THE COLLAGEN OF CHICK EMBRYONIC NOTOCHORD*

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Received May 9, 1973

SUMMARY

Notochords, isolated from 2 1/2 day chick embryos, were cultured in the presence of ^3H proline and the labeled proteins co-purified with chick skin carrier collagen. The purified material, most of which eluted from CM-cellulose as a single peak in the region of the carrier collagen $\alpha 1$ chain, contained 41% of the incorporated proline as hydroxyproline and from gel filtration measurements had a molecular weight of approximately 100,000 daltons. When the material was chromatographed on DEAE-cellulose with carrier $\alpha 1$ chains from both skin [$\alpha 1$ (I)] and cartilage [$\alpha 1$ (II)], it eluted predominantly with the cartilage chains.

The interaction between embryonic notochord or neural tube and somitic mesenchyme greatly promotes the formation of cartilage both in mixed organ culture (1) and during the development of vertebral bodies in vivo (2, 3). The possibility that collagen could be involved in such embryonic interactions has been raised by several investigators (4-6). It is therefore of interest that the embryonic neural tube, one of the inducing tissues, produces a collagen (6) which consists of only one type of α chain (7) thus resembling that of cartilage [$\alpha 1$ (II)]$_3$ (ref. 8) and basement membrane (9). We have recently shown that 8 day chick embryonic vertebral bodies, derived from the differentiated somitic mesenchyme, are also producing

*This is publication #600 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 (GRBS 229).

**Recipient Faculty Research Award, American Cancer Society (PRA 107).

cartilage collagen consisting solely of a single type of α chain (10). The collagen of most other tissues so far investigated contain two different α chains, $\alpha 1$ (I) and $\alpha 2$ in a 2:1 ratio, for example the $[\alpha 1\ (I)]_2 \alpha 2$ of skin and bone.

Here we present evidence that the notochord, the other tissue capable of influencing somite chondrogenesis, also produces a collagen consisting predominantly, if not exclusively, of an $\alpha 1$-type α chain.

MATERIALS AND METHODS

Trunks were dissected from White Leghorn chick embryos, previously incubated at 38° for 2 1/2 days and suspended in a solution of 1% trypsin (Difco 1:250) in Tyrodes saline for 1 hour at 4°. The component tissues were separated by gentle pipetting and the isolated notochords (2-3 dozen) pooled and washed several times with 20% calf serum. They were cultured overnight at 37° in 1 1/2 ml of Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum, 50 μg/ml β-aminopropionitrile, 100 μg/ml ascorbic acid and 50 μCi/ml ^{3}H-proline (NEN-323).

The labeled proteins were extracted at 4° in 0.4 Γ/2 phosphate buffer pH 7.6 after which 10 to 20 mg of lathyritic chick skin collagen was added as carrier. The extract was clarified by centrifugation and the collagen precipitated by dialysis against 0.01 M Na_2HPO_4. The precipitate was dissolved in 0.5 M HAc, the collagen reprecipitated by the addition of solid NaCl to 10%, and then redissolved in 0.5 M HAc.

Carboxymethylcellulose (CM-cellulose) chromatography of the denatured collagen was performed according to Piez et al. (11) with a modified starting buffer (Figure 3, legend). DEAE-cellulose chromatography of the $\alpha 1$ chains was performed according to Trelstad et al. (12). Molecular weights were estimated by the elution position from a calibrated 90 x 2 cm column of 8% Agarose, equilibrated with 1 M $CaCl_2$ and 0.01 M tris-HCl pH 7.5 (13).

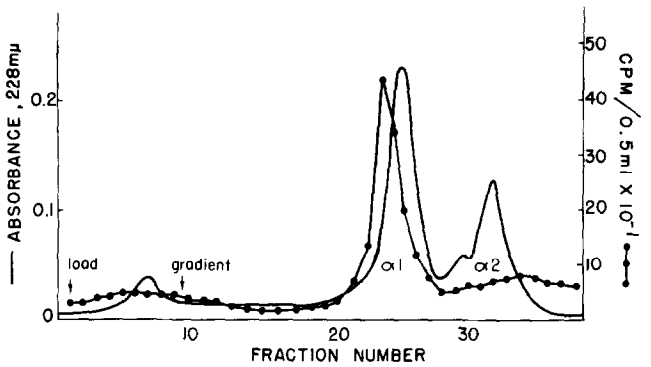

Figure 1: Chromatogram from a CM-cellulose column of the radioactive material purified from the 2 1/2 day embryonic notochords and lathyritic chick skin collagen carrier. The sample was loaded onto the column (0.9 x 5 cm jacketed at 42°) in a starting buffer of 0.02 M Na acetate pH 4.86, containing 1 M urea, and was eluted with a superimposed, linear gradient of 0 to .13 M NaCl (total gradient volume 200 ml). Absorbance, solid line; radioactivity, dotted line.

The distribution of radioactivity between proline and hydroxyproline was determined after hydrolysis (108°, 24 hours, 6 N HCl under nitrogen). The amino acids were separated (14) on a Jeolco model 5 AH amino acid analyzer from which a portion of the effluent was diverted by stream splitting for radioactivity measurement.

All radioactivity counting was performed in 10 ml of Aquasol (NEN) using a liquid scintillation counter with a tritium counting efficiency of 60%.

RESULTS

Figure 1 represents a typical profile of a chromatogram obtained by CM-cellulose chromatography of the purified, denatured radioactively labeled protein from notochords (dotted line), plus skin collagen carrier (solid line). The carrier collagen shows the $\alpha 1$ and $\alpha 2$ chains in their characteristic 2 to 1 ratio, and a small amount of the β_{12} dimer as a shoulder preceding the $\alpha 2$ fraction. The labeled notochord material elutes predominantly as a single peak (fractions 20 to 28) in the region of the carrier $\alpha 1$ chain while a small variable amount of label trails after the carrier $\alpha 2$ chain. Subsequent hydrolysis of the labeled material

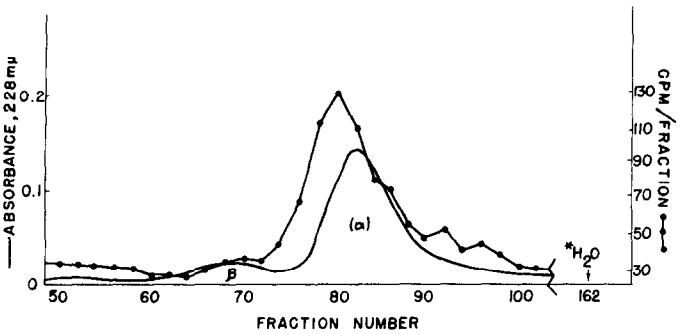

Figure 2: Elution pattern from calibrated 8% Agarose column of the $\alpha 1$ peak (fractions 20 to 28) shown in Figure 1. Absorbance, solid line; radioactivity, dotted line.

and separation on the amino acid analyzer showed that in the labeled peak coinciding with the carrier $\alpha 1$ chain, 41% of the incorporated proline had been converted to hydroxyproline. The small quantity of labeled material trailing after the main peak (Figure 1, fractions 29 to 38) was found, in different preparations, to contain some hydroxyproline (hypro:pro, 0.2-0.3).

The desalted and lyophilized labeled $\alpha 1$ chains, when chromatographed on an 8% Agarose column (Figure 2), eluted as a single peak near the α chain of the carrier (solid line). The elution position relative to that of tritiated water, indicated a molecular weight of approximately 100,000 daltons.

To further characterize the labeled material, the $\alpha 1$ chain peak from CM-cellulose, plus 5 mg of unlabeled sternal cartilage collagen α chains [$\alpha 1$ (II)] were chromatographed on DEAE-cellulose (Figure 3). Most of the labeled material (dotted line) eluted along with the cartilage α chain [$\alpha 1$ (II)] peak (after the pH 6.0 buffer change), and only a small amount of labeled material eluted with the skin carrier $\alpha 1$ chain [$\alpha 1$ (I)] (after the pH 8.5 buffer change).

DISCUSSION

We have demonstrated here that the chick notochord is producing collagen at a time during embryonic development when it is actively promoting chondrogen-

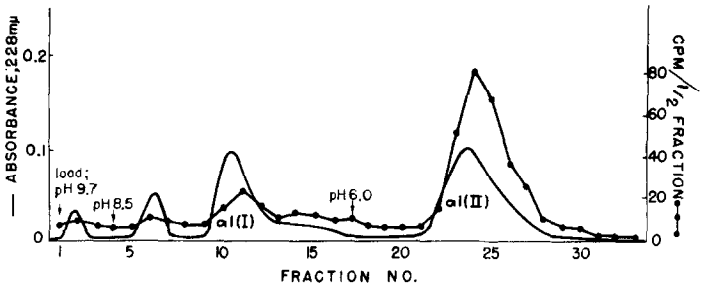

Figure 3: Chromatogram from DEAE cellulose of the $\alpha 1$ peak shown in Figure 1 and 5 mg of unlabeled cartilage type α chains. The material was loaded in .004 M tris pH 9.7 and was eluted with 2 sequential buffer changes; one pH 8.5, and the other pH 6.0. Absorbance, solid line; radioactivity, dotted line.

esis in the adjacent somitic mesenchyme (1). This collagen has approximately the same molecular weight and degree of proline hydroxylation as most collagen chains which have been described (15) and is composed predominantly, if not completely, of one type of α chain which co-chromatographs on CM-cellulose with the $\alpha 1$ chain type. Thus the collagen produced by the notochord is probably composed of 3 identical $\alpha 1$ chains $(\alpha 1)_3$ as is the collagen of cartilage $[\alpha 1\ (II)]_3$ (8) and basement membrane (9). The notochordal molecule isolated here does not, however, seem to be a basement membrane type collagen since it contains only about 1% of the total labeled hydroxyproline as the 3-isomer. All of the basement membrane collagens which have been isolated contain a large amount of 3-hydroxyproline (16, 17).

The radioactive material trailing after the carrier $\alpha 2$ peak in Figure 1 (always less than 10% of the amount of label in the $\alpha 1$ chain peak) may represent a small amount of embryonic $\alpha 2$ chain, since it does contain hydroxyproline. The late elution position of the labeled $\alpha 2$ would be consistent with our observation that the $\alpha 2$ chain of the collagen of embryonic limb buds elutes considerably later than the $\alpha 2$ chain of the chick skin carrier (in preparation). The presence of some $\alpha 2$ chain is also consistent with the observation that, on DEAE chromatography a

small amount of the notochordal $\alpha 1$ chain peak co-chromatographed with the skin $\alpha 1$ (I) (Figure 3). It may be, therefore, that some $[\alpha 1\ (I)]_2 \alpha 2$ type collagen molecules were also produced in the cultures and that either the notochord is producing two different types of collagen molecules, or a minor cellular contaminant was present in the cultures.

The observation that on DEAE cellulose most of the notochordal $\alpha 1$ chain material co-chromatographs with cartilage type α chains, does not necessarily establish the identity of notochordal collagen with that of cartilage since we know that highly hydroxylated $\alpha 1$ (I), for example, can also elute late from DEAE columns (12). This result does, however, raise the possibility that both the cartilage and notochordal molecules are the same, in which case the "inducing" tissue (notochord) would be producing the same molecule found in the responding tissue (vertebral bodies) after differentiation (10). Alternatively, the notochordal molecule may represent a differentiated function of notochord tissue itself and be a collagen different from those previously described. To test this we are currently comparing cyanogen bromide peptide profiles of the collagens (18) of the embryonic notochord, skin and vertebral cartilage.

ACKNOWLEDGMENT

We wish to thank Bryan Toole for helpful suggestions during this study.

REFERENCES

1. Lash, J.W., In "Epithelial-Mesenchymal Interactions" (eds. R. Fleischmajer and R. Billingham), p. 165 (1968).
2. Holtzer, H. and Detwiler, S., J. Exp. Zool. 123, 335 (1953).
3. Watterson, R.L., Fowler, I. and Fowler, B.J., Amer. J. Anat. 95, 337 (1954).
4. Konigsberg, I.R. and Hauschka, S.D., In "Reproduction: Molecular, Subcellular and Cellular" (ed. M. Locke), p. 243 (1965).
5. Grobstein, C., Nat. Can. Inst. Monogr. 26, 279 (1967).
6. Cohen, A.M. and Hay, E.D., Develop. Biol. 26, 578 (1972).
7. Trelstad, R.L., Kang, A.H., Cohen, A.M. and Hay, E.D., Science 179, 295 (1972).
8. Miller, E.J. and Matukas, V.J., Proc. Nat. Acad. Sci. U.S.A. 64, 1264 (1969).

9. Kefalides, N.A., Biochem. Biophys. Res. Comm. 45, 226 (1971).
10. Linsenmayer, T.F., Trelstad, R.L., Toole, B.P. and Gross, J., Biochem. Biophys. Res. Comm. In press.
11. Piez, K.A., Eigner, E.A. and Lewis, M.S., Biochemistry 2, 58 (1963).
12. Trelstad, R.L., Kang, A.H., Toole, B.P. and Gross, J., J. Biol. Chem. 247, 6469 (1972).
13. Piez, K.A., Anal. Biochem. 26, 305 (1968).
14. Kang, A.H., Biochem. 11, 1828 (1972).
15. Trelstad, R.L., Kang, A.H., Igarashi, S. and Gross, J., Biochemistry 9, 4993 (1970)
16. Grant, M.E., Kefalides, N.A., and Prockop, D.J., J. Biol. Chem. 247, 3539 (1972).
17. Kang, A.H. and Trelstad, R.L., J. Cell. Biol. 55, 129a (1972).
18. Miller, E.J., Woodall, D.L. and Vail, M.S., J. Biol. Chem. 248, 1666 (1973).