IMMUNOLOGICAL AND BIOCHEMICAL STUDIES OF COLLAGEN TYPE TRANSITION DURING IN VITRO CHONDROGENESIS OF CHICK LIMB MESODERMAL CELLS

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ABSTRACT

This work describes an approach to monitor chondrogenesis of stage-24 chick limb mesodermal cells in vitro by analyzing the onset of type II collagen synthesis with carboxymethyl-cellulose chromatography, immunofluorescence, and radioimmunoassay. This procedure allowed specific and quantitative determination of chondrocytes in the presence of fibroblasts and myoblasts, both of which synthesize type I collagen. Chondrogenesis was studied in high-density cell preparations on tissue culture plastic dishes and on agar base. It was found that stage-24 limb mesenchymal cells initially synthesized only type I collagen. With the onset of chondrogenesis, a gradual transition to type II collagen synthesis was observed. In cell aggregates formed over agar, type II collagen synthesis started after 1 day in culture and reached levels of 80–90% of the total collagen synthesis at 6–8 days. At that time, the cells in the center of the aggregates had acquired the typical chondrocyte phenotype and stained only with type II collagen antibodies, whereas the peripheral cells had developed into a "perichondrium" and stained with type I and type II collagen antibodies. On plastic dishes plated with 5×10^6 cells per 35-mm dish, cartilage nodules developed after 4–6 days, but the type II collagen synthesis only reached levels of 10–20% of the total collagen. The majority of the cells differentiated into fibroblasts and myoblasts and synthesized type I collagen. These studies demonstrate that analysis of cell specific types of collagen provides a useful method for detailing the specific events in the differentiation of mesenchymal cells in vitro.

Chick limb mesodermal cells, taken from stage-24 (14) embryonic limb buds, may develop chondrogenic, fibrogenic, and myogenic functions in vitro (for reviews, see references 17 and 34). Chondrogenic differentiation is favored by culturing mesodermal cells on tissue culture plastic dishes at high cell densities according to Caplan (2, 3) or over agar (16).

The onset of cartilage formation has been followed by studying the uptake of [^{35}S]sulfate into acid mucopolysaccharides *in situ* (32) and in vitro (7, 36), and by the appearance of polygonally shaped chondrocytes or translucent, metachromatically staining cartilage nodules (2, 4, 7, 31). With the discovery of type II collagen, a protein almost unique to hyaline cartilage (23), a new biochemical parameter has become available which is specific to the chondrogenic phenotype and may, indeed, be used to monitor its appearance. The purpose of this work was to introduce a

sensitive and specific assay of this protein suitable for studying the regulation of cartilage differentiation in cell culture experiments. Type II collagen or $[\alpha1(II)]_3$ is a genetically distinct collagen species and differs from type I collagen $[\alpha1(I)]_2\alpha2$ or type III collagen $[\alpha1(III)]_3$ by its amino acid composition and sequence, carbohydrate content, extent of hydroxylation and cross-linking, and antigenic properties. It can be distinguished routinely from type I collagen by the absence of $\alpha2$ chains on carboxymethyl (CM)-cellulose chromatography or disc gel electrophoresis and by its distinct cyanogen bromide peptide pattern (21, 22).

More recently, an immunohistological method has been developed which allows precise and highly sensitive identification of various collagen types in tissues or cell cultures. This method is based on the findings of Hahn et al. (12, 13) who were able to isolate specific antibodies to calf type II collagen which did not cross react with type I collagen. Antibodies specific to collagen types I, II, and III have been used with the immunofluorescence technique to localize these collagen types in normal and pathological tissues in several species (8, 9), and antibodies against chick type I and type II collagen have been prepared to study the occurrence of these collagen types during normal development of the chick embryo (39, 40). In this paper, the synthesis of type I and type II collagen of stage-24 limb bud cells has been followed in vitro under culture conditions which allow chondrogenic or fibrogenic/myogenic differentiation, by the use of a combination of biochemical and immunological methods.

MATERIALS AND METHODS

Cell Cultures

Chick limb mesodermal cells were obtained from 4-day (stage-23–24 [14]) hind limb and fore limb buds of White Leghorn chick embryos. The limb buds were dissected in Simm's Balanced Salt Solution and treated for 30 min with 0.25% trypsin and 0.1% crude collagenase (Worthington Biochemical Corp., Freehold, N.J., CLSPA) in Ca, Mg-free saline, pH 7.5, at 37°C, and afterwards were dissociated by vigorous shaking. The cells were filtered through a 20-μm nylon filter and washed with Ham's F12 medium (Seromed, München, West Germany) containing 10% fetal calf serum, 100 U/ml of penicillin-streptomycin, 183 μg of glutamine and 200 μg/ml of sodium ascorbate (subsequently referred to as complete F12 medium).

Cells were cultured in complete F12 medium in 35-mm Falcon tissue culture dishes over 1% agar (4) at densities of 5×10^6 cells per dish, or without agar, at densities of 10^6 and 5×10^6 cells per dish. Cells were maintained at 37°C in 95% air/5% CO_2 and fed by daily change of medium (2 ml per dish).

Biochemical Collagen Assays

Cell cultures were labeled during the first 4, 12, and 24 h and thereafter on alternate days for 24 h with 50 μCi of L-[2,3-^{3}H]proline (New England Nuclear, Dreieichenhain, West Germany, 35 Ci/mmol) per dish in F12 medium without proline, containing the additives listed above plus 50 μg/ml of β-aminopropionitrile. To isolate the labeled collagen, combined cells and medium were made 1 M in NaCl by adding an equal volume of 2 M NaCl, containing 0.1 M Tris·HCl, pH 7.5, 0.015 M EDTA, 0.01 M *N*-ethylmaleimide (NEM), and 0.005 M phenyl methylsulfonyl fluoride (PMSF) (26). Samples were kept frozen until used.

CM-Cellulose Chromatography

The samples were dialyzed exhaustively against 1 M NaCl, 0.05 M Tris HCl, pH 7.5, and precipitated together with 8 mg of carrier type I collagen from chick skin by 25% ammonium sulfate. The precipitates were dissolved in 0.5 M acetic acid and digested with pepsin (0.3 mg/ml) for 6 h at 15°C at pH 2. The pepsin digest was precipitated with 25% ammonium sulfate or 15% KCl, pH 8.5. The precipitates were dissolved in 0.5 M acetic acid and dialyzed against 0.08 M sodium acetate buffer, pH 4.8.

For CM-cellulose chromatography (29) an equal volume of 4 M urea was added, and after denaturing at 45°C for 20 min, the samples containing $0.5–2 \times 10^6$ cpm ^{3}H were applied to a (1.5×10 cm) column of CM-cellulose (CM 52, Whatman Chemicals, Div. W. & R. Balston, Maidstone, Kent, England), equilibrated with 0.04 M sodium acetate, pH 4.8, and 4 M urea (1). Elution was carried out at 40°C, using a linear gradient from 0 to 0.12 M NaCl over a total volume of 400 ml. The eluate was monitored at 206 nm with an LKB Uvicord III (LKB Instruments, Uppsala, Sweden), and collected in 6-ml fractions. Radioactivity was determined by liquid scintillation counting of 1-ml aliquots mixed with 10 ml of aquasol (NEN).

The relative amounts of type II and type I collagen were calculated from the ratios of total radioactivity eluting with the $\alpha1$ and $\alpha2$ fractions, according to the formula:

$$\%\ \text{type II} = \frac{\alpha1\ [\text{cpm}] - 2 \cdot \alpha2\ [\text{cpm}]}{\alpha1\ [\text{cpm}] + \alpha2\ [\text{cpm}]} \times 100$$

The formula is based on the experience that pure type I collagen yields a chain ratio of $\alpha1{:}\alpha2 = 2{:}1$, and the assumption that all radioactivity eluting in the $\alpha1$ fraction above the two-fold amount eluting in the $\alpha2$ fraction is due to $\alpha1(II)$.

Cyanogen Bromide Cleavage

The ^{3}H-labeled α-fractions from CM-cellulose chromatographies as well as unlabeled α-chains from chick bone, skin and cartilage collagen were cleaved with cyanogen bromide (CNBr) in 70% formic acid at 30°C for 4 h as described by Epstein et al. (6).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Whole α-chains obtained by CM-cellulose chromatography and CNBr peptides were identified by SDS-PAGE in 0.1% SDS on 5% or 10% acrylamide gels, respectively. The ^{3}H-labeled samples were separated on 10 × 0.6 cm gels in 0.1% SDS, 0.1 M sodium phosphate, pH 7.0, and 0.5 M urea according to Goldberg et al. (11). After electrophoresis, the gels were cut in 1-mm slices, and the radioactivity was determined, after digestion of the slices with 0.5 ml of 30% H_2O_2 for 18 h at 55°C, by liquid scintillation counting.

DNA-Analysis

Aliquots taken before dialysis were sonicated 3 × 15 s on ice and assayed for DNA by the fluorometric method with diaminobenzoic acid as described by Santoianni and Ayala (30).

Hydroxyproline

Aliquots of the ^{3}H-labeled, dialyzed samples were hydrolyzed with 1 ml of 6 N HCl at 108°C for 24 h under nitrogen atmosphere. The hydrolysate was chromatographed on a M-82 (Beckman Instruments, Munich, West Germany) ion exchange resin (0.9 × 55 cm), using a regular amino acid analysis program without ninhydrin. The radioactive [^{3}H]hydroxyproline and [^{3}H]proline of the eluate were determined in a liquid scintillation counter after adding 10 ml of aquasol to 1-ml aliquots of the fractions.

Immunological Techniques

PREPARATION OF ANTIBODIES: Antibodies against acid-soluble type I collagen from chick bone, pepsin-soluble type II collagen from chick sternal cartilage, and pepsin-soluble type III collagen from chick skin were prepared in rabbits and guinea pigs as described previously (reference 39 and Footnote 1). Type specific antibodies were obtained from the sera by crossabsorption on the heterologous collagen types and by affinity chromatography on the homologous collagen types. The specificity of the antibodies was tested by means of the passive hemagglutination-inhibition test and immunofluorescence (39).

IMMUNOFLUORESCENCE TECHNIQUE: Cell cultures grown on plastic dishes were rinsed with phosphate-buffered saline and incubated with rabbit or guinea pig anti-collagen type I or type II or type III antibodies for 30 min at room temperature in a moist chamber. The dishes were washed with phosphate-buffered saline and counterstained with the respective fluorescein conjugated anti-rabbit or guinea pig γ-globulins for another 30 min. After thorough washing, the cultures were sealed with glycerol/phosphate buffered saline 9:1 under a cover slip. For sectioning of the cartilage nodules which had formed on the plastic dishes after 4–5 days, the multilayer was carefully removed, randomly folded, and mounted on a block of liver. Frozen sections of 6–8 μm were cut on a cryotome (Südwestdeutsche Laboreinrichtung und Elektrotechnik GmbH, Mainz, West Germany), treated with 2% testicular hyaluronidase (Serva, Heidelberg, West Germany) for 20 min, and stained with antibodies as described above. Similarly, cell aggregates which had formed over agar were mounted and sectioned for immunofluorescence staining. Double staining with rabbit anti-type II collagen antibodies and guinea pig anti-type I collagen antibodies (or vice versa) was performed as previously described (39, 40), using fluorescein conjugated rabbit anti-guinea pig γ-globulin antisera (Behringwerke AG, Marburg-Lahn, West Germany) and trimethyl-rhodamine conjugated goat anti-rabbit γ-globulin antisera (Nordic Pharmaceuticals, Tilburg, The Netherlands).

Radioimmunoassay

To quantitate the amounts of ^{3}H-labeled type I and type II collagen present in medium and cell extracts within a time course experiment, 10–50 μl aliquots of the dialysed extracts corresponding to 20,000–50,000 cpm were precipitated with 25 μl (10–15 μg) of rabbit anti-type I collagen antibodies or anti-type II collagen antibodies. 100 μl of buffer (0.15 M NaCl, 0.05 M Tris·HCl, pH 7.5, 1% bovine serum albumin) were added and the solution was allowed to stand for 1 h at room temperature. The antibody-antigen complex was precipitated together with 50 μl of rabbit nonimmune serum by adding 200 μl of goat anti-rabbit γ-globulin serum. The precipitates were allowed to stand overnight at 4°C; they were then centrifuged, washed, and counted by liquid scintillation counting as described earlier (18, 38). To determine the amount of anti-collagen antibodies necessary for quantitative precipitation of the labeled collagen of a certain aliquot, from the sample with the highest number of cpm per milliliter within an experiment aliquots were precipitated with increasing amounts of antibodies. The amount of antibodies necessary to precipitate 90% of the precipitable counts was used routinely in all samples.

RESULTS

Collagen Synthesis during Differentiation over Agar

When dissociated chick limb mesodermal cells of stage-24 were cultured over agar according to

[1] H. Herrmann and K. von der Mark. Manuscript in preparation.

Levitt and Dorfman (16), they formed aggregates of various sizes within 24 h. After 3 or 4 days, the cells became translucent and chondroblast-like in appearance. Frequently, the primary aggregates merged with one another to form large masses of cells. During the first 24 h, sections of the aggregates reacted only with anti-type I collagen antibodies. Type I collagen was observed until the 4th day in culture (Fig. 1*a*). After 48 h, staining with anti-type II collagen antibodies revealed positive though weak reaction similar to that for type I collagen (not shown). On the 3rd or 4th day in culture, the amount of intercellular material within the aggregates increased markedly and stained heavily for type I and type II collagen (Fig. 1*b*).

After 6–8 days in culture the type I collagen disappeared from the center of the aggregates but remained in a "perichondrium" surrounding the cell aggregates (Fig. 2*a*). In the center, hyaline cartilage had developed which stained with type II collagen antibodies only (Fig. 2*b*). The cells attained typical chondrocyte-like morphology, and the extracellular matrix stained metachromatically with Giemsa dyes (not shown). At 6–8 days also, the first positive reaction with anti-type III collagen antibodies was observed in the perichondrium (not shown). This indicates that the cells of the perichondrium are fibroblast-like cells (10).

These findings were confirmed by a quantitative biochemical collagen determination, using CM-cellulose chromatography and hydroxyproline analysis. Fig. 3*a* shows a CM-cellulose chromatogram of collagen extracted from stage-24 limb bud cells, cultured immediately after preparation for 4 h over agar in the presence of [^{3}H]proline. The α1:α2 ratio was 2:1 as expected for the type I collagen molecule; it increased very rapidly with time, reaching levels of 18:1 or more after 8 days in culture (Fig. 3*b*). Such a ratio corresponded to approximately 85% type II collagen (Fig. 5). The presence of α1(II) in the α1 fraction of the CM-cellulose chromatography shown in Fig. 3*b* was confirmed by CNBr cleavage and SDS-PAGE of the peptide mixture. The CNBr peptide pattern of the α1 fraction of Fig. 3*b* was similar to that of ^{3}H-labeled α1(II) from chick sternal cartilage (Fig. 4). Small amounts of α1(I) present in the α1 fraction of Fig. 3*b* were not detectable in the CNBr peptide pattern. When the α2 fraction was cleaved with CNBr, the resulting peptide mixture was typical for α2 from chick bone collagen (not shown). CNBr peptides of α1(III) which cochromatographs with α2 on CM cellulose could not be detected, although immunofluorescent staining with type III collagen antibodies of the aggregates suggested the presence of type III collagen.

The increase in the ratio of type II/type I collagen synthesis on agar with time is depicted in Fig. 5. The ratios were calculated on the basis of the α1:α2 ratios obtained by CM-cellulose chromatography. The diagram demonstrates that, on agar, close to 90% of the collagen synthesized after 10–12 days was type II collagen.

The total collagen synthesis per cell increased rapidly on the 4th day of culture (Fig. 6*a*). The maximum [^{3}H]hydroxyproline synthesis was reached after 8–10 days, and was followed by a decrease to low levels of collagen synthesis. Type II collagen synthesis increased faster and reached higher levels than type I collagen synthesis, but there was also considerable stimulation of type I collagen synthesis which reached its maximum on the 6th day in culture, when the perichondrium formed (see Fig. 2*a*).

These results obtained by a combination of hydroxyproline analysis and CM-cellulose chromatography were confirmed by a radioimmunoassay. Fig. 6*b* shows a typical time course of type I and type II collagen-bound radioactivity, which was precipitable with an excess of type I and type II collagen specific antibodies. The maximum of type II collagen synthesis per cell occurred between the 8th and 10th day in culture, as is also shown in Fig. 6*a*. However, relatively less type I collagen synthesis was determined with the radioimmunoassay between the 4th and 6th day in culture (see Discussion).

Minor differences in time and amount of maximal collagen synthesis per cell were observed among individual experiments, but from the results it is obvious that on agar the majority of the cells develop the chondrogenic phenotype.

Collagen Synthesis during Differentiation in High Density Cultures on Tissue Culture Plastic Dishes

When seeded at densities below 10^6 cells per 35-mm dish (referred to as "low density cultures"), limb bud mesenchyme cells developed partially fibrogenic and myogenic functions, but in no case chondrogenic function, within 12 days in culture. When a low-density culture was stained with anti-type I collagen antibodies, undifferentiated mesenchymal cells gave rise to weak posi-

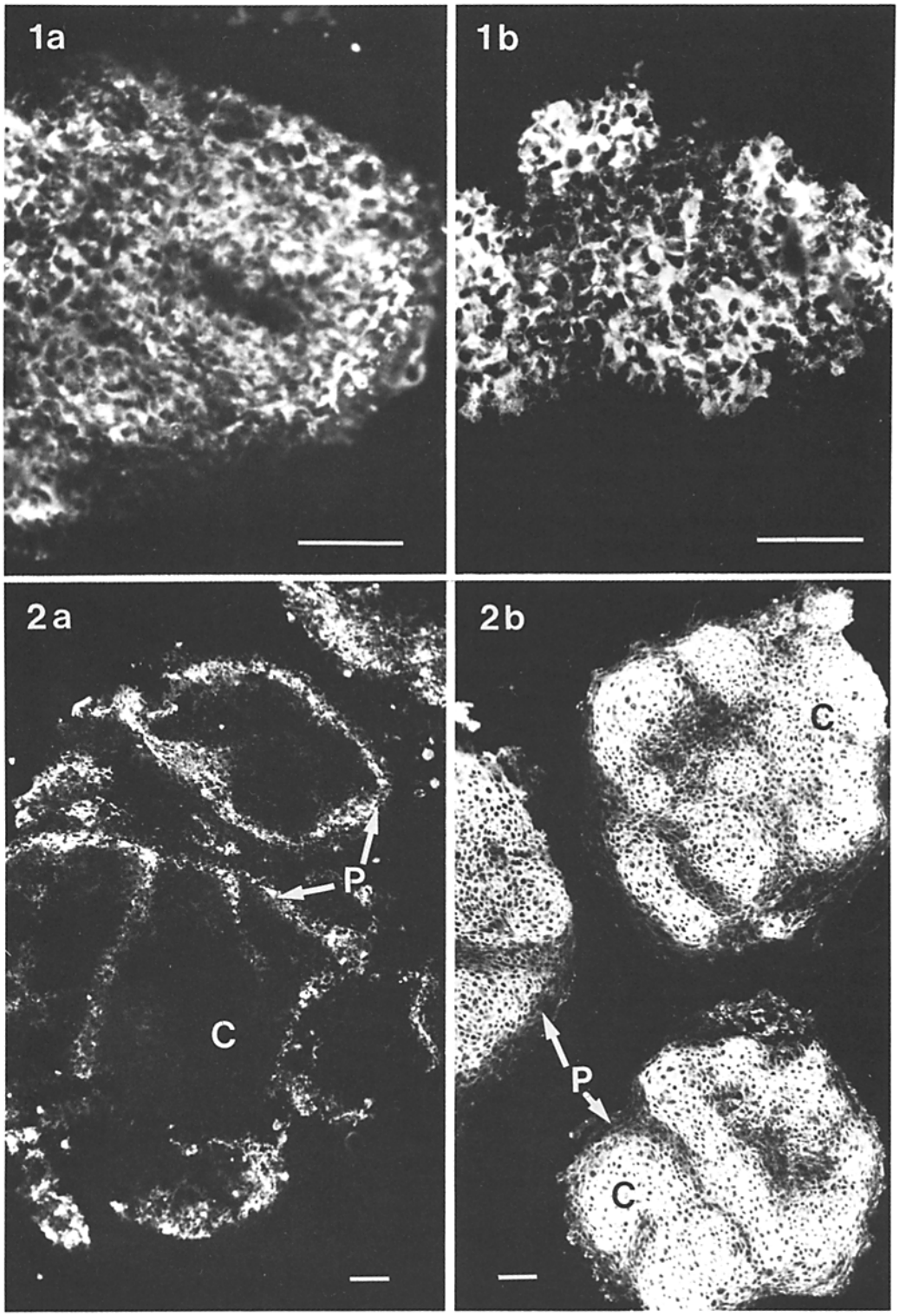


FIGURE 1 Immunofluorescent staining of frozen sections of an aggregate formed by stage-24 limb bud mesenchymal cells after 4 days in culture on agar. Section "a" was stained with antibodies to type I collagen, section "b" with antibodies to type II collagen. Although mesenchymal type I collagen synthesis is still ongoing (a), most cells have already developed chondrogenic functions as indicated by type II collagen synthesis (b). ×160. Scale bar, 100 μm.

FIGURE 2 Immunofluoresence staining of aggregates as in Fig. 1, after 6–8 days in culture: A perichondrium (P) has formed around the cell aggregate which stains with antitype I collagen antibodies (a), whereas the type I collagen of the center of the aggregates has disappeared. It is replaced by a hyaline cartilage matrix (C) which stains with anti-type II collagen antibodies (b). × 60. Scale bar, 100 μm.

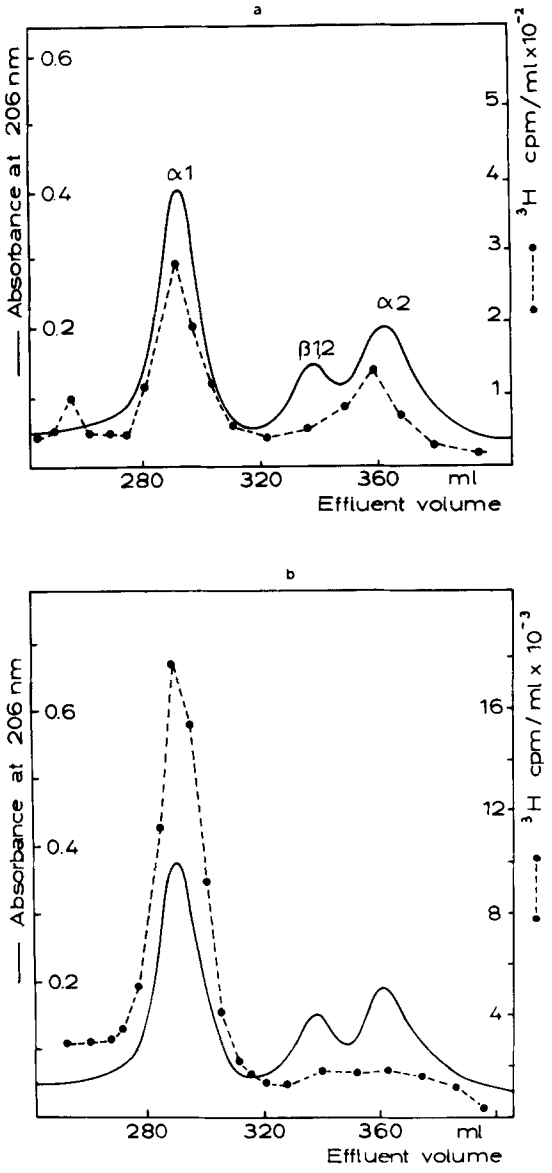


FIGURE 3 CM-cellulose chromatography of ^{3}H-labeled, pepsin-treated collagens (●----●), extracted from chick limb bud cell cultures on agar. Solid line: carrier type I collagen from chick skin. (*a*) Collagen obtained from cells which were incubated for 4 hours after dissociation in the presence of [^{3}H]proline. The α1:α2 ratio of the radioactivity was 1.9:1, indicating the predominance of type I collagen. (*b*) Collagen obtained from cells which were cultured for 8 days over agar and labeled for the last 24 h with [^{3}H]proline. The α1:α2 ratio of 18:1 indicates predominance of type II collagen.

tive fluorescence, and after 3 or 4 days, myoblasts, recognizable by their characteristical spindle shape (Fig. 7), and fibroblasts showed strong fluorescence. But no cells could be detected that showed positive reaction with anti-type II collagen antibodies within 12 days of culture.

When seeded at densities above confluence (5 × 10^6 cells per 35-mm dish, referred to as "high density cultures" according to Caplan [2]), mesenchymal cells regularly started to develop cartilage nodules between the 4th and 5th day of culture. These nodules which stained metachromatically with toluidin-blue or Giemsa-dyes could be specifically labeled with anti-type II collagen antibodies (Fig. 8), whereas the majority of the surrounding

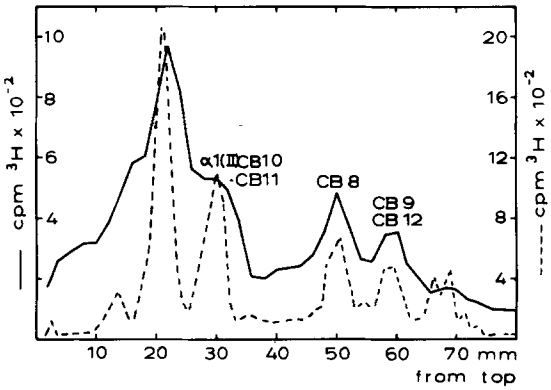


FIGURE 4 Radioactivity profile on SDS gel electrophoresis (10% acrylamide) of the ^{3}H-labeled α1 fraction of Fig. 3*b* after CNBr cleavage (——). Superimposing of the radioactivity profile of CNBr peptides of ^{3}H-labeled chick sternal cartilage α1(II) (——) indicates that the α1 fraction of Fig. 3*b* consisted mainly of α1 (II).

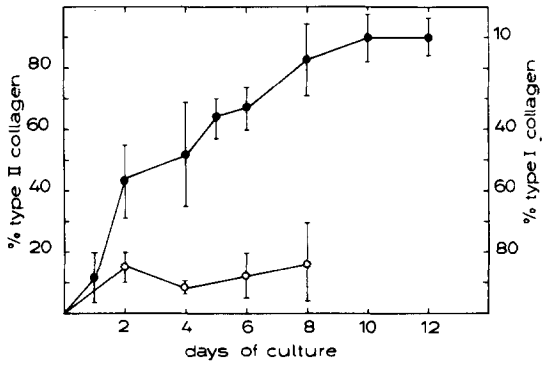


FIGURE 5 Time course of type II/type I ratios of the collagens synthesized by mesenchymal cells on agar (—●—) and on high-density plastic cultures (—○—). Cells were labeled on alternate days with [^{3}H]proline, and the type II/type I ratios of the extracted collagens were calculated from the α1:α2 ratios obtained by CM-cellulose chromatography. The values are mean values of five separate cell culture experiments. Standard deviation is given by the vertical bars.

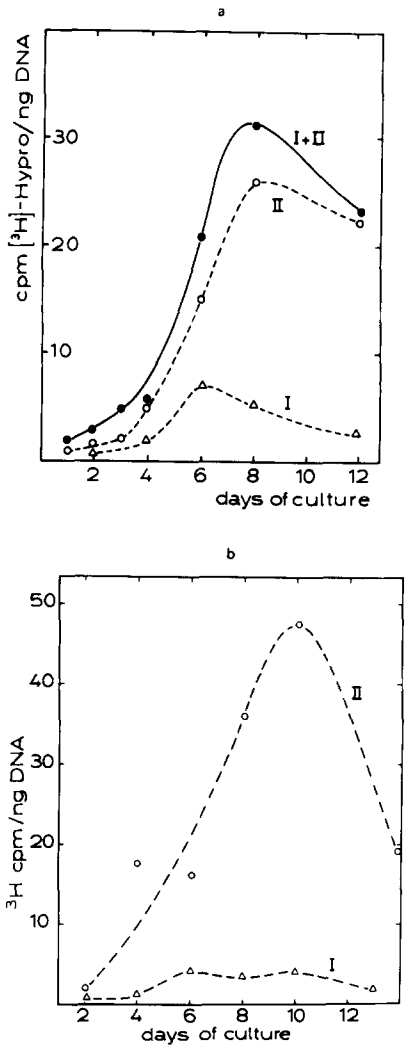


FIGURE 6 Experiment on time course of type I and type II collagen synthesis of limb bud cell cultures on agar, determined by combination of CM-cellulose chromatography and [^{3}H]hydroxyproline analysis (*a*) and by a radioimmunoassay (*b*). Collagen was extracted from cell cultures after 24-h labeling periods with [^{3}H]proline on alternate days as described in Materials and Methods. (*a*) Total collagen synthesis per cell, expressed by [^{3}H]hydroxyproline synthesis per nanogram DNA (●——●). The values for type I collagen bound [^{3}H]hydroxyproline (△-----△) and type II collagen bound [^{3}H]hydroxyproline (○-----○) were calculated from the α1:α2 ratios of CM-cellulose chromatographies. (*b*) Immune-precipitation of ^{3}H-labeled type I collagen and type II collagen with specific rabbit anti-type I collagen antibodies (△——△) or anti-type II collagen antibodies (○-----○). The collagen-antibody complexes were precipitated with goat anti-rabbit γ-globulins. The precipitable ^{3}H cpm were normalized to the DNA content of the sample. The time courses shown under (*a*) and (*b*) were obtained from one cell culture experiment which was typical among four experiments of the same type. Shifts of ±1.5 days in the time of maximal collagen synthesis were observed among individual experiments.

cell layer stained only with anti-type I collagen antibodies. This was confirmed by immunofluorescence double staining of cross-sections through the randomly folded cell layer (Fig. 9).

CM cellulose chromatography of collagen from high-density cultures revealed that, in contrast to the agar cultures, close to 90% of the collagen synthesized after 6–8 days on tissue culture plastic dishes was type I (Fig. 5). The maximum of total collagen synthesis was reached between the 5th and 8th day (Fig. 10*a*). A similar result was obtained by the radioimmunoassay: Type I collagen synthesis increased more rapidly than type II collagen synthesis (cf. Fig. 10*a* and *b*), and, after 8 days, more than 4 times as much type I collagen could be precipitated as type II collagen. This demonstrated that, even in high-density cultures, fibrogenic or myogenic differentiation was stimulated more than 4-fold over chondrogenic differentiation (see Discussion).

DISCUSSION

In this study it was demonstrated that the onset of chondrogenesis can be followed quantitatively by biochemical and immunological determination of type II collagen synthesis. The appearance of this cartilage specific protein established the beginning of chondrogenic expression of stage-24 limb bud cells in vitro 2 days after dissociation, which is 2 days earlier than determined by morphological or histological criteria (2, 16, 31). In cell cultures on agar or in high-density cultures on plastic dishes, type II collagen synthesis increased concomitant with the appearance of metachromatically staining cartilage nodules or translucent polygonally shaped chondrocytes. By immunofluorescence with collagen type specific antibodies, it was possible to gain new insight into the process of cell differentiation, since the technique is capable of following the phenotypic expression of single cells.

It was shown that in the cell aggregates formed on agar, apparently all cells synthesized type I collagen up to 4 days, while synthesis of type II collagen had already started. This suggested a gradual and continuous transition from type I to type II collagen synthesis during cartilage differ-

entiation, and confirmed the hypothesis that chondrogenic differentiation is indicated not only by the onset of type II collagen synthesis but also by a transition from type I to type II collagen synthesis (39).

The ratios of type I and type II collagen synthesis after 6–8 days determined by two independent methods were 80–90% type II for agar cultures and 10–20% for cultures on plastic dishes. From these figures we concluded that 80–90% of the cells on agar or 10–20% of the cells on plastic differentiated into chondrocytes, assuming that all cells developing from limb mesenchymal cells synthesized the same amount of collagen per cell in vitro. This assumption was supported by an estimate of the ratio of cells staining with type I or type II collagen antibodies in a number of sections of agar and plastic cultures.

With Ham's F12 medium with 10% fetal calf serum, in tissue culture plastic dishes, only 10–20% of the cells differentiated into chondrocytes at a cell density of 5×10^6 cells per 35-mm dish. Other authors (2, 3, and footnote 2) reported that, at this cell density, 80–90% of the cells become chondrocytes, which in our hands was achieved only at plating densities of $10\text{–}20 \times 10^6$ cells per dish. This discrepancy may be explained by the use of embryo extract, which could not be applied in our studies, since embryo extract contains collagen. Embryo extract collagen, however, interfered with the immunofluorescence technique, as it absorbed to the cell surfaces (Footnote 3).

The question may be raised, whether the two culture conditions (agar cultures or high-density cultures on plastic dishes) stimulated development of chondroblasts (agar) or fibroblasts (plastic), or whether they allowed selective survival of the one or the other already committed cell type. According to Searls and Janners (33), limb bud cells before stage-25 are not committed to chondrogenic or fibrogenic/myogenic differentiation; they develop according to their position within the limb bud. Therefore, the latter interpretation is unlikely. The results suggest rather that the mesenchymal cells develop in response to their environmental conditions: fibroblasts cannot develop within the aggregates formed over agar because of the anaerobic conditions, nor can they grow on an agar surface to which they cannot attach; they find, however, suitable conditions in monolayer culture on tissue culture plastic dishes, or on the surface of cartilage aggregates as perichondral fibroblasts. (A sorting-out process (27) is unlikely since no cell movement has been observed within a cartilage matrix.)

These results and those reported earlier (2, 7, 16, 31) suggest that chondrogenic development occurs only when mesenchymal cells are allowed to stay in contact with one another and thus build up their own matrix and condition their environment. This implies that cell-cell contacts, contacts between cells and matrix components, or a soluble factor such as a "conditioned medium factor" (31, 36, 37) are required for condroblast differentiation.

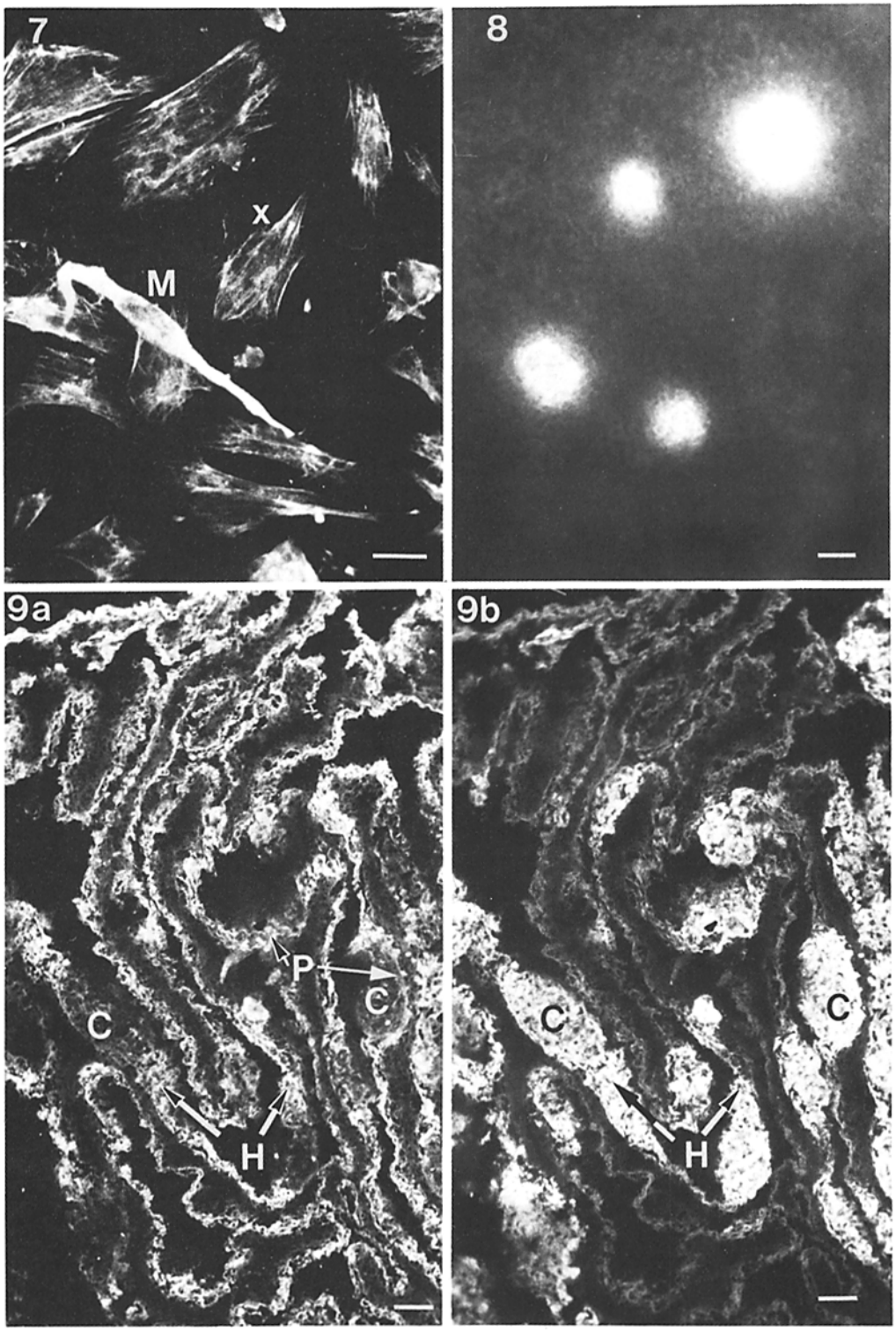
Besides hyaline cartilage, three epithelial tissues are known which also synthesize type II collagen during early embryonic stages: notochord (19, 39), neural retina (28), and cornea (35). On the other hand, senescent chondrocytes are also able to synthesize type I collagen in vitro under certain culture conditions (25, 20). Since, in limb bud cell cultures, neither one of the epithelial tissues mentioned above was present, and since the cell cultures were not maintained long enough to allow the chondrocytes to switch to type I collagen synthesis, the synthesis of type II collagen was used as a specific indicator for chondrogenesis.

Also, type I collagen is not a protein unique for one cell type; it is synthesized by fibroblasts, myoblasts, osteoblasts, odontoblasts, smooth muscle cells, corneal epithelial cells, etc. In this study, no attempts were made to identify further the nature of the type I collagen-secreting cells which developed in vitro from limb mesenchyme. According to their morphology and the reports in the literature (17, 34), we assume that the majority of these cells are fibroblasts and myoblasts. Additional marker proteins will have to be used to discriminate between myogenic phenotype and fibrogenic phenotype, e.g. the myosin light chain, which is different in both cell types.

The biochemical determination of type II collagen by CM-cellulose chromatography and hydroxyproline analysis is quantitative and rather specific but limited, for three reasons: (*a*) the $\alpha 1{:}\alpha 2$ ratio obtained from CM-cellulose chromatograms may be erroneous because of the presence of $[\alpha 1(I)]_3$ molecules which have been found in cell cultures under certain conditions (24, 20); they coelute with normal $\alpha 1$(II). (*b*)

[2] A. Dorfman. Personal communication.

[3] K. von der Mark. Unpublished observation.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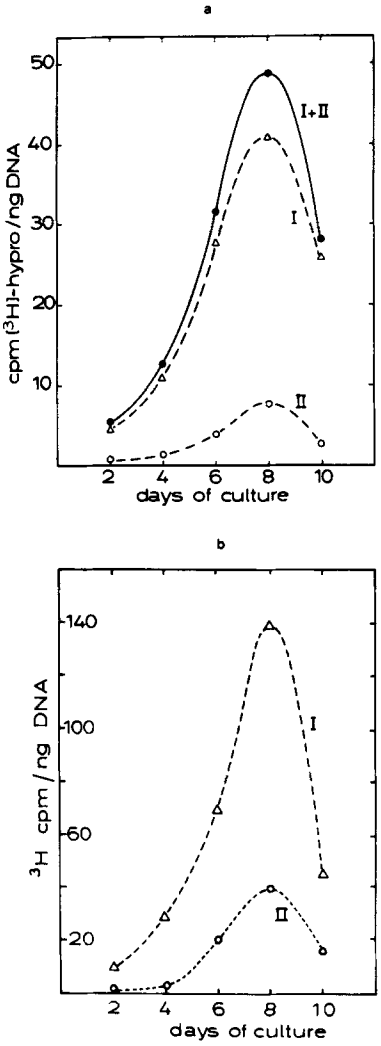

Chick $\alpha 1[(III)]_3$ molecules which co-elute with $\alpha 2$ on CM-cellulose chromatography (15) may decrease the $\alpha 1{:}\alpha 2$ ratios. (*c*) Small proportions of type II collagen (below 10%) giving rise to $\alpha 1{:}\alpha 2$ ratios below 2.5:1 cannot be accurately determined by this method.

For these reasons, the radioimmunoassay seems to be the most promising approach for the quantitative determination of the synthesis of type I and type II collagen, possibly also of type III collagen, in cell cultures. The radioimmunoassay as described requires less than 1% of labeled collagen required for the classical biochemical methods, and considerably less experi-

FIGURE 10 Time course of the syntheses of type I and type II collagen of high density limb bud cell cultures on falcon plastic, determined by CM-cellulose chromatography and [^{3}H]hydroxyproline analysis (*a*) and by radioimmunoassay (*b*). Collagen was extracted from cell cultures after 24-h labeling periods with [^{3}H]proline on alternate days as described in Materials and Methods. (*a*) Total collagen synthesis per cell, expressed by [^{3}H]hydroxyproline synthesis per nanogram DNA (●——●). The values for type I collagen bound [^{3}H]hydroxyproline (△——△) and type II collagen bound [^{3}H]hydroxyproline (○——○) were calculated from the $\alpha 1{:}\alpha 2$ ratios of CM-cellulose chromatographies. (*b*) Immune precipitation of ^{3}H-labeled type I collagen and type II collagen with specific rabbit antitype I collagen antibodies (△——△) or anti-type II collagen antibodies (○——○). The collagen antibody complexes were precipitated with goat anti-rabbit γ-globulin. The precipitable ^{3}H cpm were normalized to the DNA content of the sample. The time courses shown under (*a*) and (*b*) were obtained from one cell culture experiment which was typical among four experiments of the same type.

FIGURE 7 Immunofluorescent staining of a low-density culture (10^6 cells per 35-mm dish) of limb bud cells after 4 days on plastic dishes with anti-type I collagen antibodies. Undifferentiated mesenchymal cells (*X*) show weak membrane-bound fluorescence; single myoblasts (*M*) and fibroblasts (not shown) exhibit a more intense fluorescence. No positive reaction with anti-type II collagen antibodies was obtained within 12 days in culture. × 400. Scale bar, 20 μm.

FIGURE 8 Immunofluorescent staining of a high-density cell culture (5×10^6 cells per 35 mm dish), grown on falcon plastic for 8 days, with anti-type II collagen antibodies. Cartilage nodules show positive reaction; the surrounding cell layer does not show positive reaction but stains with anti type I collagen antibodies (not shown). × 60. Scale bar, 100 μm.

FIGURE 9 Immunofluorescent staining of a frozen section through the randomly folded cell layer shown in Fig. 8. The section was double stained with guinea pig anti-type I collagen antibodies (*a*) and rabbit anti-type II collagen antibodies (*b*) as described in Materials and Methods. The cartilage nodules (*C*) stain with anti-type II collagen antibodies only (*b*). Perichondrium (*P*) and the cell layer between the nodules stain with type I collagen antibodies only. Hybrid matrix (*H*) stains with both antibodies. × 160. Scale bar, μm.

mental effort. It relies entirely, however, on the specificity of the antibodies. The described direct precipitation test required that excess of antibodies be used. In contrast to the radioimmunoinhibition assay, only the collagen synthesized during the pulse-labeling period was determined. Equal incorporation of [^{3}H]proline into type I and type II collagen during the pulse period was assumed, in which case the amounts of precipitable type I collagen-bound radioactivity and type II collagen-bound radioactivity should be directly comparable.

The time courses of the synthesis of type I and type II collagen obtained by radioimmunoassay, on the one hand, and CM-cellulose chromatography and hydroxyproline analysis, on the other hand, agreed in their general features. Slight discrepancies can be explained by time shifts in maximal collagen synthesis among several experiments and by the observation that after a 24-h pulse period some of the peptide-bound [^{3}H]hydroxyproline was contained in material smaller than α chain size, as determined by SDS-PAGE (not shown). This material, which is in all likelihood collagen-derived by proteolytic activity, was accounted for in the hydroxyproline analysis, whereas in the radioimmunoassay only antigenic intact molecules were detected.

The use of genetically distinct collagen types as cell specific markers offers new aspects and several methodological advantages for experimental studies of connective tissue cell differentiation. Selective identification of type I and type II collagen by biochemical and immunological methods allows specific determination of chondrogenic, fibrogenic, or osteogenic development of chick limb bud mesoderm. By the immunofluorescence technique, cells exhibiting chondrogenic versus fibrogenic or myogenic phenotype can be individually identified. The radioimmunoassay enables one to follow quantitatively the onset of chondrogenesis of chick limb mesenchymal cells in vitro, and might thus provide a sensitive method for studying the mechanism and regulation of cartilage differentiation.

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