SYNTHESIS OF TYPE I AND TYPE II COLLAGEN BY EMBRYONIC CHICK CARTILAGE

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Summary. Embryonic chick articular and keel cartilage was found to synthesize two types of collagen. The amount of Type I collagen synthesis decreased from 60% to nearly 10% during the embryonic period studied, thus suggesting not only coexistence of both collagen types in the same tissue, but also a developmental transformation from predominantly Type I synthesis to Type II synthesis with cartilage development and maturation. Radioautographs suggested that all chondrocytes were equally active in collagen synthesis and failed to show any significant non-cartilagenous tissue contamination. Therefore variation in collagen type synthesis must be a product of some unknown genetic regulatory mechanism within the cartilage tissue.

Introduction

The collagen present in cartilagenous tissues represents a genetically distinct macromolecular species consisting of three identical polypeptide chains (1-3). This macromolecule $[\alpha_1(II)]_3$ may readily be distinguished from collagen of skin, bone and tendon since, in the latter case, two α_1 and one α_2 polypeptide chains provide the basic macromolecular structure. The identification of α_2 polypeptide chains in collagen samples therefore suggests the Type I collagen, whereas in the case of cartilage collagen (Type II), no α_2 should be present. The α_1 polypeptide chains of Type I and Type II can also be differentiated, the latter containing greater amounts of hydroxylysine and hexose-linked hydroxylysine (1-4) as well as different methionine distribution within the polypeptide chain (5,6). These properties provide for different elution properties on DE-52 cellulose and cyanogen bromide peptide fragmentation.

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The consistent appearance of small amounts of Type I collagen in cartilage collagen preparations (1,2,7) has raised the possibility that two collagen types, Type I and Type II, may coexist as an integral part of this tissue in much the same manner as Type I, and still another genetically distinct collagen, Type III, coexists in skin and dentine (8,9). The recent report that adult human articular cartilage synthesizes predominantly Type II collagen while osteoarthritic articular cartilage synthesizes only Type I collagen further emphasizes that variation in collagen type, i.e. Type I vs. Type II, may be involved with some cartilagenous diseases (10).

This investigation was therefore designed to determine whether the Type I collagen previously found with Type II in cartilage preparations was the result of contamination from surrounding tissue or an integral part of the cartilage tissue itself. The use of short-term organ culture provided a method by which the synthetic product of the cartilage tissue at a specific time could be determined instead of analysis of the total tissue which represents a much longer period of growth. With this method, it was established that both collagen types are synthesized simultaneously by embryonic and post-hatched chick articular and keel cartilage. Furthermore there was an apparent transformation from predominantly Type I to predominantly Type II collagen synthesis with embryonic development.

Methods and Materials

Cartilage collagen synthesis was investigated by incubating sterile keel or articular cartilage from the proximal tibia in modified Eagle's media (Gibco) supplemented with 150 μg/ml ascorbate and 10% fetal calf serum (Gibco). Lathyrism was induced by incubation in 64 μg/ml of β-aminoproprionitrile fumarate (BAPN[1], Aldrich Chemical Co.). Tissues were preincubated in ascorbate and BAPN for one hour prior to addition of 200 μc/ml of [2,3^{3}H]-L-proline (New England Nuclear, 1 c/mm) and 10 μc/ml of [^{14}C]-L-lysine (New England Nuclear, 260 mCi/mm) at 37.5°C and 5% CO_2, 95% air. Unless otherwise specified, tissues were

[1] BAPN = β-aminoproprionitrile.

incubated with radioactive label for 12 hours. These conditions were determined to be suitable based on linear incorporation of [^{3}H]-L-proline, [^{3}H]-thymidine, [^{3}H]-uridine and histological appearance of the tissue. The fact that less than 1% of the collagen was found in the media and less than 1% of the TCA[1] soluble [^{3}H]-L-proline was hydroxylated indicated little if any collagen turnover during the 12-hour interval.

After incubation, the tissues were washed twice with physiological saline and extracted with 1.0 NaCl, pH 7.0 for three days at 4°C to obtain newly synthesized lathyritic collagen. This salt-soluble collagen was chromatographed on CM cellulose according to the procedure of Miller (4) to obtain separation of α_1 and α_2 polypeptide chains. Relevant fractions were pooled, dialyzed against 3 per cent acetic acid and freeze-dried.

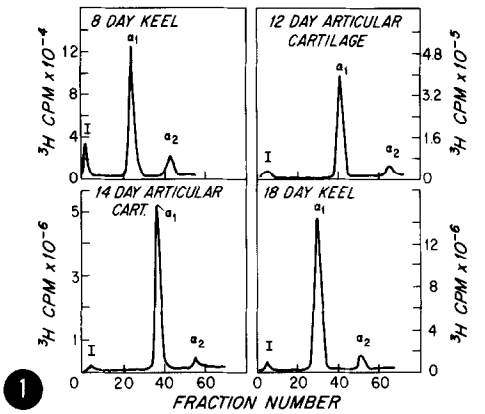
In order to ascertain the presence of both α_1(I) and α_1(II) in the isolated α_1 peak, samples were chromatographed on DE-52 cellulose according to the procedure of Trelstad et al. (11) except that 1.3 M urea was included in all the buffers.

Results and Discussion

Embryonic chick keel and articular cartilage collagen synthesis was measured using short-term organ culture methods which allowed identification of the collagen produced at a specific time of development. The simultaneous incorporation of [^{3}H]-proline and [^{14}C]-lysine into α chains enabled analysis of both collagen types as well as degree of hydroxylation of lysine and proline residues.

The use of BAPN as a lathyritic agent insured all the newly synthesized collagen could be extracted with 1 m NaCl. This material, together with large amounts of unlabelled lathyritic chick skin carrier collagen, was separated into its respective α_1 and α_2 components by CM cellulose chromatography (fig. 1). The initial peak (I) was found to contain mainly non-collagenous proteins (hydroxyproline : proline = 1:20). The total counts eluting with lathyritic

[1] TCA = trichloroacetic acid = CCl_3COOH



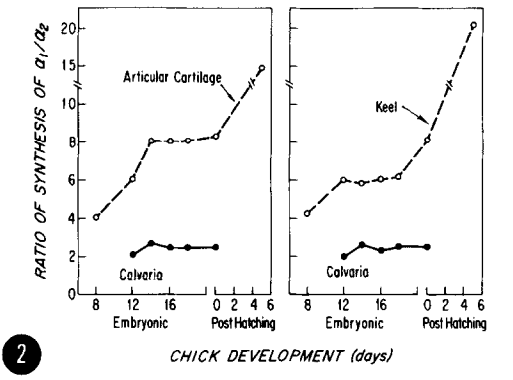


Figure 1. CM-cellulose chromatography of newly synthesized collagen from lathyritic chick articular and keel cartilage. Tissue was incubated with [^{3}H]-proline and BAPN for 12 hours, then extracted with 1 M NaCl. Chromatography was achieved using 0.9 x 10 cm columns of CM cellulose at 42°C previously equilibrated with 0.03 M sodium acetate buffer, pH 4.8 in 1.3 M urea. The linear salt gradient of 125 ml of the above starting buffer and 125 ml limiting buffer (starting buffer plus .1 M NaCl) at a flow rate of 30 ml per hour was used. The position of α_1 and α_2 polypeptide chains were determined by 230 mμ absorption of 25 mg carrier collagen (lathyritic chick skin) co-chromatographed with the newly synthesized 1 M NaCl extract. The total counts under each peak was used to determine the α_1:α_2 ratio.

Figure 2. A graphical representation of the α_1:α_2 ratio of newly synthesized collagen from embryonic chick articular and keel cartilage at different ages. The α polypeptide chains were isolated by CM-cellulose chromatography and total counts of each chain measured. The α_1:α_2 ratio was plotted with respect to embryonic age with bone collagen (calvaria) with a normal α_1:α_2 synthetic ratio of 2:1 used as a control.

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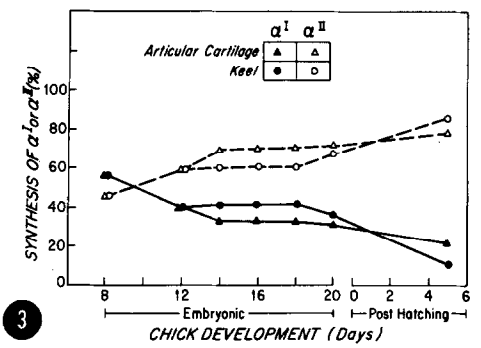
chick skin carrier α_1 and α_2 were used to determine the corresponding α_1:α_2 ratio. When plotted with respect to age of the embryo, a progressive increase in α_1 content over that of α_2 was noted (fig. 2). Approximately parallel curves were found for both articular and keel cartilage collagen. With the assumption of one α_2 chain per two α_1 chains in Type I collagen and three α_1 chains in Type II collagen, the α_1:α_2 ratio was used to determine the per cent Type I collagen vs. Type II collagen (fig. 3). From this graph it is readily apparent that a transformation from Type I synthesis to Type II synthesis does exist going from approximately 40% Type II at 8 days to 60-70% with 18-day cartilage. Since fusion of mesenchymal cells to form the keel occur at day 7.5 to 8 (12), this represents the earliest possible chondrogenic tissue available for this organ.



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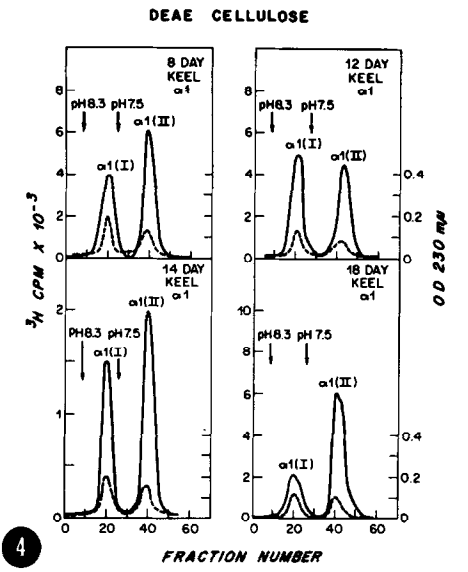

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Figure 3. Schematic representation of Type I vs. Type II newly synthesized collagen from embryonic chick articular and keel cartilage with respect to age. The content of each collagen type was calculated from the previous $\alpha_1 : \alpha_2$ ratio (fig.2) with the assumption that Type I collagen, $[\alpha_1(I)]_2\alpha_2$, was the only source of α_2, and the macromolecular structure of Type II collagen was $[\alpha_1(II)]_3$.

Figure 4. DE-52 cellulose chromatography of newly synthesized α_1 collagen from embryonic chick articular and keel cartilage. The α_1 polypeptide chains were previously isolated by CM-cellulose chromatography, then dialyzed against 3 per cent acetic acid, freeze-dried, and dialyzed again against 0.004 M Tris·HCl, pH 9.7 in 1.3 M urea before application on a 0.9 x 5 cm DE-52 cellulose column at 42°C. The eluting buffers consisted of the following: a) starting buffer, 0.004 M Tris·HCl, pH 9.7 in 1.3 M urea, b) first step change, 0.004 M Tris·HCl, pH 8.3 in 1.3 M urea which eluted $\alpha_1(I)$ and c) second step change, 0.004 M Tris·HCl, pH 7.5 in 1.3 M urea to elute $\alpha_1(II)$. The position of $\alpha_1(I)$ and $\alpha_1(II)$ was determined by 230 mμ absorption using 10 mg lathyritic chick skin $\alpha_1(I)$ and 10 mg pepsin solubilized calf articular $\alpha_1(II)$ cartilage collagen as carrier. Total counts under each peak were used to determine Type I:Type II collagen ratio assuming two $\alpha_1(I)$ polypeptide chains per Type I macromolecule and three $\alpha_1(II)$ polypeptide chains per Type II macromolecule. The per cent synthesis of Type I collagen is a solid line, Type II collagen a dotted line. Recoveries were equivalent to those obtained by CM-cellulose chromatography as reported by Trelstad, et al. (11).

These results therefore demonstrate synthesis of Type II cartilage collagen during very early chondrogenesis of the keel, but not as an all-or-none phenomenon. During this period of embryological cartilage development, a progressive increase of Type II collagen over Type I was noted so that by 5 days post-hatch, 80-90% of the collagen produced is represented by the Type II cartilage collagen.

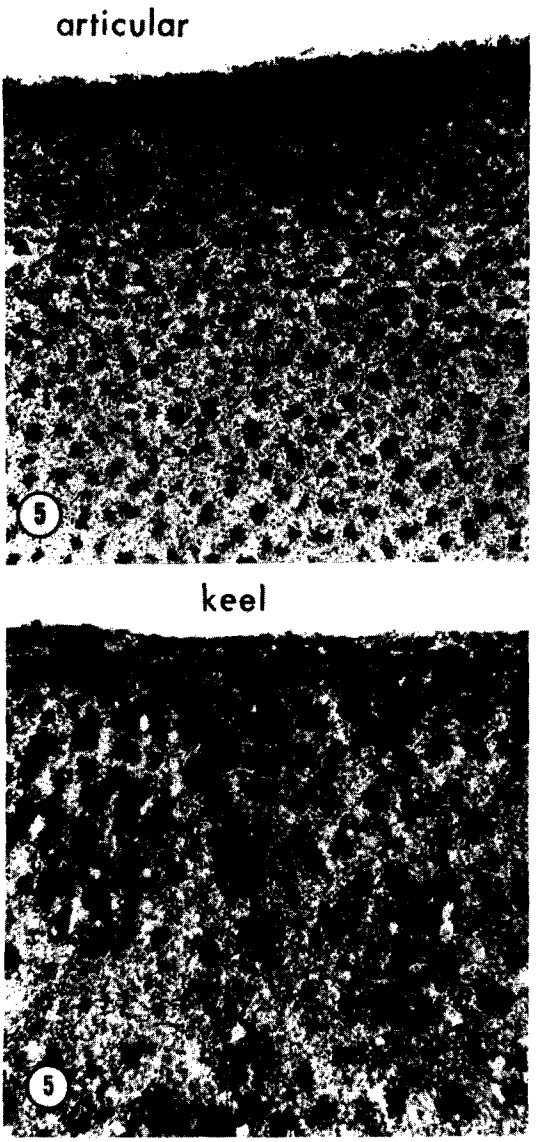

Figure 5. Autoradiographs of Bouins fixed [^{3}H]-proline labelled 14-day embryonic keel and articular cartilage, using Kodak NTB-2 emulsion, reveal a relatively uniform grain density and no perichondrial or non-cartilagenous contaminants. As collagen represents the dominant proline labelled protein (75%, see fig.1) and 40% of this collagen synthesis is Type I, Type I collagen synthesis is normal in these cartilages. The lack of increased grain density along the tissue edge obviates response to tissue damage as a source of Type I collagen synthesis.

In order to further substantiate the identification of Type I and Type II collagen, the α_1 components previously isolated (fig. 1) were further separated into α_1(I) and α_1(II) by DE-52 chromatography (fig. 4). The ratio of α_1(I) to

Table 1

Hydroxylation of Collagen α Chains[1]

	α_1(I)	α_1(II)	α_2
Proline	253,100	496,900	55,200
Hydroxyproline	112,000	219,200	124,000
% Hydroxylation	44.6	44.1	44.3
Lysine	2,410	5,030	459
Hydroxylysine	126	3,076	148
% Hydroxylation	19.2	61.3	32.2

(1) Eleven-day embryonic chick keel were incubated with [^{3}H(3,4)]-L-proline and [^{14}C]-L-lysine for 12 hours. Separation of α_1 and α_2 chains was accomplished by CM-cellulose chromatography and the pooled α_1 chains further separated into α_1(I) and α_1(II) by DE-52 cellulose chromatography. Automatic amino acid analysis using a split effluent stream analyzer was used to separate amino acids of acid hydrolyzate of each α chain so that radioactive labelled proline, hydroxyproline, lysine and hydroxylysine could be measured.

α_1(II) in this case agreed very well with the amount of Type I and Type II collagen calculated from the α_1:α_2 ratio (fig. 3). For instance, at 16 days, the articular cartilage α_1:α_2 ratio was 8:1, representing 30% Type I collagen and 70% Type II. The α_1(I) to α_1(II) ratio by DE-52 chromatography was 75% Type II and 25% Type I.

The final criterion used to substantiate the identification of α_1(I) and α_1(II) was based on the degree of lysine hydroxylation (table 1). The DEAE-cellulose purified α_1(I) and α_1(II) showed the same level of hydroxylation of [^{14}C]-lysine residues *in vitro* (17.2% and 61.3%, respectively) as Trelstad *et al.* (2) had reported for amino acid compositions of α_1(I) and α_1(II) chains. The same agreement was found for α_2 chains, having 32.2% of lysines hydroxylated. All three chains had the expected level of proline hydroxylation (44%). Thus synthesis of both Type I and Type II collagen has been established by three independent criteria: (1) ratio of synthesis of α_1 to α_2 chains, (2) identification of α_1(I) and α_1(II) chains, and (3) level of lysine hydroxylation. Auto-

radiographs of 14-day keel and articular cartilage labelled with [^{3}H]-proline (fig. 5) reveal that Type I collagen synthesis by these cartilages is a normal synthetic event due neither to perichondrial or non-cartilagenous contamination nor a response to tissue injury.

The above study has therefore demonstrated that two collagen types, Type I and Type II, are synthesized simultaneously by embryonic and post-hatch chick cartilage. There was a direct correlation between the ratio of Type I vs. Type II synthesis with respect to age or embryonic development. An obvious analogy which can be drawn here lies in the similar transformation found with fetal vs. adult hemoglobin. If Type I cartilage collagen contains a different affinity or association with proteoglycan components, it may very well play an important role in the initial cartilage matrix deposition. If this were the case, certain cartilagenous diseases may well be a function of this initial cartilage collagen, Type I vs. Type II collagen ratio.

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