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Deposition of Type X Collagen in the Cartilage Extracellular Matrix

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In cultured chick embryo chondrocytes, type X collagen is preferentially deposited in the extracellular matrix, the ratio between type II and type X collagen being about 5 times higher in the culture medium than in the cell layer. When the newly synthesized collagens deposited in slices from the epiphyseal cartilage of 17-day-old embryo tibiae were isolated, type X collagen was always the major species. In agreement with this result the mRNA for type X collagen was the predominant mRNA species purified from the same tissue. When the total collagen (unlabeled) deposited in the epiphyseal cartilage was analyzed, it was observed that type X collagen represented only 1/15 of the type II collagen recovered in the same preparation. The possible explanations for these differences are discussed.

Key words: type X collagen, chondrocyte differentiation, extracellular matrix

We have shown that cultured chondrocytes, obtained from tibiae of 17-day-old chick embryos, synthesize, in addition to type II and other minor collagens, a new low molecular weight (64 K) collagen [1] recently classified as type X [2]. The synthesis of the same collagen has been described also by two other groups starting from tibial [3] and sternal chondrocytes grown in tridimensional collagen gels [4]. The native type X collagen contains a triple helix domain and a noncollagenous globular domain located at one end of the molecule [5]. By limited pepsin digestion the noncollagenous domain is removed and the molecule is converted to a form with 45-K molecular weight chains (Fig. 1) [6,7]. When correctly hydroxylated, type X collagen is deposited in the extracellular matrix of cultured chondrocytes. Apparently, in the extracellular matrix the conversion to the 45-K form does not occur, but there is evidence that within few hours type X collagen is matured into a 30-K fragment released in the medium [8].

Type X collagen must be considered a marker of chondrocytes at late stages of differentiation. It is not synthesized by chondrocytes from tibiae at early stages of

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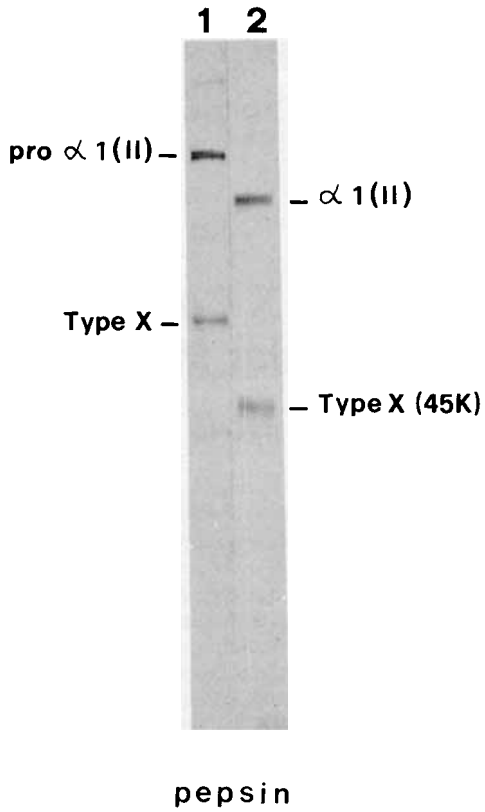

Fig. 1. Limited pepsin digestion of the collagens secreted by cultured chondrocytes. The medium of cells continuously labeled with [^{3}H] proline for 12 hr was dialyzed and analyzed on SDS gel electrophoresis before and after digestion with 0.1 mg/ml pepsin at 4°C for 8 hr.

development but is the major product of chondrocytes concentrated at the diaphysis of 9-day-old and at the epiphysis of 17-day-old embryo tibiae, regions characterized by a remodeling of the cartilage matrix leading to the replacement of the cartilage by the bone [9]. In addition the synthesis of type X collagen and other specific differentiation markers is blocked in Rous sarcoma virus-infected chondrocytes [10]; the synthesis of type X collagen is also blocked in MC29 virus-immortalized chondrocytes still synthesizing type II collagen and cartilage specific proteoglycans [11].

The evidence that type X collagen is deposited in the extracellular matrix in vivo has been obtained by the purification of the 45-K form from the pepsin digest of the epiphyseal cartilage of 17-day-old embryo tibiae [2,8,12].

MATERIALS AND METHODS

Culture and Labeling of the Cells

Chondrocytes were obtained from tibiae of 17-day-old chick embryos and grown in the absence of ascorbic acid as described [1].

For [^{35}S]methionine labeling, chondrocytes were grown for 24 hr in the presence of 50 μg/ml ascorbic acid. Cells were then preincubated for 2 hr in methionine-free medium containing 1% dialyzed fetal calf serum, 50 μg/ml ascorbic acid, and

100 μg/ml β aminopropionitrile; [^{35}S]methionine was added at a concentration of 20 μCi/ml. After 6 hr the medium was collected, clarified by centrifugation at 12,000 rpm for 5 min, extensively dialyzed at 4°C against 0.5 N CH_3COOH, and digested with pepsin (1 mg/ml) at 4°C overnight. The cell layer (including the extracellular matrix) was washed twice with phosphate-buffered saline (PBS), scraped from the dish, and extracted with pepsin (1 mg/ml in 0.5 N CH_3COOH) at 4°C overnight.

Labeling of Cartilage Slices

After removal of the perichondrium, slices were obtained from different zones of tibiae of 17-day-old embryos. The slices were continuously labeled with [^{3}H]-proline for 24 hr using the same conditions used for labeling the cell monolayers. Labeled collagens were pepsin-extracted from the slices following the procedure described by Burgeson and Hollister [13].

Carboxymethyl Cellulose Chromatography

The carboxymethyl cellulose (CM 52 Whatman) column (0.5 × 11 cm) was equilibrated overnight with 0.02 M Na acetate, 4 M urea pH 4.8, and maintained at 42°C. The sample was supplemented with chick embryo calvaria collagen as carrier, dialyzed overnight against the starting buffer, and denaturated at 42°C for 40 min just before layering on the column. Elution was achieved by a linear gradient from 0 to 0.1 M NaCl over a total volume of 200 ml. The flow rate was 15 ml/hour and the volume of the fractions was 2 ml. Type I collagen marker was prepared from chick embryo calvaria essentially as in Bornstein et al [14].

Purification of Type II and X Collagens From Epiphysis of 17-Day-Old-Tibiae

The outline of the purification procedure is in Figure 4. Further details are in reference [2].

Purification of Collagen mRNA's From Epiphysis of 17-Day-Old Tibiae and In Vitro Translation

The RNA's were isolated from the cartilage frozen in liquid nitrogen and powdered with a pestle by a guanidine extraction method [15].

The preparation and the general properties of wheat germ translation system have been described by Erickson and Blobel [16]. The translation mixture contained 0.25 vol wheat germ extract, 20 mM Hepes pH 7.35, 3.5 mM Mg acetate, 75 mM K acetate, 4 mM DTT, 1 mM ATP, 0.2 mM GTP, 0.7 mM spermidine, 7.8 mM creatinphosphate, 40 μg/ml creatinphospho-kinase, 38 μM each nonradioactive aminoacid, 0.8 mCi/ml [^{3}H]proline (~ 120 Ci/mmol), or [^{35}S]methionine (> 1,000 Ci/mmol); the incubation was for 2 hr at 27°C.

Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed under reduced conditions as described by Laemli [17] and modified by Bonatti and Descalzi-Cancedda [18]. The gel concentration was 9%. In some cases, before loading on the gel, aliquots of the samples were digested with 12 μg/ml of highly purified bacterial collagenase in 50 mM Tris-HC1 pH 7.6; 5mM $CaCl_2$.

RESULTS

Type X Collagen is Preferentially Deposited in the Extracellular Matrix of Cultured Chondrocytes

We have already shown that type X collagen synthesized by chick embryo chondrocytes is deposited in the extracellular matrix [8]. Here we report a more precise quantitation of the relative distribution between the cell monolayer and the culture medium of the newly synthesized type X collagen compared to the distribution of the newly synthesized type II collagen. A preferential association of type X

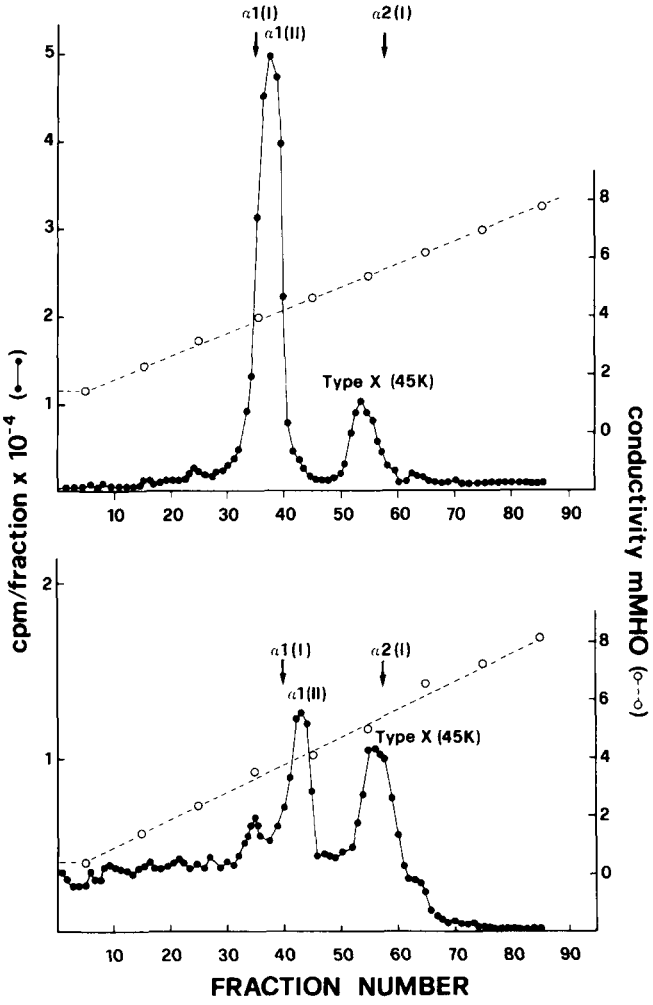

Fig. 2. Collagens recovered from the medium and the cell monolayer of cultured chondrocytes. About 1×10^7 chondrocytes grown as monolayer were labeled for 6 hr with [^{35}S]methionine. After pepsin digestion, labeled collagens from culture medium and cell monolayer were denatured and applied to a CM-cellulose column equilibrated with 0.2 M Na acetate, 4 M urea pH 4.8 at 42°C. Elution was performed with a linear gradient between 0 and 0.1 M NaCl. Arrows indicate the positions of type I collagen chains as determined reading the adsorbance at 226 nm. Upper panel: collagens from the culture medium. Lower panel: collagens from the cell monolayer. In the experiment of the figure the relative distribution of total collagens (type II and type X) between the culture medium and the cell layer pools was about 2:1.

collagen with the cell monolayer was observed (Fig. 2). The ratio between the radioactivity in the type II collagen peak and the radioactivity in the type X peak was in fact 4:1 in the chromatography of the sample from the culture medium and 1:1 in the chromatography of the sample from the cell extract.

Prevalent Deposition of the Newly Synthesized Type X Collagen in Epiphyseal Cartilage Slices

A prevalent deposition in the extracellular matrix of the newly synthesized type X collagen compared to the newly synthesized type II collagen was observed in the collagens extracted from sliced epiphysis of 17-day-old embryo tibiae incubated with [^{3}H]proline for 24 hr (Fig. 3).

Recovery of Type X and Type II Collagen From Epiphyseal Cartilage

Type X collagen is deposited in the epiphyseal cartilage of 17-day-old embryo tibiae and from these regions of the bone can be purified as 45-K form [2]. With the purification procedure outlined in Figure 4 [see also 2], from the same pepsin extract it is possible to isolate both type II and X collagens. At variance with the results obtained looking at the distribution of the newly synthesized collagens in cultured chondrocytes and in slices of tissue, with this procedure we always recovered a larger amount of type II collagen than type X collagen. In a typical experiment, starting from 200 gm of wet cartilage, we recovered 76 mg of type II collagen and 5 mg of type X collagen.

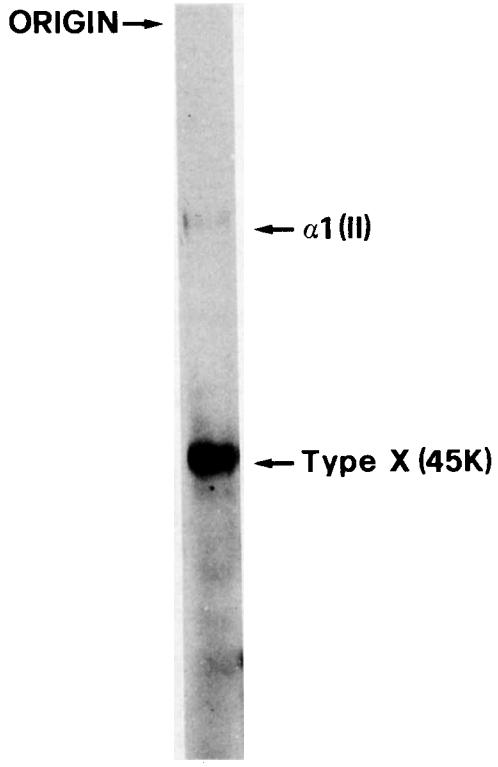

Fig. 3. Analysis of [^{3}H]collagens extracted from sliced epiphysis of 17-day-old tibiae incubated for 24 hr with [^{3}H]proline. Collagens were pepsin-extracted according to Burgeson and Hollister [13] and analyzed on a 9% polyacrylamide SDS gel.

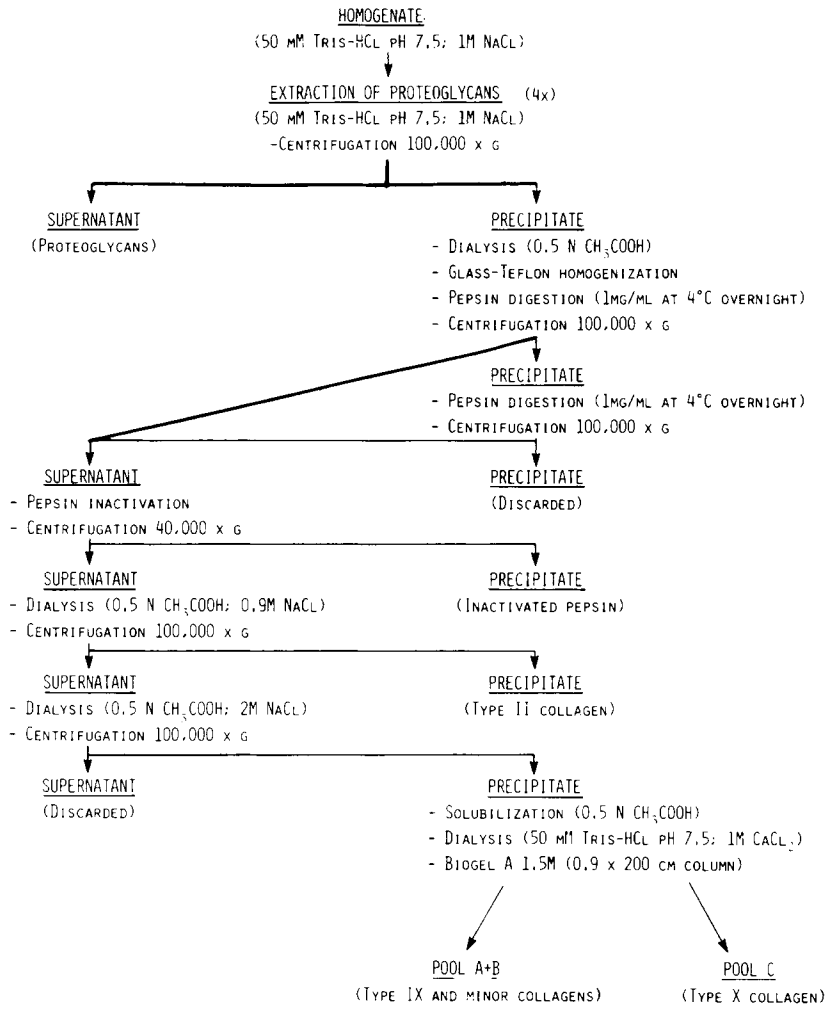

Fig. 4. Outline of the purification procedure used for the isolation of type II and type X collagens from the epiphysis of 17-day-old embryo tibiae.

Recovery From Epiphyseal Cartilage and "In Vitro" Translation of Collagen mRNA's

To rule out that the apparent discrepancy between the result obtained comparing the relative distribution of the newly synthesized collagens (labeled) and the unlabeled collagens in the embryonic bone could be due to a specific activation of the type X collagen gene during the incubation of the tissue slices, we decided to look at the level of translatable mRNA's for type II and type X collagens in the epiphyseal cartilage of 17-day-old tibiae.

The RNA's were purified from the cartilage by a guanidine extraction method and translated in a cell-free wheat germ system. The analysis of the proteins synthesized in vitro under the direction of these RNA's is presented in Figure 5. Several

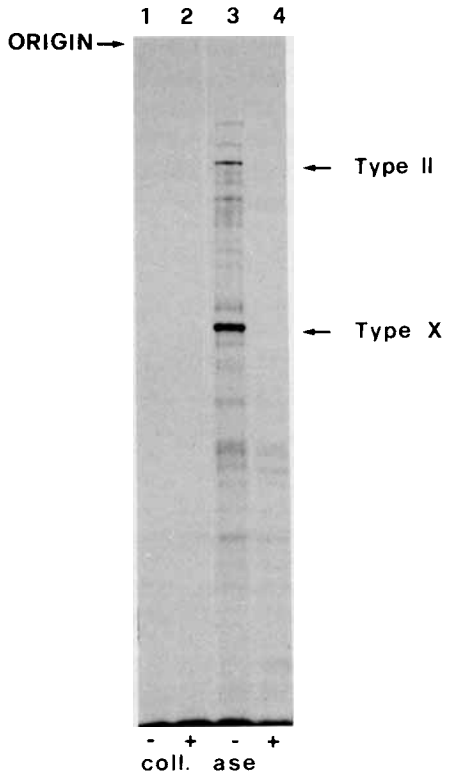

Fig. 5. Gel electrophoresis of proteins synthesized in vitro by cartilage cell RNA. The RNA isolated from epiphysis of 17-day-old tibiae was translated in a cell-free wheat germ system in the presence of [^{3}H] proline. Lanes 1–2, no RNA added; lanes 3–4, RNA added. When indicated, an aliquot of the sample was collagenase-digested before loading on the gel.

proteins, including type II collagen, are clearly detectable, but type X collagen is certainly the major translation product.

DISCUSSION

In the matrix of hyaline cartilage, the presence of several collagens, in addition to the predominant type II, has been reported. These include 1α, 2α, 3α chains [19,20], HMW, and LMW collagens [19, M_1 and M_2 collagens in 20] recently classified as type IX collagen, type X collagen (reviewed in the introduction of this article), and other minor collagens [13,21,22]. The role of these molecules as well as their interactions with the other components of the extracellular matrix are still under investigation. Some of these collagens codistribute in the cartilage with type II collagen. The synthesis of type X collagen is instead restricted to zones of bones, like diaphysis of 9-day-old and epiphysis of 17-day-old tibiae, characterized by the presence of an osteochondral interface.

In this paper we have shown that type X is the predominant collagen species synthesized and deposited in the epiphysis of 17-day-old embryo tibiae, but that when

the total (unlabeled) collagen is purified from the same region, type X collagen represents only 1/15 of the type II collagen recovered. In cultured chondrocytes, after its deposition in the extracellular matrix, type X collagen is rapidly degraded. The data presented are compatible with a similar situation occurring in vivo. Alternatively, the data can be explained assuming an activation of the type X collagen gene in the late stages of bone formation (possibly contemporary to an inhibition of type II collagen gene expression) and a deposition of this molecule in the preexisting matrix rich of type II collagen.

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