecipitated by the addition of NaCl to a final concentration of 10% and redissolved in 0.5 M HAc.

Carboxymethylcellulose (CM-cellulose) chromatography of the thermally denatured collagen was performed using 0.9 x 5 cm columns jacketed at 42° C according to Piez et al. (5) modified to use a starting buffer of 0.02 M Na acetate pH 4.86, containing 1 M urea and a superimposed, linear gradient of 0 to .13 M NaCl (total gradient volume 200 ml).

DEAE-cellulose chromatography of the $\alpha 1$ chains isolated by CM-cellulose chromatography was performed according to Trelstad et al. (6). The material was loaded in .004 M tris pH 9.7 and was eluted with a stepwise pH gradient as described in the legend of Figure 4.

Molecular weights were estimated from the elution positions from a calibrated 90 x 2 cm column of 8% Agarose, equilibrated with 1 M $CaCl_2$ and .05 M tris-HCl pH 7.5 as described previously (7).

The distribution of radioactivity between hydroxyproline and proline within the isolated α chains was determined after hydrolysis at 108° for 24 hours in 6N HCl. The amino acids were separated and identified on a Jeolco model 5AH amino acid

analyzer using the single column method (8). A portion of the effluent was diverted by stream splitting and collected for radioactivity measurement in 10 ml of Aquasol (NEN) using a Beckman model LS-230 scintillation counter.

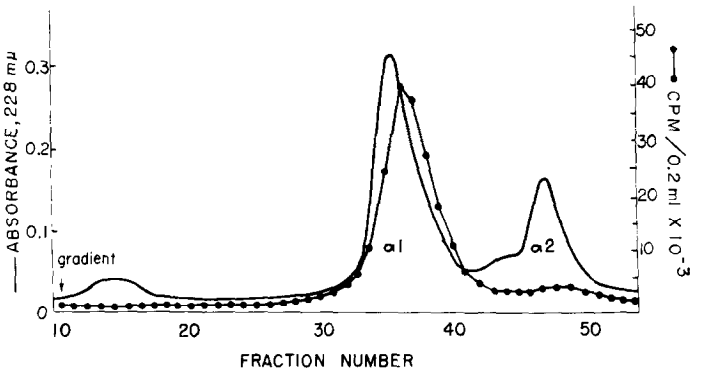

Figure 2: Chromatogram from a CM-cellulose column of the radioactive material isolated from the 8-day vertebral bodies and unlabelled chick skin collagen carrier. Absorbance, solid line; radioactivity, dotted line.

RESULTS

Figure 1 is a representative example of the radioactivity profile obtained from CM-cellulose chromatography of the denatured collagen extracted from 8-day tibial diaphyses. The carrier (solid line) shows the $\alpha 1$ and $\alpha 2$ peaks in their characteristic 2 to 1 ratio, and a small amount of the crosslinked dimer β_{12}. The radioactively labelled fraction elutes as a single peak coinciding with the $\alpha 1$ of the carrier. In none of the cultures examined was any radioactivity present in the $\alpha 2$ region. Analysis of the relative labelling of hydroxyproline and proline in the isolated $\alpha 1$ chain, by hydrolysis and separation on the amino acid analyzer, indicated that 45% of the incorporated proline had been converted to hydroxyproline. This is the value commonly found for pure collagen α chains (2).

The CM-cellulose chromatograms obtained from the purified, radioactively labelled collagen from the 8-day vertebral bodies (Figure 2) were similar to those of the tibial diaphysis. Almost all the radioactivity eluted as a single peak, again

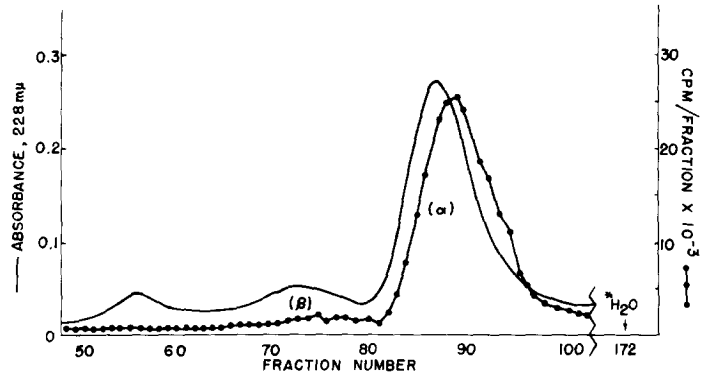

Figure 3: Elution pattern from calibrated 8% Agarose column of unlabelled chick skin carrier collagen and a portion of the radioactive material from the radioactivity peak shown in Figure 1.

coinciding with $\alpha 1$ chain of the skin carrier, with only a trace in the $\alpha 2$ region.

Portions of the radioactive peaks representing $\alpha 1$ (Figure 1) were desalted, lyophilized, dissolved in 1 M $CaCl_2$ and applied to an 8% Agarose column along with 5 mg of chick skin collagen and tritiated water. The radioactively labelled α chain eluted as a single peak in the region of the carrier α chains (Figure 3).

Further identification of the α chains was accomplished by chromatography of the radioactive peak eluted from CM-cellulose, plus 5 mg of unlabelled chick sternal cartilage α chains, on a warm DEAE-cellulose column at alkaline pH (6). The carrier profile (Figure 4, solid line) shows a small peak eluting with the pH 8.5 buffer which represents the $\alpha 1$ (I) of the skin carrier collagen. The larger peak eluting after the pH 6.0 buffer step represents the carrier cartilage collagen $\alpha 1$ (II) chains. Essentially all of the radioactivity eluted with the cartilage $\alpha 1$ (II) chains.

DISCUSSION

We have shown here that in the 8-day chick embryo both the diaphyseal region of the tibia and the vertebral bodies are producing a collagen similar or identical to the predominant species in adult cartilage. The radioactivity profiles from CM-cellulose chromatography revealed the collagen to consist solely of one type of α

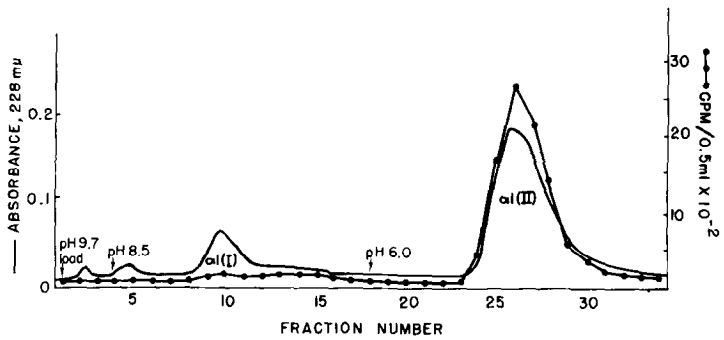

Figure 4: Chromatogram from DEAE-cellulose of 10% of the material eluting in the $\alpha 1$ region of the CM-cellulose chromatogram in Figure 1 and 5 mg of unlabelled cartilage type α chains. The material was loaded in .004 M tris pH 9.7 and was eluted with 2 sequential buffer changes; one pH 8.5, the other pH 6.0. Absorbance, solid line; radioactivity, dotted line.

chain which elutes with the $\alpha 1$ chain of the skin collagen carrier. In chromatograms of the vertebral bodies the small, variable amount of radioactivity which eluted in the position of the carrier $\alpha 2$ chain most probably was derived from some contaminating, non-chondrogenic mesenchymal cells (which we have found to produce $(\alpha 1)_2 \alpha 2$ collagen).

Additional evidence that the $\alpha 1$ chains represent $\alpha 1$ (II) is provided by co-chromatography of the labelled material with cartilage carrier $\alpha 1$ (II) on DEAE-cellulose. However, since $\alpha 1$ (I) chains rich in hydroxylysine may also elute late(6) from DEAE columns,a cyanogen bromide peptide profile of the labelled collagen would further assist in its characterization (Miller et al.) (9).

Studies in progress indicate several transitions in collagen type during limb development: from $(\alpha 1)_2 \alpha 2$ in the undifferentiated limb bud mesenchyme (prior to 4 1/2 days incubation); to solely $\alpha 1$ $[(II)]_3$ in the tibial diaphysis by 8 days as shown here; to the appearance of $[\alpha 1 (I)]_2 \alpha 2$ by 10 days, associated with the appearance of bone matrix.

So far, in both the adult and in the embryo, all of the cartilaginous tissues that have been examined have been found to produce $[\alpha 1 (II)]_3$ collagen. It seems, there

fore, that the production of $[\alpha 1\ (II)]_3$ is indeed one of the differentiated functions of cartilage, both in the stable cartilaginous regions in the adult and in the transitory chondrogenic regions in the embryo. The possibility that $[\alpha 1\ (II)]_3$ might be produced by non-cartilaginous tissues has been suggested (10) but as yet has not been confirmed.

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