CHONDROGENIC DIFFERENTIATION OF LIMB BUD CELLS IN COLLAGEN GEL CULTURE

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ABSTRACT

Chick limb bud mesenchyme cells were isolated from stage 22-23 embryos and embedded within exogenous collagen gels at low cell density. They grew to form a colony of round cells *in situ* and differentiated into cartilage with an overt metachromatic matrix. Synthesis of sulfated glycosaminoglycan increased markedly with time and most were deposited within the collagen gel matrix. The cells primarily synthesized type I collagen followed by predominant synthesis of cartilage-specific type II collagen, during the collagen gel culture. The control cells seeded in the monolayer cultures at the same initial density never showed chondrogenic activity. The results indicate that the three-dimensionally enclosed environment of collagen gel culture is required for the limb bud mesenchyme cells to effectively accumulate synthesized matrix macromolecules and to differentiate into cartilage.

Differentiation of cartilage is an important key event in limb development and has been extensively studied using *in vitro* culture systems (1, 7, 9, 24, 27). It has been demonstrated that dissociated prechondrogenic limb mesenchyme cells could differentiate into cartilage *in vitro* (7, 12, 24, 27). Chondrogenic differentiation is favored by culturing mesenchyme cells at densities greater than confluency, or as a pellet. However, cells plated at low density become fibroblastic and fail to differentiate into cartilage. These findings indicated the importance of cell association or cell-cell interaction in chondrogenesis (7, 21, 27). Recently, we found that chick embryonic chondrocytes cultured within exogenous collagen gels could maintain their differentiated phenotype *in vitro* (13, 30). This collagen gel culture system provided the cells with a three-dimensionally enclosed environment to which matrix macromolecules effectively accumulated. We now report that limb mesenchyme cells cultured within exogenous collagen gels eventually differentiated into cartilage, as confirmed by morphological and biochemical analyses. An enclosed environment, rather than close cell-cell associa-

tion, seemed to play an important role in chondrogenesis.

MATERIALS AND METHODS

Cell Culture

Fore- and hind-limb buds of stage 22-23 White Leghorn chick embryos (10) were dissected with watchmakers forceps, under a dissecting-microscope. Limb buds were washed three times with phosphate-buffered saline without Ca^{2+} and Mg^{2+} , and incubated in culture medium (Ham's F-12 medium) containing 0.1% collagenase (CLS II, Worthington, Freehold, NJ) and 0.25% trypsin (Difco Lab., Detroit, MI) for 30 min at 37°C. Enzyme-treated limb buds were washed three times with culture medium and the loosened ectoderm was removed with cataract knives. Fresh culture medium, supplemented with 10% fetal calf serum (FCS), was then added to the remaining tissue and the preparation was triturated through Pasteur pipet 10 to 15 times to disrupt the tissue into single cells. Dissociated mesenchyme cells were filtered through four layers of lens paper, collected by centrifugation,

washed three times with culture medium and counted in a hemocytometer.

Released limb bud mesenchyme cells were embedded in 1 ml of collagen gels (1×10^6 cells/ml) in 35 mm plastic dishes by previously reported methods (13, 30) and each gel was overlaid with 2 ml of culture medium supplemented with 10% FCS. Control monolayer cultures were prepared at the same cell density in 35 mm plastic dishes. Dishes were incubated at 37°C under 95% air/5% CO₂ and 1.5 ml of the medium was replaced daily.

Morphologic Examination

Phase photomicrographs of the cultures were taken at various times during incubation. For histological examination, the collagen gel cultures were fixed in Bouin's solution, dehydrated in alcohol, and embedded in paraffin. Sections were stained with toluidine blue or hematoxylin-eosin. Monolayer cultures (cultures on plastic dishes) were stained without embedding in paraffin, after fixation and dehydration.

Synthesis of [³⁵S]Sulfated Glycosaminoglycan (GAG)

Cultures were labeled with sodium [³⁵S]sulfate (10 µCi/ml; New England Nuclear, Boston, MA) for 24 h in 3 ml of culture medium at selected times of culture. Cell-layer and the medium were collected separately, frozen and thawed. The sulfated GAG was extracted as previously described (11) and was precipitated with cetylpyridinium chloride on a Millipore membrane (23) prior to liquid scintillation counting in Insta-Gel (Packard, Downer's Grove, IL).

Isolation of Newly Synthesized Collagens

Cultured cells were labeled for 24 h on days 1 and 8 with L-[¹⁴C]proline (7 µCi/ml, New England Nuclear) in 3 ml of fresh culture medium supplemented with ascorbic acid (50 µg/ml). Cells and medium were brought together, collected and stored frozen until use. The samples were digested with pepsin (100 µg/ml, Sigma) in 0.5 M acetic acid for 48 h at 4°C in the presence of carrier collagen (0.5 mg, bovine skin, acid-soluble). Labeled collagen was precipitated by adding solid NaCl to give a final concentration of 2 M. The precipitate was collected by centrifugation at 10,000 g for 60 min, dissolved in 1.0 M NaCl, 0.05 M Tris-HCl buffer (pH 7.4) to inactivate pepsin, dialyzed exhaustively against dilute

acetic acid at 4°C, and lyophilized. Additionally, a portion of the lyophilized sample was digested with purified bacterial collagenase (Advance Biofactures, Lynbrook, NY) as previously described (28) to test for collagenous character of the extracted samples.

Sodium Dodecyl Sulfate (SDS)-Gel Electrophoresis

Lyophilized samples were resolved by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (16) using a 6% acrylamide gel and processed for fluorography with sodium salicylate (8). Cyanogen bromide (CNBr) cleavage was performed according to Miller *et al.* (20) and analyzed by electrophoresis on 15% acrylamide gels.

Two-dimensional CNBr peptide mapping was performed using a modification of the method of Barsh and Byers (3, 29) and processed for fluorography using 2,5-diphenyloxazole as a scintillator (5).

RESULTS

Freshly isolated limb mesenchyme cells had a rounded or ovoid appearance within the collagen gels (Fig. 1a). Within two days of culture, some of these cells seemed apparently larger in size (Fig. 1b) and an accumulation of metachromatic matrix was first detected around these larger cells at this stage (Fig. 1c). Daily observations showed that the cells were viable and grew to form colonies of round cells, which exhibited concentrated accumulation of pericellular metachromatic matrix (Fig. 1, d and e). In contrast, when limb bud mesenchyme cells were cultured on a plastic dish (monolayer culture), they initially showed a polygonal morphology followed by gradual transformation to flat and fibroblastic appearance. No metachromatic matrix was detected during the 12-day culture period (Fig. 1f).

The rate of sulfated GAG synthesis was used as a quantitative marker of chondrogenic differentiation. The GAG synthesis in collagen gel culture increased markedly with time, and GAG predominantly accumulated within collagen gels, that is, within cell-layers (Fig. 2). Only 17–20% of the synthesized GAG was released into the medium after 7–12 days of culture. In control monolayer cultures, however, the rate of GAG synthesis was maintained at a low level during the entire culture period. No significant increase was observed in monolayer cultures below confluency and only in high density cultures was the

Fig. 1 Morphology of chick limb mesenchyme cells in culture. a, b: Phase-contrast micrographs in collagen gel culture, soon after gelation and on day 2, respectively. c, d, e: Micrographs in collagen gel culture, on days 2, 4 and 12, respectively (toluidine blue staining; arrow, cell; arrowhead, metachromatic matrix). f: 12-day monolayer culture (toluidine blue staining). a, b: $\times 600$. c: $\times 150$. d-f: $\times 260$

increase of GAG synthesis observed (data not shown).

The analysis of collagen synthesis revealed another biochemical aspect of chondrogenic differentiation. Cultures were labeled with L- $[^{14}\text{C}]$ proline for 24 h on day 1 and on day 8, and radiolabeled collagens were analyzed by SDS-gel electrophoresis. On day 1, newly synthesized collagen in both collagen gel culture and monolayer culture was predominantly type I which consists of two identical $\alpha 1(\text{I})$ chains and one $\alpha 2$ chain (Fig. 3, a and b, and Fig. 4, c and d). Changes in collagen synthesis occurred during 8 days in collagen gel culture, but not in monolayer culture. As shown in Fig. 3c, the collagen synthesized by 8-day collagen gel culture contained very small amounts of $\alpha 2$ chain, indicating the relative decrease of type I collagen synthesis and possible

increase of type II collagen which consists of three identical $\alpha 1(\text{II})$ chains. The initiation of type II collagen synthesis, during collagen gel culture, was confirmed by the CNBr peptide profiles (Fig. 4f). Using the two-dimensional CNBr peptide mapping method, the presence of type II collagen peptides in 8-day collagen gel culture is clearly shown among type I collagen (Fig. 5). Moreover, the cells grown in collagen gels can synthesize cartilage-specific minor collagenous chains which include 1α , HMW (or M1), and N (6, 22, 28, 29) (Fig. 3c). These chains were completely digestible with purified bacterial collagenase indicating the collagenous character of these chains.

In the control monolayer culture, the synthesis of type I collagen persisted in the 8-day cultures (Fig. 3d), and this was also confirmed by

Fig. 2 Rate of glycosaminoglycan synthesis after labeling with [^{35}S]sulfate for 24 h. Cell-layer ($\circ$) and the medium ($\triangle$) in collagen gel culture. Cell-layer ($\bullet$) and the medium ($\blacktriangle$) in monolayer culture. Each value represents the average of three cultures.

Fig. 3 Fluorogram of [^{14}C]proline-labeled collagen synthesized by cultured chick limb mesenchyme cells. Collagen gel culture (a) and monolayer culture (b) on day 1; collagen gel culture (c) and monolayer culture (d) on day 8

Fig. 4 Fluorogram of cyanogen bromide peptides derived from synthesized collagen and standard collagens. Standard type I (a) and type II collagen (b). Monolayer (c) and collagen gel culture (d) on day 1. Monolayer (e) and collagen gel culture (f) on day 8

CNBr peptide analysis (Fig. 4e). Bands with the mobility of HMW and N were never detected.

DISCUSSION

Disaggregated limb mesenchyme cells fail to differentiate into cartilage in conventional monolayer cultures, and become chondrogenic only when the cells are seeded at high density or above confluency (7, 12, 27). In addition to cell density, requirements of the cell-cell and cell-matrix interaction, and rounded cell shape in chondrogenesis have also been documented (7, 17, 21). Our present study showed that dispersively embedded single limb bud cells within collagen gels could divide and form colonies of round cells and eventually differentiate into cartilage. High cell density or close cell-cell proximity was not required in this culture system. We have previously demonstrated that chick chondrocytes were phenotypically stable *in vitro* when

Fig. 5 Two-dimensional cyanogen bromide peptide mapping from 8-day collagen gel culture. The first dimension gel is shown at the top. The direction of the first (5% acrylamide gel) and the second dimension (12.5% acrylamide gel) electrophoresis is indicated by horizontal and vertical arrows. Lane on the right shows the cyanogen bromide-cleaved peptides of standard type II collagen.

they were three-dimensionally enclosed within exogenous collagen gels (13). This enclosed environment again seemed to be important during chondrogenic differentiation of limb bud mesenchyme cells in the present study.

The importance of round cell shape in chondrogenesis has been suggested (2). Even in high density cultures, the rounded cell shape plays an important role, since the central cells of aggregates could form a rounded morphology in such a compact associated state (2). In our study, in which the prechondrogenic mesenchyme cells were cultured as rounded or ovoid cells within collagen gels, partly supports the role of such spherical cell shape in chondrogenesis.

Cell-matrix interaction is another requirement for chondrogenesis (17, 26). It has been demonstrated that exogenous chondroitin sulfate stimulated chondrogenesis (15). In the pres-

ent study, the synthesis of GAG increased significantly with time and most GAG was deposited in the cell-matrix (within the gels). This may have stimulated the chondrogenesis of limb bud mesenchyme cells.

Type II collagen is another matrix macromolecule of cartilage which is used to define the expression of chondrogenic phenotype (19). Chick limb bud mesenchyme before stage 25 predominantly synthesizes type I collagen and *de novo* synthesis of type II collagen is found biochemically at stage 25/26 in the limb core (18). Our present data demonstrated that stage 22–23 limb bud cells primarily synthesized type I collagen. During the collagen gel culture, however, they began to synthesize type II collagen, which indicated the chondrogenesis. This is in accord with the recent observation by Solursh *et al.* who demonstrated the chondrogenesis from single limb mesenchyme cells by the appearance of immunofluorescence stain against type II collagen, using a similar collagen gel culture method (25). These findings suggest the transition of collagen types *in vitro*. However, it is difficult to exclude the possibility of selective survival of presumptive chondroblasts which begin to synthesize type II collagen and selective death of other type I collagen-producing cells during collagen gel culture.

Cartilage specific disulfide-bonded collagenous chains, HMW (22, 28) and N (29), also appeared in the 8-day collagen gel culture of limb mesenchyme cells, but never in the monolayer cultures. The appearance of these cartilage-specific molecules supports the chondrogenic expression in collagen gel culture, although the detailed role of these molecules remains to be determined.

It has been demonstrated that exogenous collagens stimulate chondrogenesis *in vitro* (14). The possibility exists that the chondrogenesis in our cultures was initiated by embedding the cells within the exogenously added collagen gel matrix. However, using agarose gels, instead of collagen gels, limb bud mesenchyme cells again differentiated into cartilage when they were placed in a three-dimensionally enclosed state (unpublished data). This enclosed environment may also play an important role during the re-expression of the differentiated phenotype from dedifferentiated chondrocytes (4). Our present culture method provides a microenvironment in which cells can obtain spherical cell shape, and the collagen gels act as a useful framework in which synthesized matrix macromolecules can effectively accumulate. The collagen gel culture

described here provides a useful means of investigating environmental or cellular factors during cartilage differentiation.

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