Type X Collagen Synthesis by Chick Sternal Cartilage and Its Relationship to Endochondral Development

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ABSTRACT Our morphological studies have demonstrated that the appearance of localized, paired zones of primary calcification on either side of the midline of the 19-d embryonic chick sternum is heralded by the development of paired, translucent zones 2 d previously.

Histological studies demonstrated that the majority of chondrocytes within these translucent zones are hypertrophic, and that the zones are surrounded by a margin of flattened nonhypertrophic cells.

The discrete localization of these paired areas of hypertrophic chondrocytes and subsequent endochondral bone development allows for the direct correlation of the histological and biochemical characteristics of the zones sequentially during development and makes it possible to precisely match the synthetic activity to the cellular morphology, thereby eliminating possible minor but critical variations in developmental staging that could otherwise arise.

Our studies have demonstrated that there is a direct spatial and temporal correlation between the degree of cellular maturation and the synthesis of type X collagen, and that the sudden and profound initiation of type X collagen synthesis on days 16–17 of development occurs concurrently with the attainment of hypertrophic characteristics by the majority of cells within the translucent zone.

Before acquisition of these hypertrophic characteristics, the cells of this precalcification zone synthesize only type II and the minor cartilage collagens. Chondrocytes isolated from these regions in more immature sternae (i.e., 11+ d embryos) were found to synthesize high levels of type X collagen within 4 d of culture within collagen gels even though hypertrophic development and type X collagen synthesis by cells within this region would not normally have been apparent *in ovo* for several more days.

These data indicate that there is a direct correlation between the development of hypertrophic characteristics and the synthesis of type X collagen, and that the maturation of chondrocytes in precalcification zones may be regulated by matrix components and/or stimulated by culture within collagen gels.

We have previously shown that chondrocytes isolated from areas of the developing chick sternum that will eventually be replaced by bone, synthesize large amounts of a unique low molecular weight collagen in cell culture (7). This species, which we originally termed G collagen, was shown to be composed of chains with an M_r of 59,000. Controlled incubation with pepsin or chymotrypsin digested an apparently nonhelical peptide that appeared to be largely confined to one end of the molecule, leaving a triple helical region composed of chains of M_r 45,000 (5, 6). This collagen species was shown to be distinct from the interstitial collagens, including type II, as well as the recently isolated minor collagens of cartilage (1, 17, 18, 20, 25, 26, 28) by cyanogen bromide and proteinase peptide mapping. In common with the minor collagens of cartilage, G collagen was found to be very soluble in acetic acid solutions being precipitated at NaCl concentrations >1.2 M. An apparently identical molecule has recently been shown to be synthesized by regions of the embryonic chick tibiotarsus containing hypertrophic chondrocytes (22), by chondrocytes isolated from this tissue (3, 4, 21, 23), and by organ cultures

of slices of rabbit growth plate cartilage (19), and has now been classified as type X collagen.

During the process of endochondral development, chondrocytes pass through a series of morphological changes that progressively lead to their hypertrophy and eventually to their apparent degeneration and the vascular and cellular invasion of their vacated lacunae. The developing chick sternum has provided a convenient tissue in which to study the changes in synthetic activity of chondrocytes involved in this process. The first signs of endochondral ossification in the sternum occur relatively late in development (16–18 d *in ovo*) by which stage the sternum is large enough to dissect free of adherent tissues. The sternum is bilaterally symmetrical around its longitudinal axis allowing both histology and biochemistry to be performed on comparable regions on either side of the midline of the same tissue.

To more clearly examine the relationship of the endochondral development of chondrocytes with type X collagen synthesis in the intact tissue, we have undertaken an exhaustive morphological study of endochondral ossification in the chick sternum, and by monitoring the stage of development of each sternum, we have been able to directly compare the histology of the cartilage with its collagen synthesis after short-term tissue culture.

We have shown that calcification of the chick sternum is preceded by the development of a distinct nidus of cartilage containing an almost pure population of hypertrophic chondrocytes that synthesize type X collagen as the major species in short-term culture. The initiation of type X collagen synthesis and hypertrophic development occurs abruptly within a 24-h period between days 16 and 17 of development. Furthermore, we have been able to show that chondrocytes isolated from areas of presumptive calcification in much more immature sternae will, if cultured within collagen gels, synthesize high levels of type X collagen several days before the onset of the calcification process, and type X collagen synthesis would be apparent in tissue of the same developmental age *in ovo*.

MATERIALS AND METHODS

Tissue Culture: Sternae were dissected from chick embryos at various stages of development between 11 and 21 d. Before the 11th day of development, sternae were found to be too small and poorly developed to permit the necessary precise dissection. The dissected sternae were freed of adhering perichondrium and divided into presumptive calcification regions (cephalic half) and permanent cartilagenous regions (caudal half) as described previously (7). The presumptive calcification region was divided in half longitudinally in the saggital plane. One half was used for histological examination, whereas from the other half, which was to be used for tissue culture, a translucent nidus (see Fig. 1 *a*) of cartilage hypertrophy and incipient calcification was dissected free of adjacent tissue. The small pieces of sternal cartilage from each region were incubated in Dulbecco's modified Eagle's medium (DME)[1] (0.1 ml) (Gibco NZ Ltd., Auckland, New Zealand) containing 5-[^{3}H]proline (10 μCi) and ascorbate (100 μg/ml) in 5% CO_2/95% air at 37°C for 24 h.

Cell Culture: Chondrocytes were prepared from sternal cartilage of chick embryos of various stages of development as described previously (5). The sternae were cleaned of surrounding perichondrium, divided into presumptive calcification regions and permanent cartilagenous regions, and digested with bacterial collagenase/trypsin. Released chondrocytes were washed with DME and plated on plastic (7×10^4 cells/cm^2) or within collagen gels (5×10^5 cells/ml) and incubated in DME containing 16% fetal calf serum (vol/vol) and ascorbate (50 μg/ml) in 5% CO_2/95% air at 37°C. Cells maintained in culture on plastic or within collagen gels were labeled at selected times by incubation with DME containing fetal calf serum (16%), ascorbate (100 μg/ml), and 5-[^{3}H]proline (20 μCi/ml) (Amersham, UK) for 24 h.

Extraction and Isolation of Newly Synthesized Collagens: Newly synthesized collagens were isolated from the incubation medium and the cell-matrix layer of cell cultures or tissue and culture medium of short-term tissue cultures as described previously (7). Medium proteins were precipitated with $(NH_4)_2SO_4$ (176 mg/ml) in the presence of proteinase inhibitors, and matrix proteins were extracted with pepsin (1 mg/ml) (Sigma Chemical Co., St. Louis, MO) in 0.5 M acetic acid followed by 0.1 M Tris HCl (pH 7.4) containing 0.4 M NaCl.

In experiments in which very small pieces of cartilage were incubated, the medium and tissue were not separated but extracted directly with pepsin–acetic acid followed by Tris/NaCl as described above. In these experiments, the pepsin and Tris/NaCl extracts were combined and dialyzed extensively against Tris/NaCl buffer containing unlabeled proline (10 mg/ml).

As described previously, > 90% of the radiolabeled proteins were routinely extracted from cell cultures or incubated sterna (7).

Electrophoretic Analyses: Radiolabeled proteins were resolved after denaturation by PAGE according to the method of Laemmli (12) using a separating gel of 8% and a stacking gel of 3%. All gels were run under reducing conditions. The procedure of Bonner and Laskey (2) was used to impregnate the gel with 2,5-diphenyloxazole, and gels dried onto filter paper were exposed to X-ray film (Agfa-Gavaert Curex RP2, Agfa-Gevaert Inc., Belgium) at −70°C.

To determine the ratio of radioactivity incorporated into specific proteins, strips corresponding to exposed bands on the fluorograms were cut from the dried gels. Attached paper was removed by rehydration in H_2O, and the strip of gel was dissolved by incubation in a solution (0.3 ml) of H_2O_2 (100 vol containing 0.3% NH_4OH) in a humidified atmosphere for 16 h at 37°C. Scintillation fluour was added and radioactivity determined by scintillation counting.

Since we believe that type X collagen is synthesized exclusively by chondrocytes, the radioactivity present in type X collagen bands is expressed as a percentge of the total radioactivity present in cartilage collagens. Synthesis of $\alpha 2$ chains is thought to be indicative of the synthesis of type I collagen by developing bone present at later stages of development. The incorporation of radioactivity into type I collagen is deducted from the incorporation of radioactivity into type II collagen using the formula

$$\text{II} = \alpha 1 - 2\,(\alpha 2),$$

where II represents the radioactivity present in type II collagen; $\alpha 1$, the radioactivity in the $\alpha 1$ bands; and $\alpha 2$, the radioactivity in $\alpha 2$ bands. The value for incorporation into type II collagen was then used to determine the radioactivity in type I collagen as a percentage of the total radioactivity in cartilage collagens. When significant, the radioactivity incorporated into 1α and 2α bands was included in the total radioactivity in cartilage collagens.

Histology: The stage of development was monitored by histological examination of one saggital half of each sternum. Hand processed paraffin sections taken at right angles to the long axis of the sternum at a number of points along the suspected nidus of hypertrophic cartilage or equivalent areas in younger sternae were stained with hematoxylin and eosin or toluidine blue (1% aqueous).

Cleared Preparations: To determine the site of initial calcification and subsequent bone development, whole sternae were cleared in KOH by the Spalterholz technique (27) and stained with alizarin before sequential dehydration and storage in glycerin. Cleared specimens of sternae were viewed with transmitted light and photographed on a Nikon Multiphot low power photomicrography set-up (Nippon Kogaku K.K., Japan).

RESULTS

Gross and Histological Appearance of the Developing Chick Sternum

After ~17 or 18 d of development, small areas of translucent cartilage appear near the cephalic end of the chick sternum on either side of the keel (Fig. 1 *a*). These occur in areas where the first signs of calcification appear (Fig. 1 *b*), but precede calcification by 1 or 2 d. Sternae were divided in half longitudinally as shown in Fig. 1 *a*. The area of translucent cartilage or equivalent areas in younger sternae were carefully dissected and used to determine the type of collagen synthesized after short-term tissue culture. The contralateral half

[1] *Abbreviation used in this paper*: DME, Dulbecco's modified Eagle's medium.

was used to monitor development of the sternum.

Photomicrographs of transverse histological sections of sternae dissected from 16–20-d chick embryos from the area indicated by the arrow in Fig. 1 *a* are shown in Figs. 2 and 3.

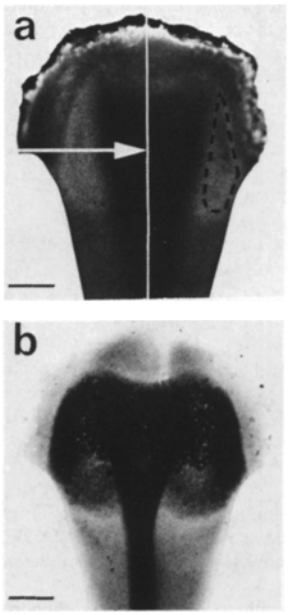
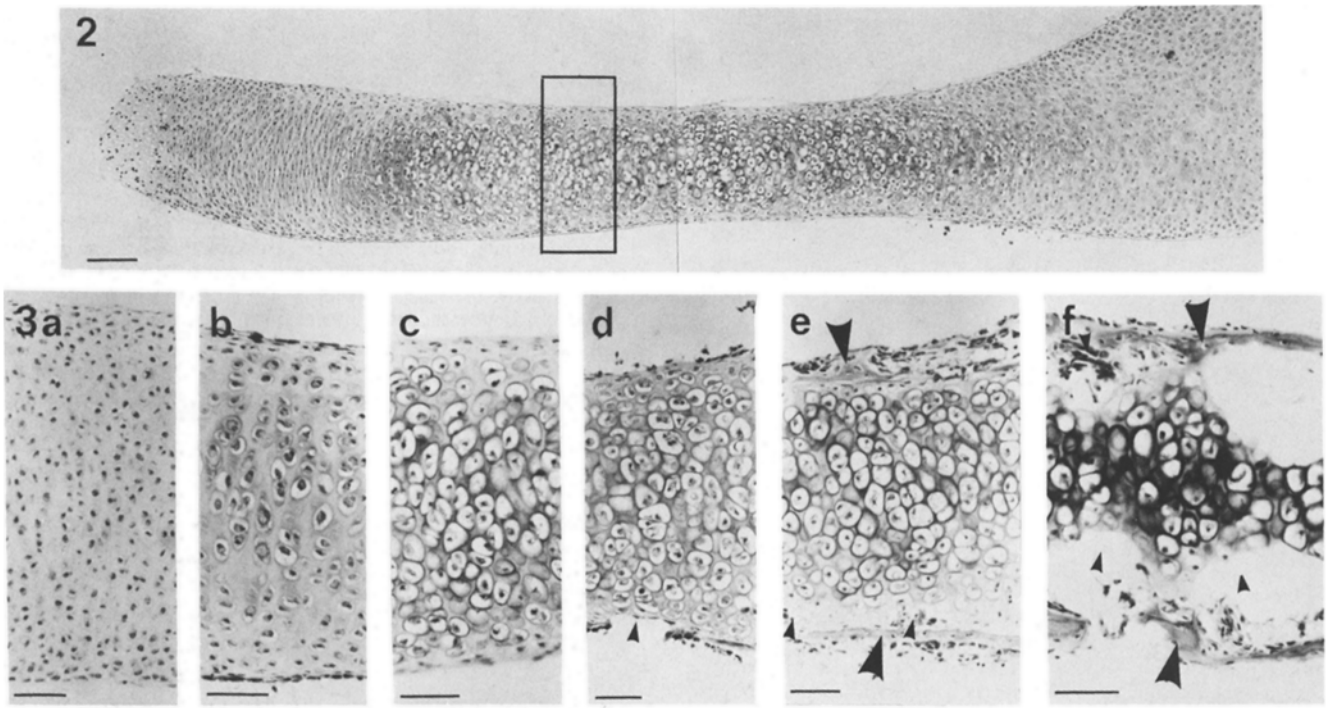


FIGURE 1 Planar views of the cephalic region of the embryonic chick sternae. (*a*) Photographed by transmitted light, unstained sternum dissected from a 17-d embryo, showing the more translucent areas of cartilage. Sternae were divided in half as shown by the solid white line. One half was used for histology. Transverse histological sections were taken from the region shown by the arrow. The region enclosed by the dashed line shows the area of cartilage for tissue culture and collagen synthesis studies. (*b*) 19-d embryonic chick sternum cleared with the Spalterholtz technique and stained for calcium with alizarin. Note that calcification has occurred directly over the more transparent areas seen in Fig. 1*a*. Bar, 1 mm. × 6.

Sternae from 16-d embryos normally do not show any visible translucent areas of cartilage and contain large numbers of fairly uniform small chondrocytes embedded in an amorphous extracellular matrix. Between 16 and 17 d of development, small numbers of hypertrophic chondrocytes appear in this region of the cartilage (Fig. 3 *b*). Any change in translucency of the cartilage is very difficult to detect at this stage of development. After ~17 d of development, clearly visible areas of translucent cartilage similar to that shown in Fig. 1 *a* appear. Histological examination at this stage reveals large numbers of hypertrophic chondrocytes flanked by a small zone of elongated cells at the outer limiting edge of the translucent area (Figs. 2 and 3 *c*) that are reminiscent of the flattened cells of the proliferative zone of the epiphyseal growth plate. After a further 24-h development, the area of translucent cartilage becomes larger and populated almost exclusively with hypertrophic chondrocytes. Occasionally, small areas of peripheral vascular invasion are also visible at this time (Fig. 3 *d*). After 19 d of development, the pericellular area surrounding hypertrophic chondrocytes appears distinctly basophilic after hematoxylin and eosin staining, areas of vascular invasion are common, and perichondrial bone formation is often observed (Fig. 3 *e*). By 20 d of development, the cartilage becomes grossly eroded by vascular invasion, and perichondrial bone formation is prominent (Fig. 3 *f*).



FIGURES 2 and 3 Histological section of the developing chick sternum. (Fig. 2) Section of a 17-d embryonic sternum taken along the region shown by the arrow in Fig. 1*a*. Elongated cells are present at the edge of the translucent region shown in Fig. 1*a*. Between this region and the midline of the sternum nearly all cells are hypertrophic. Hematoxylin and eosin stain. Bar, 100 μm. × 73. (Fig. 3) Photomicrograph of higher magnification of regions corresponding to the rectangle shown in Fig. 2 taken from the sternae of a series of chicks ranging from 16–20 d of development. (*a*) Section of a sternum from a 16-d embryo showing small chondrocytes surrounded by an amorphous matrix. Bar, 50 μm. × 150. (*b*) 16-d embryo exhibiting a slightly more advanced stage of development. Some hypertrophic chondrocytes surrounded by smaller chondrocytes are seen. Bar, 50 μm. × 190. (c) 17-d embryo showing that the vast majority of cells are hypertrophic. Bar, 50 μm. × 180. (*d*) 18-d embryonic chick sternum, showing a uniform population of hypertrophic chondrocytes and an area of vascular invasion (arrowhead). Bar, 50 μm. × 140. (e) 19-d chick embryo sternum showing hypertrophic chondrocytes surrounded by a more basophilic pericellular matrix; areas of vascular invasion (small arrowhead) and perichondrial bone (large arrowhead). Bar, 50 μm. × 150. (*f*) 20-d chick embryo sternum showing cartilage grossly eroded by vascular invasion (small arrowheads); a very strongly basophilic matrix surrounding the remaining hypertrophic chondrocytes and prominent perichondrial bone formation (large arrowheads). Bar, 50 μm. × 190.

Collagen Synthesis by Sternal Cartilage in Short-term Tissue Culture

To determine the type of collagen synthesized, hypertrophic regions (or equivalent areas in young sternae) were dissected from a large number of sternae and cultured individually for 24 h in the presence of 5-[^{3}H]proline. Newly synthesized radiolabeled proteins were extracted with pepsin–acetic acid followed by NaCl/Tris and subjected to PAGE and fluorography (Fig. 4).

The radiolabeled proteins synthesized by pieces of sternae taken from the areas contralateral to those shown in histological section in Fig. 3, *a–f* are shown in Fig. 4, lanes *a–f*. The stage of development of all sternae examined biochemically was assessed histologically. The average incorporation of radioactivity into type X collagen expressed as a ratio of the total radioactivity incorporated into all cartilage collagens is shown in Fig. 5.

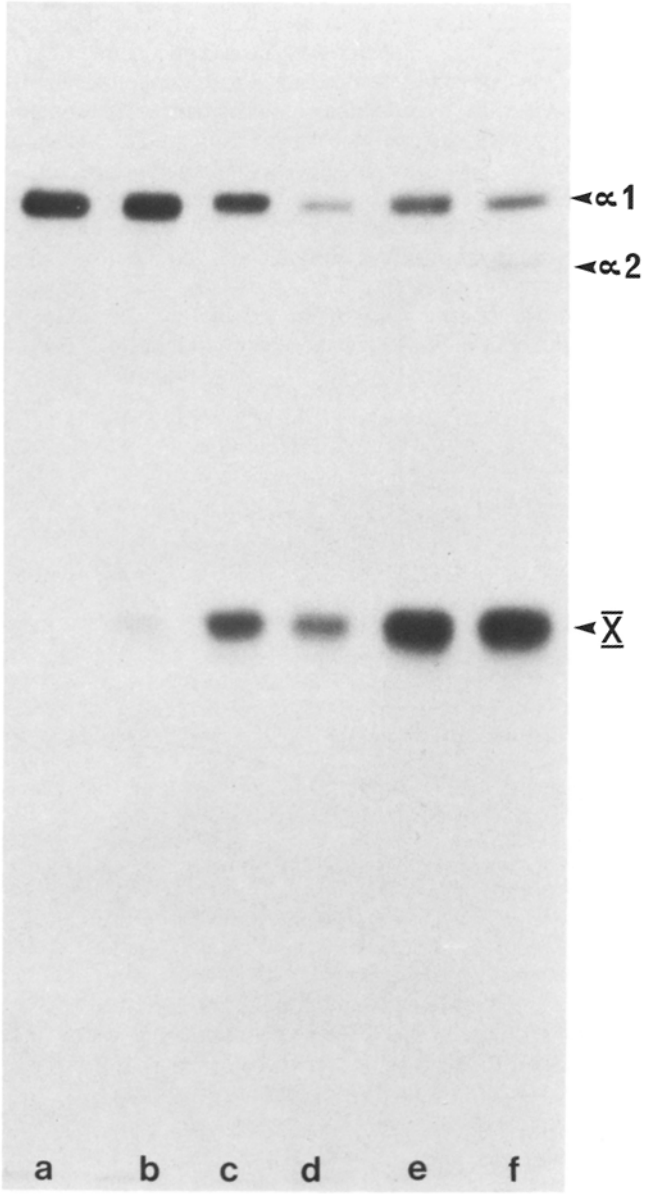


FIGURE 4 Fluorogram of [^{3}H]proline-labeled proteins extracted from regions of sternae contralateral to those depicted in histological section in Fig. 3, after brief incubation in tissue culture. Areas of translucent cartilage or equivalent areas in more immature sternae were incubated for 24 h in DME containing [^{3}H]proline and proteins, extracted with pepsin–acetic acid followed by NaCl/Tris, analyzed on an 8% polyacrylamide gel. Lanes *a–f*, cartilage sections shown in Fig. 3, *a–f*. Migration positions of the type I collagen α chains and type X collagen chains are shown. All lanes contain 10,000 cpm except lane *d*, which contains 5,000 cpm.

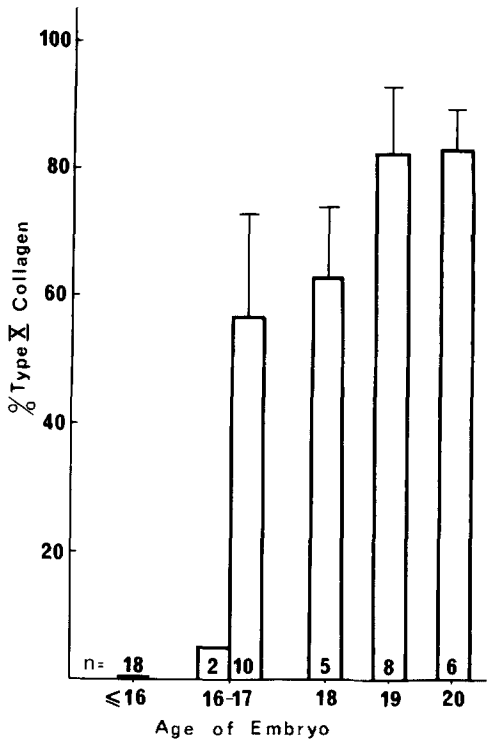


FIGURE 5 The effect of embryo maturity on the proportion of type X collagen synthesized by dissected areas of chick sternal cartilage. Radiolabeled proteins were extracted from hypertrophic or presumptive hypertrophic cartilage after incubation in tissue culture and subjected to PAGE as described in Fig. 4. Bands revealed by fluorography were cut from dried gels and the radioactivity determined by scintillation counting after digestion with H_2O_2. The radioactivity present in type X collagen is expressed as a percentage of total radioactivity present in collagen bands. The number of cartilage pieces analyzed is shown by the numbers at the bottom of the figure (*n*). Bars show the standard deviation.

It was found that there was a close direct spatial and temporal correlation between the cellular morphology as observed histologically and the type and amount of collagen produced by the cartilage sample.

Before the development of hypertrophic chondrocytes, cartilage samples dissected from the cephalic region of the sternum synthesized only collagen chains with the mobility of α1 chains (presumably α1[II]) on PAGE (Fig. 4). Cartilage with small numbers of hypertrophic chondrocytes (Fig. 3*b*) synthesized a small but detectable amount of type X collagen (Fig. 4, lane *b*), representing an average of 5% of the total collagen synthesized. It was difficult to assess the exact proportion of hypertrophic chondrocytes in these sterna without examining serial sections of the whole cephalic half of the sternum, as the distinct area of translucent cartilage was not yet apparent. However, in tissues showing a well defined area of hypertrophic cartilage and which, histologically, contained a large or predominant number of hypertrophic chondrocytes, type X collagen was the major collagenous species synthesized

after 24 h in culture, representing from 57 ± 16% of the total collagen synthesized by day 17 sternae to 83 ± 6% by day 20 sternae (Fig. 5). Hypertrophic regions from 20-d (five of the six examined) and occasionally 19-d (two of the eight examined) sternae also synthesized chains migrating in the position of the $\alpha 2$ chain (Fig. 4), consistent with the synthesis of type I collagen associated with new perichondrial bone formation. Type I collagen synthesis varied greatly between sternae ranging from 4–27% of total collagen synthesis, consistent with the variation in bone production observed histologically.

Type X collagen was not detected in extracts of similarly incubated cartilage dissected from the cellular areas surrounding the hypertrophic centers or from the caudal half of the sternae at any stage of development examined.

Collagen Synthesis by Chondrocytes in Cell Culture

To investigate the intrinsic potential of chondrocytes to synthesize type X collagen, cells were isolated from the cephalic half of sternae dissected from chicks at various embryonic stages of development and cultured either on plastic or within collagen gels. All chondrocytes grew well in culture both on plastic and within collagen gels and showed very similar growth kinetics whether cultured on plastic or within collagen gels, comparable with those cells isolated from 18-d embryonic chick sterna described previously (7). Although chondrocytes isolated from more immature sternae tended to assume a more fibroblastic appearance at comparatively earlier stages of culture, particularly when cultured on plastic, all cultures appeared to show similar growth kinetics, and when monitored by DNA estimation after 7 d in culture, they had attained similar densities (data not shown).

After various times in culture, the type of collagen synthesized was examined by PAGE of radiolabeled proteins extracted from cultures incubated with 5-[^{3}H]proline for 24 h. Type X collagen synthesis was observed in all cultures of chondrocytes isolated from the cephalic region of sternae of all stages of development examined. Readily detectable levels of type X collagen were found in the medium of cultures of chondrocytes isolated from 11-d embryos, the youngest examined, after only 4 d in culture within collagen gels. After 7 d in culture, type X collagen was the major radiolabeled collagenous species present in the culture medium (Fig. 6). The identity of the radiolabeled species migrating between the $\alpha 1$ and $\alpha 2$ chains in these cultures is at present unknown.

Since most of the newly synthesized type X collagen is present in the culture medium, the proportion of type X collagen expressed as a percentage of total collagen synthesized (present in both the medium and matrix) is lower than that seen in the culture medium alone, particularly with cultures of chondrocytes from younger embryos (Fig. 7, cf. Fig. 6). Chondrocytes, isolated from the cephalic half of the sternum from embryos at all stages of development examined, synthesized significant quantities of radiolabeled type X collagen after only 4 d of culture within collagen gel, and this increased considerably after 7 d of such culture. However, chondrocytes cultured in plastic showed lower levels of type X collagen synthesis (Fig. 7), tended to assume a more fibroblastic appearance, and began to synthesize type I collagen at earlier culture times than did similar cells cultured within collagen gels (Fig. 6). Chondrocytes isolated from the caudal region of sternae did not synthesize detectable levels of type X collagen under either of the culture conditions used and regardless of the stage of development of the parent sternae.

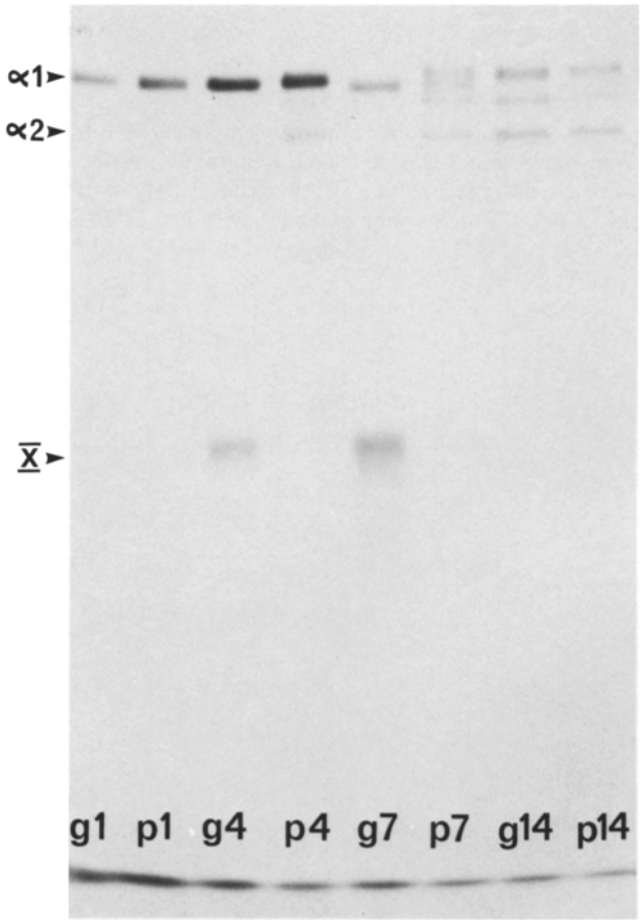


FIGURE 6 Fluorogram of radiolabeled proteins isolated from the medium of cultures of chondrocytes obtained from the cephalic region of 11-d chick embryo sternae. After selected times in culture, cells were labeled for 24 h with [^{3}H]proline, and the medium proteins precipitable at 30% $(NH_4)_2SO_4$ saturation were analyzed on an 8% polyacrylamide gel. Samples from cultures grown within collagen gels or on plastic are labeled *g* and *p*, respectively. Duration of culture is shown by the accompanying number at the foot of the fluorogram. The migration position of the type I ($\alpha 1$ and $\alpha 2$) and type X (45,000-mol-wt species) collagen chains are indicated. Samples in Tris HCl (0.1 M) pH 7.4 containing NaCl (0.4 M) were incubated with chymotrypsin (300 μg/ml) for 4 h before denaturation and PAGE to remove the extension peptides of the types I and II collagen precursors, thus reducing the number of bands present and simplifying the fluorogram. Type X collagen was degraded to the 45,000-mol-wt species during this digestion.

DISCUSSION

Previous studies have shown that chondrocytes isolated from presumptive calcification areas of the embryonic chick sternum (7) or long bone (23) synthesize high levels of type X collagen when cultured under conditions thought to preserve the endochondral development of isolated chondrocytes, namely culture within collagen gels (7) or a specialized long-term secondary culture on plastic (23). The suggestion that hypertrophic chondrocytes may synthesize type X collagen at a late stage of their development was supported by observations of slices of developing chick long bone taken near the calcification front (4, 22); slices of rabbit costochondral

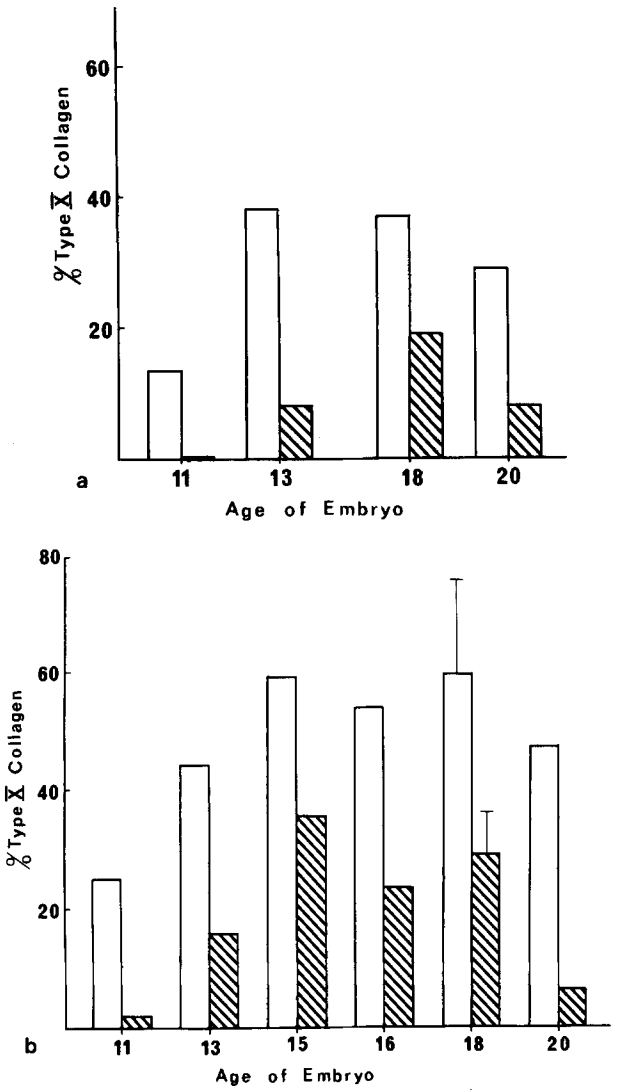


FIGURE 7 The influence of embryo maturity on the proportion of type X collagen synthesized by cultures of chondrocytes isolated from the cephalic region of their sternae. Chondrocytes were isolated from the cephalic region of sternae dissected from chick embryos ranging in maturity from 11–20 d and cultured on plastic or within collagen gels for 4 or 7 d. Radiolabeled proteins were isolated from the medium and matrix after incubation with [^{3}H]-proline for 24 h and subjected to PAGE. The proportion of radioactivity present in all collagens was determined as described in Fig. 5 and is expressed as a percentage of total radioactivity present in all collagen bands (i.e., medium plus matrix/cell layer). (*a*) The proportion of type X collagen synthesized by 4-d cultures. (*b*) The proportion of type X collagen synthesized by 7-d cultures. The values represent averages determined from duplicate cultures except 7-d cultures from 18-d embryos, in which six cultures were analyzed. Results are expressed as standard deviations (*bars*). □, cultures within collagen gels; ▨, cultures on plastic.

growth plate (19) and cephalic regions of the embryonic chick sternum (7), which contained large numbers of hypertrophic chondrocytes and synthesized type X collagen in short-term tissue culture. The level of type X collagen synthesis by intact slices of tissue, however, appeared to be considerably less than that achieved in cell culture. This could have been due to technical difficulties in precisely dissecting some of the tissues and the difficulty in precisely identifying the morphological and cellular characteristics of cultured and analyzed tissue.

In this study, we have identified two small areas of cartilage in the developing chick sternum that contain an almost pure population of hypertrophic chondrocytes and represent the initial sites of endochondrial calcification in this tissue. As these are distinct areas of translucent cartilage, they can be accurately dissected free of surrounding tissue, and since the sternum is bilaterally symmetrical, the two areas are precisely equivalent and can be used for simultaneous histology and tissue culture. Identification of the collagens synthesized in dissected areas of cartilage after brief tissue culture has shown that the appearance of hypertrophic chondrocytes is coincident with the onset of type X collagen synthesis.

The initiation of type X collagen synthesis appears to be very sudden. Within a day of being first detected, type X collagen synthesis reaches levels close to the observed maximum, representing as much as 80% of the total collagen synthesized in brief tissue culture (Fig. 5). This abrupt change indicates that type X collagen synthesis is restricted to a very specific stage of chondrocyte development and skeletal morphogenesis that immediately precedes and accompanies the calcification of the cartilagenous template. In the developing sternum in contradistinction to the developing growth plate, the maturation of most of the chondrocytes appears to be synchronized during early endochondral development, which leads to a much more dramatic and observable increase in type X collagen synthesis in this tissue.

The abrupt onset of type X collagen synthesis between 16 and 17 d of development may well explain the failure of Kielty et al. (10) to extract this collagen from the sternae of 17-d chick embryos even though they could extract type X collagen from the tibiotarsal cartilage of the same chicks. The 17-d sternae examined may not have yet initiated type X collagen synthesis or accumulated a sufficient amount to be detected.

Most studies of endochondral development have concentrated on the epiphyseal growth plate of the developing long bone. This tissue contains a linear sequence of chondrocytes at all stages of development and maturation ranging from proximal zones of predominantly resting cells to distal zones of degenerative hypertrophic chondrocytes. The cells undergo this orderly sequence of development as vascular invasion and cartilage destruction proceeds in the direction of the epiphysis, resulting in endochondral bone formation and thus, long bone lengthening. The phase of sternal development described in this paper developmentally precedes that which is seen in the growth plate and is equivalent to the primary center of ossification. Nevertheless, the sequence of chondrocyte development and maturation appears to be similar in both situations. In the developing sternum, the areas of hypertrophic cartilage develop very rapidly to occupy almost all of its cephalic half and become surrounded by perichondrial bone within 2–3 d of their initial appearance. This rapid and almost complete transformation and maturation of the greater proportion of the chondrocyte population of this region probably accounts for the much higher level of type X collagen synthesized by hypertrophic sternal cartilage than was synthesized in similar but more heterogenous regions dissected from the developing long bone or rib growth plate. It is also likely that the degree of maturity of the hypertrophic chondrocytes may influence the level of type X collagen synthesis observed. Higher levels of type X collagen synthesis were observed after brief tissue culture of hypertrophic cartilage dissected from 19- to 20-d embryos than were observed in cultures of cartilage

from the 17- and 18-d embryos (Fig. 5), even though the number of hypertrophic chondrocytes was essentially similar. The regions of growth plate cartilage studied previously probably contain fewer and/or less mature hypertrophic chondrocytes that can synthesize maximal levels of type X collagen.

Without an analysis of the rates of collagen synthesis and cell numbers in the dissected regions of the sternae, it is not possible to determine whether the increased proportion of type X collagen seen after 18 d of development is due to a decline in type II collagen synthesis, an increased synthesis of type X collagen, or changes in both types II and X collagen synthesis. It seems unlikely, however, that the large changes seen between the initiation of type X collagen synthesis at 16–17 d and the close to maximal type X collagen synthesis at 18 d of development are due to a rapid decline in type II synthesis, as this would require a decrease in the rate of total collagen synthesis of ~90%. It is apparent that this does not occur as the average radioactivity incorporated into collagens after a brief culture of dissected areas of cartilage was similar for all developmental stages examined. Furthermore, the rate of collagen synthesis per cell determined from the amount of radioactivity incorporated into collagen after 7 d in culture within collagen gels was not significantly affected by the developmental age of the embryo from which the chondrocytes were isolated. Similarly, previous studies of collagen synthesis by dissected zones of the developing chick tibiotarsus in brief tissue culture have shown that those areas synthesizing type X collagen (22) displayed the highest rate of total collagen synthesis (15).

Cell culture studies showed that chondrocytes isolated from areas of sterna destined to undergo endochondral development could be induced to synthesize type X collagen in culture, particularly if cultured within collagen gels, even though, in some cases, similar cells left to develop in vivo would not become hypertrophic or synthesize type X collagen for several more days (Figs. 6 and 7). The whole sternum of the 11-d-old embryonic chick is < 5-mm long and contains a fairly uniform population of relatively closely packed small chondrocytes. There is no apparent morphological difference between chondrocytes in the cephalic, presumptive calcification region and those in the caudal, permanent cartilagenous region. Yet isolation and culture of the chondrocytes from the presumptive calcification region, particularly culture within collagen gels, initiate the synthesis of type X collagen and presumably hypertrophic development. Chondrocytes from the presumptive calcification region of early embryos required ~4 d in culture before a significant proportion of type X collagen was synthesized, and ~7 d in culture before maximal levels were seen. At first sight, it would appear that this 4-d lag period was merely a reflection of the difference in the maturational age of the cells, and that the cells had during this time matured sufficiently to complete a previously programmed time sequence. However, a similar 4-d lag period and peak in culture was also observed in the culture of 18-d chick sternal cells even though they would normally have produced type X collagen in vivo that day (7). Furthermore, the peak of type X collagen synthesis was shown to correspond to the peak of total collagenous and noncollagenous protein synthesis (5), suggesting that the lag in collagen synthesis at early culture times probably involved a recovery of the cell from disruption caused by the isolation procedure and possible re-formation of a specific pericellular matrix (7). Another possibility is that the initiation of type X collagen synthesis is brought about by accelerating cell division in culture.

These results supplement our previous findings (5–7) and indicate that cells from sites of presumptive calcification will not only continue the synthesis of type X collagen when grown within collagen gels but can also be induced to synthesize type X collagen by culture within the collagen gels at a much earlier stage of development than they would spontaneously exhibit this characteristic under in vivo conditions. It appears that chondrocytes are committed to a course of endochondral development long before they show any obvious morphological changes and that this course of development is allowed to continue in cell culture. However, the demonstration of accelerated synthesis of type X collagen by chondrocytes isolated from early embryos suggests that either matrix constituents, removed during the isolation of the chondrocytes, may act to delay endochondral development in vivo or that some constitutent or the physical nature of the type I collagen gel may serve to accelerate the process.

The loss of the capacity of chondrocytes to synthesize type X collagen under prolonged culture conditions was accompanied by new synthesis of type I collagen indicated by the presence of $\alpha 2$ chains on PAGE (Fig. 6). These results are consistent with recent reports that show that the growth of chick chondrocytes in culture conditions known to cause a switch of collagen synthesis from type II to type I collagen, i.e., growth to senescence (14), culture in the presence of BrdU (14), or transformation with Rous sarcoma virus (8), also cause a switch from the synthesis of the 1α, 2α, 3α chains to synthesis of type V collagen and the loss of the capacity to synthesize type X collagen.

This loss of cartilage collagen phenotype was markedly retarded by culture of the chondrocytes within collagen gels, and along with previous papers (5, 7), supports a recent report demonstrating the maintenance of cartilage phenotype by chondrocytes isolated from 13-d embryonic chick sternae cultured for up to 6 wk within collagen gels. Collagen gel culture, in our hands, however, produced a less dramatic stabilizing influence on the chondrocyte phenotype in culture. We often observed type I collagen synthesis after only 2 wk of culture within collagen gels (Fig. 6). Difference in the degree of stabilization may reflect differences in the techniques of chondrocyte isolation or culture conditions. Kimura et al. (11) were also able to detect the synthesis of only trace amounts of type X collagen after culture of chondrocytes within collagen gels. However, they have reported studies of collagen synthesis after only 4 d and 6 wk in culture and have studied chondrocytes isolated from whole sternae. We have shown that type X collagen synthesis represents close to 40% of the total collagen synthesized by chondrocytes isolated from the presumptive calcification region of sternae isolated from 13-d embryos after 4 d in culture (Fig. 7 *a*). Using whole sternae to prepare chondrocytes, we would expect to detect a lower proportion of type X collagen, but this should still represent ~10% of the total collagen synthesized. Kimura et al. suggest that some loss of phenotype may have occurred after 6 wk in culture since the synthesis of $\alpha 2$ chains was detected and synthesis of the 2α collagen chain appears to have been lost, indicating a switch to type I collagen and possibly type V collagen synthesis (11). Capacity to synthesize type X collagen after this time in culture may have been similarly lost.

Improvement in the preparation of tissue for examination by electron microscopy has shown that hypertrophic chon-

drocytes maintain an intact plasma membrane and contain well developed endoplasmic reticulum, Golgi, and mitochondria throughout their development (9). These findings are not compatible with the previously held concept that these cells become either effete or synthetically degenerate with development (24). Recent studies have also cast doubt on the functional role of matrix vesicles as nucleation sites for cartilage calcification (9, 13) and have shown that initial deposits of calcium phosphate appear as amorphous electron dense deposits perhaps associated with the calcium binding protein chondrocalcin (16). These recent findings indicate that some of our older concepts of cartilage calcification and bone formation may be in need of reappraisal.

Our observation of the close temporal and spatial relationship between the synthesis of type X collagen and the maturation of hypertrophic chondrocytes in zones of presumptive calcification and the initiation of type X collagen synthesis in cell culture may open up new areas of investigation, particularly into aspects such as the function of type X collagen in this process and the factors that influence the initiation and consolidation of endochondral development.

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