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HIGH DENSITY MICROMASS CULTURES OF EMBRYONIC LIMB BUD MESENCHYMAL CELLS: AN IN VITRO MODEL OF ENDOCHONDRAL SKELETAL DEVELOPMENT

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SUMMARY

To study the mechanisms regulating endochondral skeletal development, we examined the characteristics of long-term, high density micromass cultures of embryonic chicken limb bud mesenchymal cells. By culture Day 3, these cells underwent distinct chondrogenesis, evidenced by cellular condensation to form large nodules exhibiting cartilage-like morphology and extracellular matrix. By Day 14, extensive cellular hypertrophy was seen in the core of the nodules, accompanied by increased alkaline phosphatase activity, and the limitation of cellular proliferation to the periphery of the nodules and to internodular areas. By Day 14, matrix calcification was detected by alizarin red staining, and calcium incorporation increased as a function of culture time up to 2 to 3 wk and then decreased. X-ray probe elemental analysis detected the presence of hydroxyapatite. Analogous to growth cartilage developing in vivo, these cultures also exhibited time-dependent apoptosis, on the basis of DNA fragmentation detected in situ by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL), ultrastructural nuclear morphology, and the appearance of internucleosomal DNA degradation. These findings showed that cellular differentiation, maturation, hypertrophy, calcification, and apoptosis occurred sequentially in the embryonic limb mesenchyme micromass cultures and indicate their utility as a convenient in vitro model to investigate the regulatory mechanisms of endochondral ossification.

Key words: micromass cultures; endochondral ossification; cartilage maturation; calcification; apoptosis; collagen type X.

INTRODUCTION

Endochondral ossification is the process by which mesenchymal cells differentiate into chondrocytes, which proliferate, mature, and undergo hypertrophy and matrix calcification; the calcified cartilage is eventually replaced by bone. This process is seen in embryonic skeletal development, as well as in postnatal skeletal growth and fracture healing.

Several in vitro culture systems have been used to reproduce the events that occur during endochondral ossification in vivo. In general, chondrogenic cells and chondrocytes express their full phenotypic repertoire only when cultured at high density. Examples include the high density micromass cultures of embryonic limb mesenchymal cells (1,8,26–28,35,36,38), cultures of chondrocytes in agarose gels (7), gelatin-coated dishes (2,3), and alginate beads (10,25), and cultures of chondrocyte pellets (5,6,21). In all these types of cultures, three-dimensional interactions between the cells are needed for chondrogenesis, cartilage maturation, and calcification.

In the chondrocyte maturation program, the fate of terminal chondrocytes at the chondro-osseous junction remains incompletely understood. A number of studies have shown that terminal chondrocytes undergo apoptosis (9,17,39). Some authors have suggested that terminal chondrocytes undergo transdifferentiation into osteoblast-like cells (11,33,34). There is also recent evidence that some chondrocytes located at sites of first endochondral bone formation exhibit an asymmetric cell division, resulting in one daughter cell which undergoes apoptosis, while the other remains viable and reenters the cell cycle (33). It has been shown that chondrocytes that leave the proliferative pool exhibit the DNA fragmentation pattern characteristic of programmed cell death, and that terminal chondrocytes display the characteristic morphology of apoptotic cells, such as chromatin and cellular condensation (9,17,39).

In this study, we have characterized in detail the high density micromass cultures of embryonic limb mesenchymal cells (1) to investigate growth cartilage maturation. This system permits the observation of the continuous process of differentiation, maturation, and calcification (8,36,38). The chondrocytic nature of the cells in culture was characterized by morphology and extracellular matrix (ECM) production. Chondrocyte maturation was assessed on the basis of cell proliferation, cellular hypertrophy, expression of collagen type X, and calcification, as measured by ^{45}Ca incorporation, atomic absorption spectrometry, and X-ray probe microanalysis. Programmed cell death was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL), electrophoretic analysis of internucleosomal DNA degradation, and ultrastructural morphology.

MATERIALS AND METHODS

Chicken embryos. Fertilized White Leghorn chicken eggs (Truslow Farms, Chestertown, MD), were incubated at 37° C in a humidified egg incubator for 41/2 d until Hamburger–Hamilton stage 23–24.

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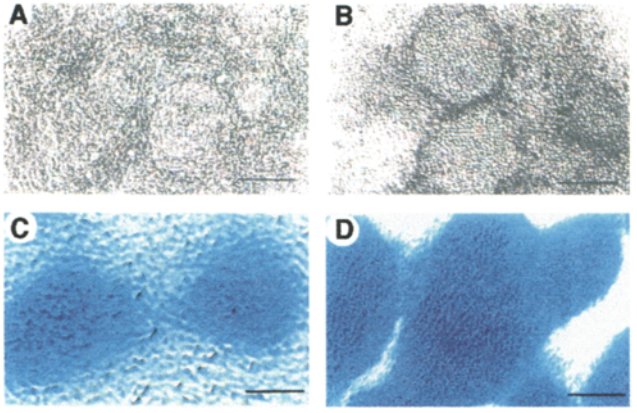

FIG. 1. Morphology of limb mesenchyme micromass cultures examined by light microscopy of whole mount cultures. *A–B*, Day 3 and Day 6, respectively, viewed by phase-contrast microscopy; *C–D*, Day 3 and Day 6, respectively, stained with Alcian Blue. By Day 3, cartilage nodules which are positive for Alcian Blue are seen, indicating that the ECM is rich in sulfated proteoglycans characteristic of cartilage. The nodules increased in size with time in culture. *Bar* = 100 μm.

Micromass limb bud mesenchymal cultures. Chick limb bud mesenchymal cells were isolated and established as micromass cultures as described previously (1,36). Cells were plated at 25–30 × 10^6 cells/ml as 10 μl micromass in 24-well tissue culture plates (Corning Glass Works, Corning, NY). The culture medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12) (GIBCO BRL, Gaithersburg, MD), containing 1.1 m*M* $CaCl_2$, 10,000 u/ml penicillin-streptomycin (Sigma Chemical Co., St. Louis, MO), 1% glucose, and 10% fetal bovine serum (FBS, HyClone, Logan, UT). From Day 2 on, the medium was supplemented with 2.5 m*M* β-glycerophosphate, 0.3 mg glutamine per ml, and 25 mg ascorbate per ml (Sigma) (8). The cultures were kept for up to 28 d and were harvested at Days 7, 14, 21, and 28.

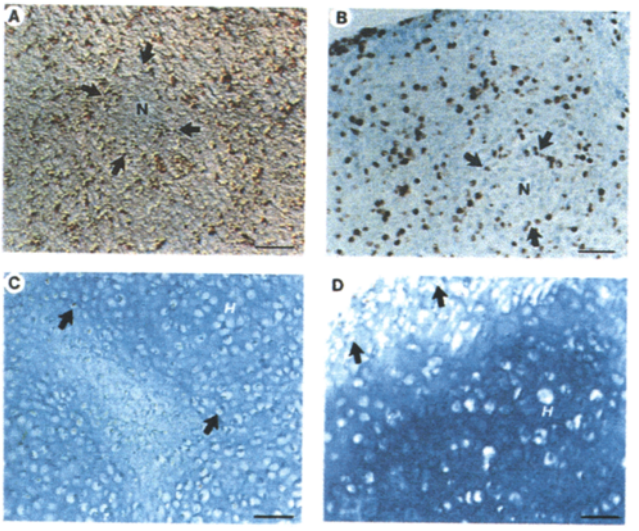

FIG. 4. Characterization of cell proliferation in limb mesenchyme micromass culture. Immunohistochemical detection of BrdU incorporation in limb mesenchyme micromass culture. *A*, Whole mount, Day 2. Sections: *B*, Day 7; *C*, Day 14; *D*, Day 21. *Arrows*, labeled cells; N, nodules; H, hypertrophic chondrocytes. In early cultures (Days 2 to 7) numerous cells stain positively for BrdU and are distributed mostly in the peripheral regions of the nodules. By Days 14 and 21, just a few labeled cells are detected and are located in the internodular regions and periphery of the cultures, and these cells, as shown by the lack of Alcian Blue counterstaining, are not chondrocytes. By Day 28, no positive cells are detected (data not shown). *Bar* = 100 μm (*A*) or 50 μm (*B–D*).

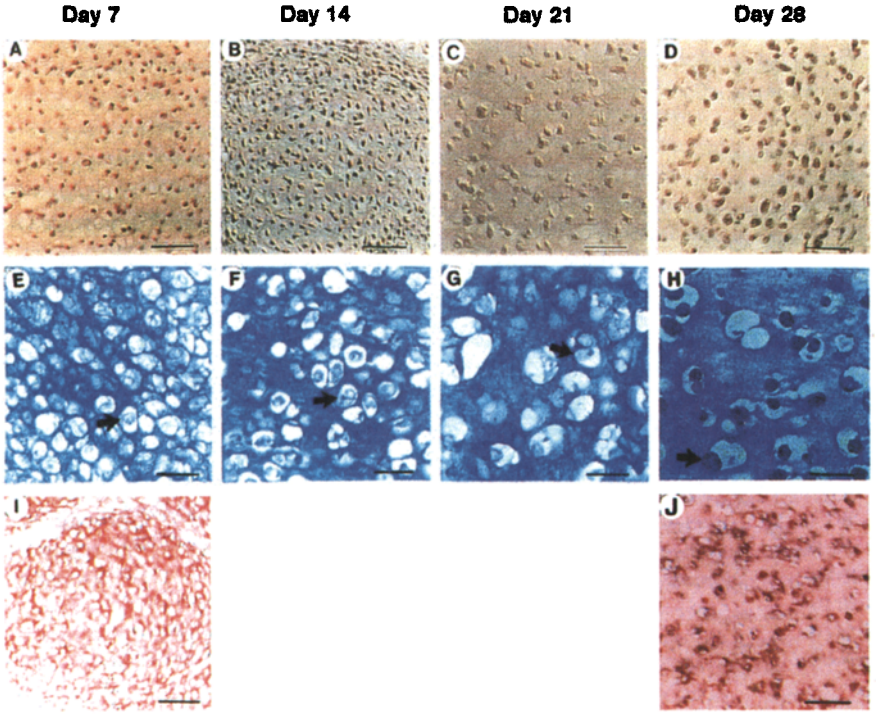

FIG. 2. Morphology of histological sections of limb mesenchyme micromass cultures examined by light microscopy. *A–D*, H/E stain; *E–H*, Alcian Blue stain. The cells stained with H/E display cuboid-like appearance and are surrounded by large amounts of ECM, similar to chondrocytes in vivo. The Alcian Blue-stained ECM, seen since early stages of culture, persists up to Day 28 and increases as a function of time. Large hypertrophic chondrocytes are seen (*arrows*), with characteristic increase in cell size (*I,J*). Immunostaining for type II collagen (*I*, Day 7; *J*, Day 28) showing persistent expression up to Day 28, increasing in amount in relation to cell number. *Bar* = 50 μm (*A–D*, and *I–J*) or 25 μm (*E–H*).

Morphology and histology of cultured cells. Until Day 7, whole mount cultures were observed routinely by phase-contrast light microscopy. Generally, because large nodules formed over the entire culture by Day 7 and significantly reduced resolution of whole mount observation, cultures maintained for 7 d or more were examined only after sectioning. The cultures harvested on Days 7, 14, 21, and 28 were fixed in 4% paraformaldehyde or HistoChoice (Amresco, Solon, OH), embedded in Paraplast-X (Oxford Labware, St. Louis, MO), and sectioned at 8 μm thickness.

Histochemical stains. The following stains (Sigma) were used: hematoxylin and eosin (H/E) for culture morphology (22), Alcian Blue at pH 1 to detect cartilage matrix sulfated glycosaminoglycans (23), and alizarin red for mineralization (22).

Immunohistochemistry (IMH). The production of collagen type II, characteristic of cartilage, was assessed by IMH with a monoclonal antibody against chick collagen type II (II-II6B3 from Developmental Studies Hybridoma Bank, Iowa City, IA) (24). The sections were deparaffinized, rehydrated, and swelled overnight with 0.1 *N* acetic acid to expose collagen fibrils, and treated with 300 U of hyaluronidase (Calbiochem, San Diego, CA) per ml for 40 min at 37° C to release proteoglycans before incubation with the primary antibody (20). The antibody was diluted at 1/50 in phosphate-buffered saline (PBS). Secondary antibody incubation and detection were done with a Streptavidin-Biotin System Histostain-SP Kit (Zymed, San Francisco, CA).

Scanning electron microscopy. Micromass cultures were plated on glass slide chambers (Corning). At specific time points, the cells were fixed in glutaraldehyde, postfixed in 1% osmium tetroxide, and then critical point dried. Specimens were sputter-coated with gold and viewed in a JEOL 35-C scanning electron microscope.

Chondrocyte Maturation

Cell proliferation. BrdU incorporation. Cells were incubated with medium containing 3 m*M* bromodeoxyuridine (BrdU; Sigma) and were harvested after 24 h. To detect sites of BrdU incorporation, sections were first treated with 0.2 HCl for 20 min to denature DNA, and then incubated with a mouse monoclonal antibody against BrdU (Sigma) at a dilution of 1:50 in PBS. The Zymed Histostain-SP Kit was used to detect BrdU, and in the sections, light Alcian Blue counterstaining (pH 1 for 2 min) was used to visualize the spatial distribution of the BrdU-positive cells within the cartilaginous nodules and internodular regions. Nonimmune mouse IgG (Sigma) was used as primary antibody for the negative control.

Hypertrophy. Cell size. Given the round shape of the cells, mean cell size (± SD) at each culture time point was estimated by our measuring cell diameter microscopically using ocular and stage micrometers (n ≥ 100 cells for each culture).

Alkaline phosphatase activity. Cells were extracted with 0.05% Triton X-100 and assayed spectrophotometrically for alkaline phosphatase activity (ALP10; Sigma). Enzyme activity was expressed as the amount of *p*-nitrophenol released from the substrate *p*-nitrophenyl phosphate (based on absorbance at 405 nm) per min per milligram of protein, as determined by micro BCA assay (Pierce Chemical Co., Rockford, IL).

Collagen type X expression. Immunohistochemistry. Rabbit-derived antibodies against chick collagen type X (gift of Dr. Maurizio Pacifici, University of Pennsylvania) were used for immunohistochemistry as described above for collagen type II. The primary antibody was used at 1:200 dilution.

Immunoblot analysis. Cultures were extracted and homogenized in 2% sodium dodecyl sulfate (SDS) in the presence of protease inhibitors (phenylmethylsulfonyl fluoride, ε-aminocaproic acid, and benzamidine hydrochloride; Sigma). Proteins were precipitated with 10% trichloroacetic acid, solubilized in 2% SDS, and assayed by the micro BCA assay. Samples (40 μg protein) were digested with pepsin (0.2 mg/ml in 0.5 *N* acetic acid; Sigma) overnight at 4° C, electrophoretically separated on a 4–15% polyacrylamide gel (Bio-Rad, Hercules, CA), and electrotransferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Blots were incubated with collagen type X antibody (1/1000 dilution), followed by alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) and the immunoreactive proteins were detected by alkaline phosphatase histochemistry. As a positive control, a protein extract from incubation Day 19 chick embryonic growth plate was used. The negative control used an irrelevant mouse IgG as the primary antibody.

Mineralization. Alizarin red staining. Paraffin-embedded sections of the cultures were stained with alizarin red to detect mineralization (22).

^{45}Ca incorporation. Cultures were incubated for 24 h with medium containing 1 μCi of ^{45}Ca (NEN/DuPont, Boston, MA) per ml, and the incorporated radioactivity was measured by liquid scintillation counting (15).

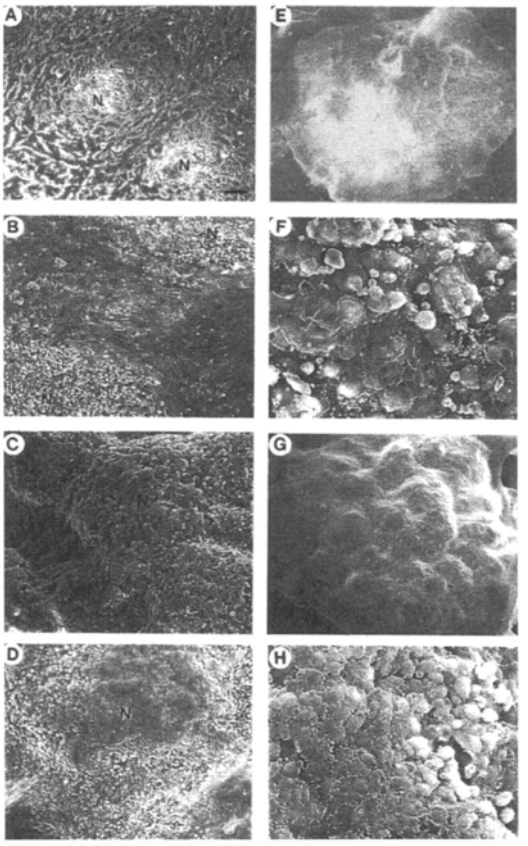

FIG. 3. Morphology of limb mesenchyme micromass cultures examined by scanning electron microscopy. *A–D*, Distinct cartilaginous nodules which increase in size during culture time (Days 7, 14, 21, and 28, respectively). *E–H*, View of the top of the cultures during later stages of culture (*E* and *F*, Day 14; *G* and *H*, Day 21), low and high magnification at each time point. N: nodules. Magnification *bar* = 100 μm (*A–D*), 500 μm (*E* and *G*), or 20 μm (*F–H*).

Calcium analysis by atomic absorption spectrometry. The cultures were desiccated by lyophilization, and ashed for 2 h at 650° C; the ash was diluted in 0.3 *N* HCl (Tracer Analysis Grade, Fisher Chemicals, Fair Lawn, NJ) and calcium content measured by atomic absorption analysis (Model 11E, Thermo Jarrell Ash Corp., Franklin, MA).

X-ray microanalysis. Cultures were fixed in glutaraldehyde, dehydrated, critical point dried, sputter-coated with carbon, and examined with a JEOL 35c scanning electron microscope equipped with a KEVEX 7000 EDAX system (Foster City, CA) for the analysis of Ca:P ratio (16).

Apoptosis. Internucleosomal DNA degradation was assessed by the following methods. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (12). Tissue sections were incubated with terminal transferase to label the 3′-end of fragmented nuclear DNA with fluorescein-conjugated dUTP. The fluorescein label was visualized by epifluorescence microscopy; the label was also subsequently detected immunohistochemically by peroxidase-labeled antibodies against fluorescein (In situ Cell Death Detection Kit, POD, Boehringer-Mannheim, Indianapolis, IN). Paraffin-embedded 8-μm histologic sections were deparaffinized, rehydrated in an ethanol series, and rinsed in PBS. The protocol recommended by the supplier was used, except for the Proteinase K step, which was not needed for cultures fixed in HistoChoice (Amresco), a noncrosslinking fixative. The sections were examined by light microscopy and the percentage of positive cells in distinct nodules of each section was calculated (n ≥ 300).

Gel electrophoresis of DNA. DNA was extracted from the cultures and

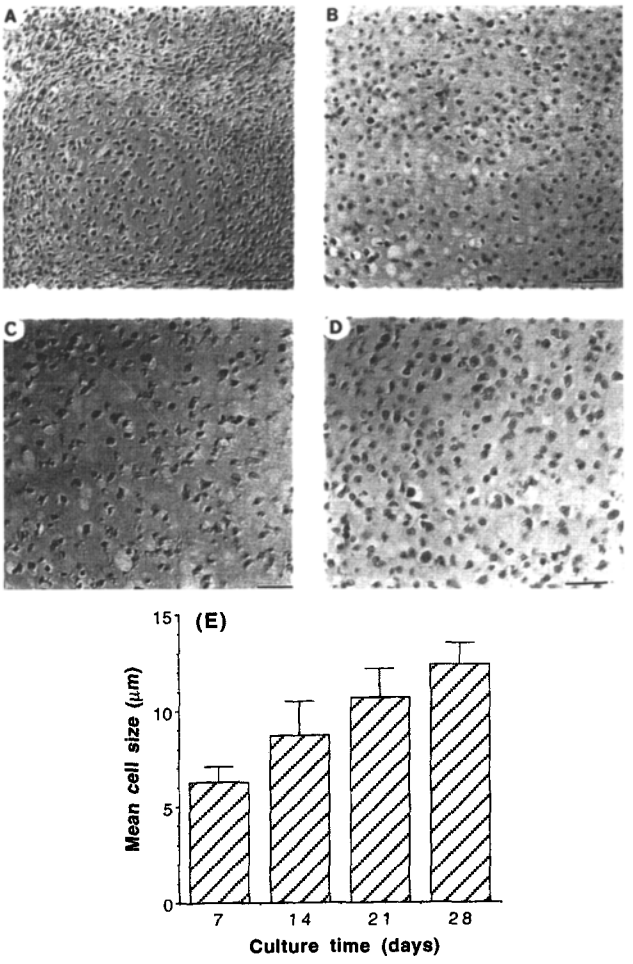

FIG. 5. Histomorphometric analysis of cell-size change in limb mesenchyme micromass culture as a function of time. Cell size was measured directly by the examination of histological sections stained with H/E: *A*, Day 7; *B*, Day 14; *C*, Day 21; *D*, Day 28. *Bar* = 50 μm. *E*, Graphic representation of change in cell size as a function of culture time (n = 100 cells). $P < 0.05$ compared to Day 7.

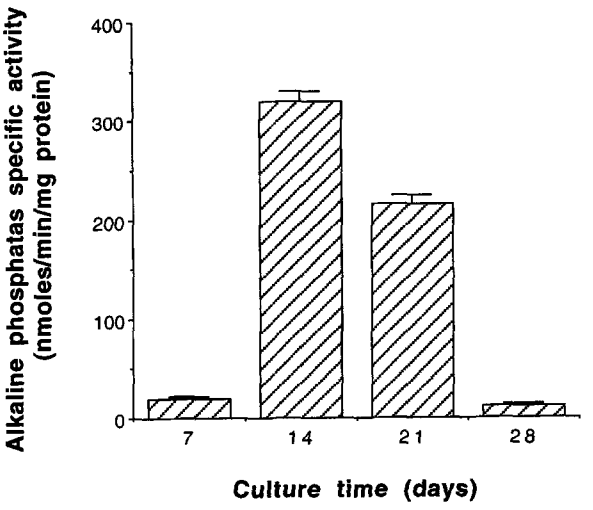

FIG. 6. Alkaline phosphatase-specific activity in limb mesenchyme micromass culture as a function of culture time. A peak of activity was detected on Day 14, followed by a decrease.

labeled with α[^{32}P]dideoxy ATP (Amersham, Arlington Heights, IL) (30,37). The presence of internucleosomal fragmented DNA was determined by electrophoretic size fractionation on a 2% agarose gel. After electrophoresis, the gels were first stained with ethidium bromide (Sigma) to visualize the size marker, dried without heat overnight, and then exposed to X-ray film at −70° C for about 4 h. As a negative control, α[^{32}P]dideoxy ATP-labeled DNA from the caudal region of chick embryonic sternum was used, since chondrocytes from this region are not hypertrophic (31).

Transmission electron microscopy. Cells grown in plastic culture dishes were scraped, rinsed, and fixed in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2 at 4° C, dehydrated, and embedded in Spurr's low viscosity epoxy resin (Polysciences, Warrington, PA). Ultrathin sections were cut at 600–800 Å thickness, placed on copper grids, stained with uranyl acetate and Reynold's lead citrate, and viewed in a JEOL 100 B transmission electron microscope.

Statistical analysis. All values were compared to the Day 7 values by paired Student's *t*-test. Statistical significance was considered at $P \leq 0.05$.

RESULTS

Morphology and ECM analysis. Cartilage nodules were observed by phase-contrast microscopy of whole mount cultures by Day 3 of culture (Fig. 1 *A*), and increased in size as a function of culture time, as observed on Day 6 (Fig. 1 *B*). The nodules stained positively for Alcian Blue (Fig. 1 *C*, Day 3; Fig. 1 *D*, Day 6), indicating that the matrix was rich in sulfated proteoglycans, characteristic of cartilage.

Light microscopy of histological sections stained with H/E showed that the cultured cells were round and totally surrounded by abundant ECM, resembling chondrocytes found in cartilage in vivo (Fig. 2 *A–D*). Increase in cell size and in the amount of ECM was seen by Alcian Blue staining over time (Fig. 2 *E–H*). Collagen type II was present in the early cultures and persisted up to Day 28 in culture (Fig. 2 *I* and *J*).

The cultures examined by scanning electron microscopy (Fig. 3) displayed conspicuous, cartilaginous aggregates that increased in size as a function of culture time (Fig. 3 *A–D*, Day 7, 14, 21, and 28, respectively). Top views of the cultures on Day 14 (Fig. 3 *E*) and Day 21 (Fig. 3 *G*) revealed the massive formation of nodules over the entire culture. Abundance of chondrocytes encased in large amounts of ECM could be seen at higher magnification on Day 14 (Fig. 3 *F*) and Day 21 (Fig. 3 *H*).

Cell proliferation. In early cultures, such as those of Days 3 and 7, numerous cells stained positively with BrdU (Fig. 4 *A*, *B*), and were primarily distributed to the periphery of the cartilaginous nodules, as detected by Alcian Blue counterstaining. With increased culture time, fewer positive cells were seen and were localized only to the internodular regions by Day 14 (Fig. 4 *C*), and Alcian Blue counterstaining showed that these cells had an only slightly stained pericellular matrix and therefore unlikely to be established chondrocytes; by Days 21 and 28, positive cells were detected only in the periphery of the cultures, not in the cartilaginous nodules (Fig. 4 *D*). These results showed that the overall rate and location of cellular proliferation in the micromass changed significantly during culture.

Hypertrophy. Morphology and ultrastructure. The chondrocytes in the center of the nodules resembled hypertrophic chondrocytes at Day 14 (Fig. 4 *C*), and on the basis of BrdU incorporation, they did not proliferate. A statistically significant increase in cell size as a function of culture time could be seen in H/E stained sections (Fig.

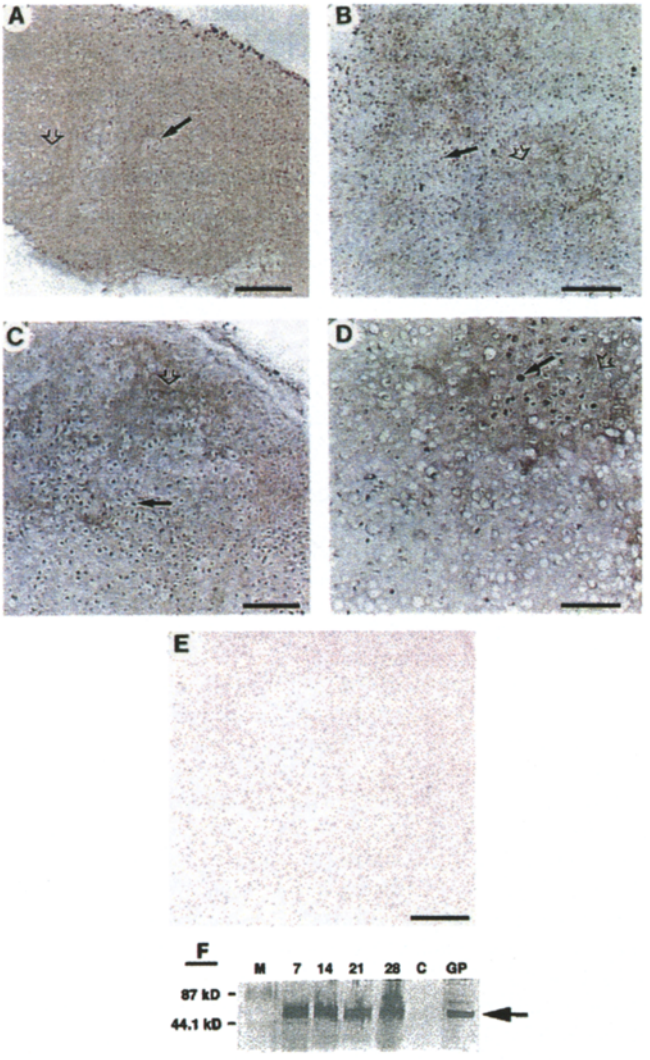

FIG. 7. Type X collagen expression in limb mesenchyme micromass culture detected by immunohistochemistry (IMH) and immunoblotting. *A*, IMH for type X collagen on Day 7; *B*, Day 14; *C*, Day 21; and *D*, Day 28. *E*, Negative control with irrelevant IgG as primary antibody. Type X collagen, a marker of chondrocyte hypertrophy, is detected by IMH in the cells (*arrows*) and in the ECM (*open arrows*) since Day 7 of culture and persists up to Day 28. The type X collagen ECM deposition is more intense in older cultures (Days 21 and 28). *Bar* = 100 μm. *F*, Type X collagen immunoblot. Type X collagen was detected (Mr approx. 70 kDa, *arrow*) in all samples (Days 7 to 28). *Lane C*: Negative control, staining with irrelevant mouse IgG. *Lane GP*: Positive control, protein extract from tibial chicken embryo growth plate.

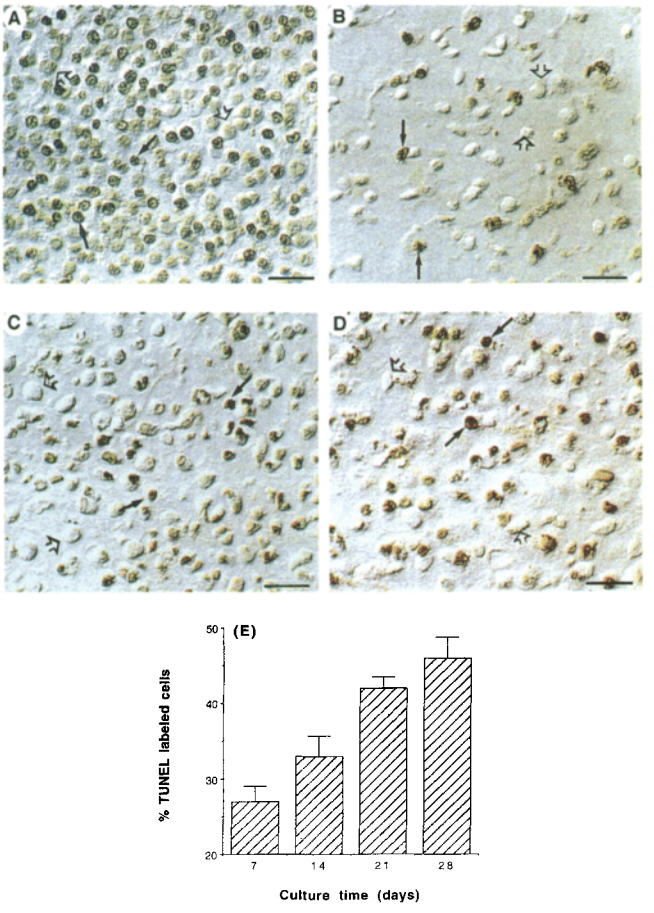

FIG. 9. Progression of apoptosis in limb mesenchyme micromass culture analyzed by TUNEL. Histological sections: *A*, Day 7; *B*, Day 14; *C*, Day 21; *D*, Day 28. *Arrows*: positive cells; *open arrows*: negative cells. *Bar* = 25 μm. *E*, Graphic representation of the percentage of TUNEL-positive cells over time.

5 *A*, Day 7; *B*, Day 14; *C*, Day 21; and *D*, Day 28) and is graphically represented in Fig. 5 *E*.

Ultrastructural analysis of the cultures revealed the presence of matrix vesicles which subsequently underwent mineralization, characteristic of hypertrophic chondrocytes. Alkaline phosphatase activity increased significantly from Day 7 to Day 14 and then started to decrease (Fig. 6). Collagen type X, a marker of hypertrophic chondrocytes, was detected in the cultures by Day 7, and its expression persisted up to Day 28, as seen by immunohistochemistry (Fig. 7 *A–D*) and immunoblot (Fig. 7 *F*).

Mineralization. Alizarin red staining was detectable by Day 14 and increased somewhat in intensity as a function of culture time (Fig. 8 *A*, Day 14; *B*, Day 28). The temporal profile of ^{45}Ca incorporation showed a statistically significant increase at Day 14, then a decrease (Fig. 8 *C*). Total calcium accumulation by the cultured cells, as determined by calcium atomic absorption analysis, increased significantly as a function of culture time until Day 28 (Fig. 8D). X-ray probe analysis also showed a Ca:P ratio of approximately 1.6, consistent with the presence of hydroxyapatite (data not shown).

Apoptosis. The presence of apoptotic cells was detected in the cultures beginning at culture Day 7. Fragmented DNA was detected by the TUNEL method from Day 7 on (Fig. 9 *A–D*), and the percentage of TUNEL-positive cells increased in a statistically significant manner over time (Fig. 9 *E*). The characteristic DNA ladder showing internucleosomal fragmentation of nuclear DNA was also observed by gel electrophoresis analysis in Day 14, 21, and 28 cultures (Fig. 10).

Finally, ultrastructural analysis of the cultured cells showed cells with prominent condensed chromatin and apoptotic bodies (Fig. 11), characteristic of cells undergoing apoptosis. Interestingly, these cells also appeared to produce abundant matrix vesicles, consistent with their hypertrophic state.

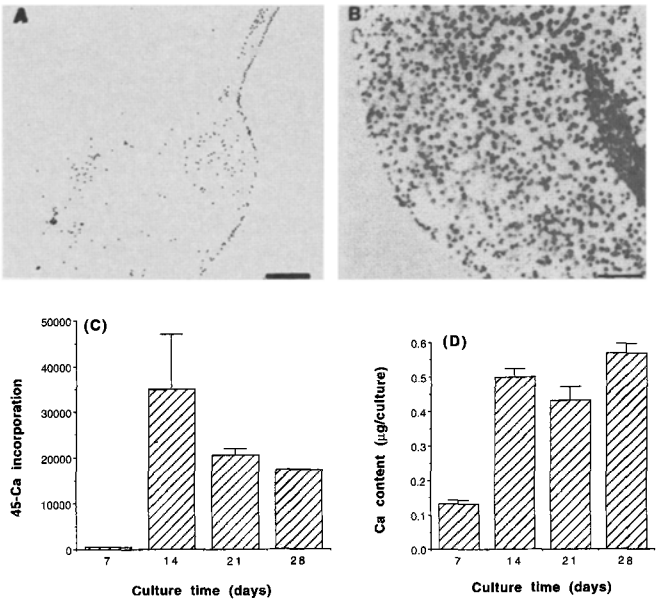

FIG. 8. Matrix mineralization in limb mesenchyme micromass culture. The analyses include: (*A,B*) Alizarin red staining of histological sections, Days 14 and 28, respectively. (*C*) ^{45}Ca incorporation as a function of culture time. (*D*) Calcium measurement by atomic absorption spectrometry as a function of culture time. By Day 14, calcification was detected by alizarin red around the cells (*reddish yellow stain*), and by Day 28 the staining appeared to increase in intensity and more extensive matrix deposition is seen. (*Note*: Day 28 cultures appeared to also show non-specific staining associated with the nuclei.) *Bar* = 100 μm. Increased Ca accumulation was detected by ^{45}Ca incorporation and Ca atomic absorption analysis by Day 7, with the maximum incorporation by Day 14.

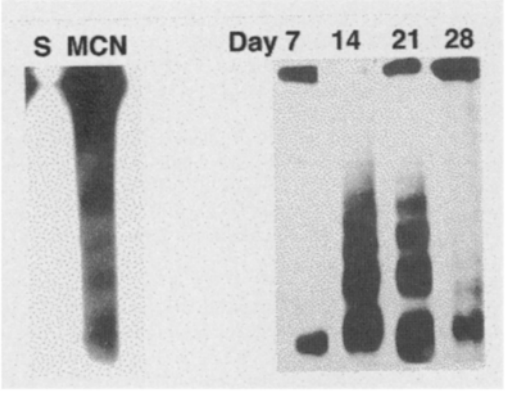

FIG. 10. Apoptosis in limb mesenchyme micromass culture analyzed on the basis of DNA fragmentation. Nuclear DNA extracted from cultures on Days 14, 21, and 28 show the DNA size ladder, typical of internucleosomal DNA fragmentation. Additional lanes: S-DNA isolated from the caudal region of day 16 chick embryonic sternum, consisting of chondrocytes which do not undergo hypertrophy, was used as a negative control; MCN-DNA samples treated with micrococcal nuclease used as positive control.

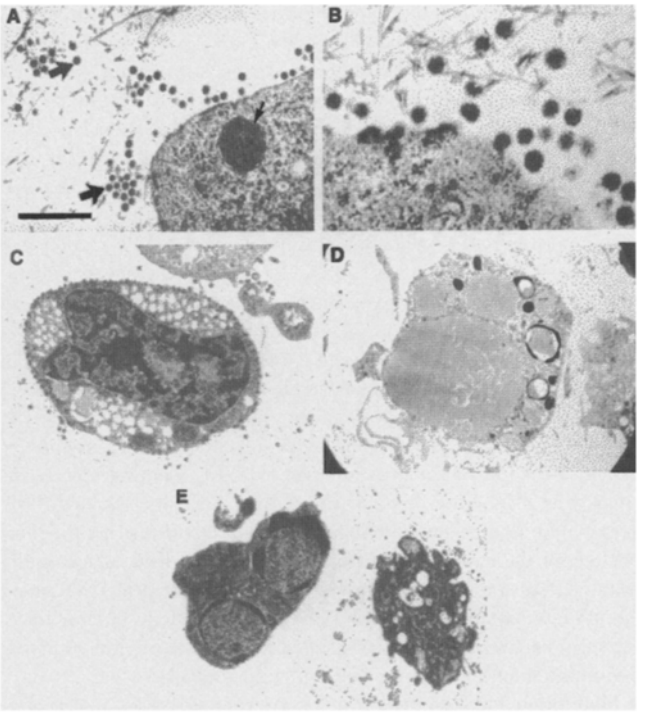

FIG. 11. Ultrastructure of limb mesenchyme micromass culture examined by transmission electron microscopy. *A*, At Day 7 the cultures showed signs of hypertrophy and apoptosis. Presence of discrete matrix vesicles (*arrows*), indicate the initiation of hypertrophy and mineralization. Also *note* the presence of an apoptotic body (*open arrow*) within the chondrocyte. *B*, Higher magnification view of a cluster of matrix vesicles with electron-dense centers associated with extracellular matrix. *C*, Appearance of condensed chromatin in a cell which is also producing abundant matrix vesicles. *D–E*, Chondrocytes showing peripheral crescents of condensed chromatin, consistent with active apoptosis. *Bar* = 1 μm (*A*), 0.33 μm (*B*), 1.33 μm (*C*), 4 μm (*D*), and 2.5 μm (*E*).

DISCUSSION

In this study we have characterized high-density micromass cultures of chick embryonic limb mesenchyme as an in vitro system to study the cartilage maturation program of endochondral skeletal development. The results clearly illustrate the applicability of the system to the study of chondrocyte development, growth, and maturation.

Chondrogenic differentiation is seen to begin by culture Day 2, giving rise to cartilaginous nodules that stain positively for Alcian Blue and collagen type II. Morphologically, by light microscopy as well as by scanning electron microscopy, the cells all display the typical cuboidal appearance of chondrocytes, surrounded by abundant extracellular matrix, and resemble chondrocytes found in cartilage in vivo.

The chondrocytes proliferate, as detected by BrdU incorporation. The number and spatial distribution of BrdU-positive cells as a function of culture time change considerably. Within the growing nodule, numerous cells stain positively with BrdU and are mainly at the periphery of the nodule. However, in the center of the nodules, where the cells resemble hypertrophic chondrocytes, they do not proliferate. With increasing culture time, fewer BrdU-positive cells are detected and are found only in the internodular regions. These cells, as seen by the lack of pericellular Alcian Blue staining, are not chondrocytes. By Day 28, no positive cells are detected.

The process of cellular hypertrophy is clearly seen in the micromass cultures, on the basis of increase in cell size as a function of culture time, the expression of collagen type X (from Day 7 on), and the onset of increased alkaline phosphatase activity. While the cultured cells within the micromass are not spatially organized as chondrocytes within the growth plate, their size at late culture ranges from 20 to 25 μm in diameter, which is comparable to and only somewhat smaller than that observed by Hunziker (19), ca. 30 μm, for early to mid-hypertrophy stage chondrocytes in situ; the slightly smaller size is expected since we did not include the ruthenium reagents of Hunziker et al. (19) in the fixative. The micromass cultures also undergo calcification, as evidenced by concomitant increased levels of ^{45}Ca incorporation and total Ca content, consistent with a requirement of high levels of alkaline phosphatase for calcification.

It is noteworthy that apoptosis is observed here as part of the cartilage maturation process in the in vitro system. The progress of apoptosis in the cultures as seen by TUNEL labeling clearly increases as a function of time. In addition, internucleosomal DNA degradation is detected electrophoretically. The ultrastructural morphology of the cells is also consistent with apoptosis, showing the characteristic presence of crescent-shaped, condensed DNA along the nuclear membrane and the presence of apoptotic bodies. Interestingly, matrix vesicles are associated with the same cell, indicating the initiation of hypertrophy and mineralization (4).

Maturation and hypertrophy of the growth cartilage is an essential part of the process of endochondral ossification, following which the cartilage matrix is resorbed and replaced by vascular and marrow elements (18,29). While the characteristics of the maturation and hypertrophy program, such as distinct morphological changes, expression of alkaline phosphatase and collagen type X, are well established, the fate of the terminal, calcifying chondrocyte remains incompletely understood (13). In vivo observations have provided evidence that terminal chondrocytes of the growth plate undergo apoptosis, (9,14,17,39) whereas others claim that a chondrocyte–osteoblast transition takes place (11,32). Because of the tissue complexity at the growth plate, it is often technically difficult to distinguish the origin of the specific cellular phenotypes, further complicated by the intrinsic anatomical differences between mammalian and avian growth plates, the two commonly studied systems.

In the micromass culture system described here, by using embryonic chondroprogenitor cells, we are able to follow the entire history of the chondrocyte from the precartilage to the calcified cartilage stage. The in vitro developmental program closely mimics that in vivo, in terms of cellular morphology, matrix characteristics, and the apoptotic program. Interestingly, Roach et al. (32–34) observed that in the avian growth plate, hypertrophic chondrocytes undergo asymmetric mitosis within the lacunae, with one cell undergoing apoptosis and the other becoming osteogenic. It is suggested by these authors and others (14) that the fate of the hypertrophic chondrocyte depends on its localization within the growth plate, specifically whether a specific region is targeted for resorption or bone formation. It is noteworthy that in the micromass system, mesenchymal cells chondrify, mature, and undergo hypertrophy as discrete cartilage nodules distributed randomly within the culture, in the absence of vascular resorption. The system should thus be highly applicable for analyzing the regulation of discrete phases in the maturation and hypertrophy program of the chondrocyte. We are currently using the limb mesenchyme micromass system described here to investigate the influence of various growth regulating hormones and factors on chondrocyte maturation.

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