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A Novel Cell Culture Model of Chondrocyte Differentiation During Mammalian Endochondral Ossification

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ABSTRACT

Endochondral ossification (EO) occurs in the growth plate where chondrocytes pass through discrete stages of proliferation, maturation, hypertrophy, and calcification. We have developed and characterized a novel bovine cell culture model of EO that mirrors these events and will facilitate in vitro studies on factors controlling chondrocyte differentiation. Chondrocytes derived from the epiphyses of long bones of fetal calves were treated with 5-azacytidine (aza-C) for 48 h. Cultures were maintained subsequently without aza-C and harvested at selected time points for analyses of growth and differentiation status. A chondrocytic phenotype associated with an extensive extracellular matrix rich in proteoglycans and collagen types II and VI was observed in aza-C–treated and –untreated cultures. aza-C–treated cultures were characterized by studying the expression of several markers of chondrocyte differentiation. Parathyroid hormone–related protein (PTHrP) and its receptor, both markers of maturation, were expressed at days 5–9. Type X collagen, which is restricted to the stage of hypertrophy, was expressed from day 11 onward. Hypertrophy was confirmed by a 14-fold increase in cell size by day 15 and an increased synthesis of alkaline phosphatase during the hypertrophic period (days 14–28). The addition of PTHrP to aza-C–treated cultures at day 14 led to the down-regulation of type X collagen by 6-fold, showing type X collagen expression is under the control of PTHrP as in vivo. These findings show that aza-C can induce fetal bovine epiphyseal chondrocytes to differentiate in culture in a manner consistent with that which occurs during the EO process in vivo. (J Bone Miner Res 2001;16:309–318)

Key words: chondrocyte, endochondral ossification, azacytidine, cell culture, growth plate

INTRODUCTION

DURING THE early stages of embryonic development in higher vertebrates, long bone formation occurs by the process of endochondral ossification (EO). During this process, chondrocytes in the central part of the cartilaginous template of the axial and appendicular skeleton enlarge and hypertrophy, secreting a unique extracellular matrix. As this cartilage matrix is degraded gradually, osteoblasts are deposited on the partially degraded cartilage. Osteoblasts secrete bone matrix and the cartilage eventually is replaced by bone. This process results in the radial growth of bone.

As the center of the cartilaginous model is converted to bone, an ossification front is formed between the newly

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synthesized bone and the remaining cartilage and growth plates are subsequently formed. Longitudinal bone growth takes place in the growth plate cartilage by EO. Within the mammalian growth plate, chondrocytes undergo a series of discrete stages of differentiation, namely, proliferation, maturation, and hypertrophy, which are under strict spatial and temporal control.[1,2] EO normally ceases at puberty but is reinitiated in cases of bone fracture repair and osteoarthritis.[3,4]

Most of the convincing data regarding the role, regulation, and importance of factors required for chondrocyte differentiation within the growth plate have come from the generation and/or examination of the phenotypes of mouse and human forms of osteochondrodysplasia.[5–7] However, targeted experiments to investigate the precise mechanisms of action of these factors have been hampered in part by the lack of a reliable mammalian cell culture model that can reproduce the chondrocyte differentiation pathway as it occurs in vivo.

Chondrocytes can be propagated using a wide variety of cell culture systems. Of the various cell culture models that have been developed to study the differentiation of chondrocytes in vitro, most have used chick chondrocytes.[8,9] Studies of mammalian chondrocyte differentiation have included the fractionation of growth plate chondrocytes by density gradient separation and the culture of each cell fraction separately,[10] three-dimensional (3D) pellet cultures,[11] and cultures of clonal chondrocytic cell lines CFK2[12,13] and the RCJ family of cell lines.[14,15] Transformed chondrocyte cell lines also have been suggested as an alternative to the use of primary chondrocytes because they represent a renewable source of material.[16–18] However, to date no robust model of chondrocyte differentiation using primary mammalian chondrocytes, where all the cells are at the same stage of differentiation, has been developed.

The work presented in this article describes the development and characterization of a mammalian cell culture model of chondrocyte differentiation that mimics events occurring during EO. This model involves the treatment of fetal bovine epiphyseal chondrocytes with 5-azacytidine (aza-C), which induces their differentiation. aza-C is a potent DNA demethylating agent and it has been reported to induce the differentiation of mesenchymal C3H10T1/2 cells to myoblasts, adipocytes, and chondrocytes.[19] Preliminary studies in our laboratory indicated that aza-C was able to induce epiphyseal chondrocytes to express type X collagen, which under normal conditions is expressed only by hypertrophic chondrocytes in the growth plate. These studies led us to examine in more detail the consequences of the treatment of fetal bovine epiphyseal chondrocytes with aza-C and we found that phenotypic changes associated with chondrocyte differentiation are mirrored in this culture system. The cells in this culture model of EO are synchronized, allowing the study of individual stages of the differentiation pathway. The potential of this model for the study of regulatory factors involved in EO was confirmed by investigating the effect of parathyroid hormone–related protein (PTHrP) on type X collagen expression.

MATERIALS AND METHODS

Chondrocyte isolation and culture

Epiphyseal cartilage was dissected from the long bones of bovine fetuses (170–235 days) well away from the growth plate and from any secondary centers of ossification. The cartilage was cleaned of adhering tissue, chopped finely, and digested for 3 h at 37°C with bacterial collagenase type IA (10 mg/ml; Sigma Chemical Co., Dorset, UK) and trypsin (0.4 mg/ml; Gibco BRL, Paisley, Scotland) in minimal essential medium (MEM; Gibco BRL) containing 10% fetal calf serum (FCS; Gibco BRL). The released chondrocytes were plated on 9-cm^2 culture dishes at a density of 0.5×10^6 cells/cm^2. The culture medium used throughout was MEM-buffered to pH 7.2 with 10 mM HEPES and supplemented with penicillin (100 IU/ml; Gibco BRL), streptomycin (100 μg/ml; Gibco BRL), gentamycin (0.01 mg/ml; Gibco BRL), L-glutamine (2 mM), L-ascorbate (25 μg/ml), 0.34% (wt/vol) sodium bicarbonate, and 10% (vol/vol) FCS. All cultures were incubated at 37°C in 5% CO_2/95% O_2 atmosphere.

aza-C treatment of chondrocyte cultures

One hour after plating, cultures were treated with aza-C (15 μg/ml in standard MEM containing 10% FCS) for 48 h. The medium was then removed and the cultures were maintained in standard MEM containing 10% FCS, which was replaced every other day throughout the culture period. Control cultures were set up without the addition of aza-C.

Determination of cell number

Each well was washed twice with phosphate-buffered saline (PBS). The chondrocytes were released by treatment with trypsin (0.4 mg/ml) and bacterial collagenase type IA (10 mg/ml) at 37°C for 1 h. After centrifugation at 250*g* for 3 minutes, the cells were resuspended in 5 ml of MEM and counted using a Coulter counter. This procedure was repeated five times per plate.

Transmission electron microscopy

Cultures were washed three times with PBS and fixed in 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3), which had been prewarmed to 37°C before use. After 2 hours, cultures were washed several times in the sodium cacodylate buffer with added 0.3 mM $CaCl_3$ and then carefully peeled from the culture dishes, cut into small rectangles (approximately 4 mm $\times$ 5 mm), and transferred to glass vials for processing. The tissue was postfixed in 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.3) for 1 hour at 4°C, rinsed in buffer, and dehydrated in an ascending alcohol series. After two 15-minute incubations in propylene oxide, the tissue was infiltrated with a 1:1 mixture of propylene oxide and Taab epoxy resin (Taab Laboratories Equipment Ltd., Aldermaston, UK) for 1 hour, and then left overnight in a 1:3 mixture of propylene oxide/resin at 4°C on a rotator. The next day, tissues were given three

1-h changes of neat resin at 48°C, placed in flat embedding molds with three or four rectangles overlying each other, and polymerized at 60°C for 72 h. Ultrathin pale gold sections were cut and mounted on copper grids and contrasted with uranyl acetate and lead citrate. Sections were examined in a EM 301 electron microscope (Philips, Hillsboro, OR, USA) at an accelerating voltage of 60 kV.

Immunohistochemical analyses

Collagen type I: Cultures were washed three times with PBS and the cells were fixed in ice-cold methanol for 10 minutes. After rinsing in 0.05 M Tris-buffered saline (TBS) (Tris, 121.1 g; NaCl, 170.0 g; 2 liters distilled water; pH 7.4–7.6), the cells were incubated with 3% H_2O_2 solution for 5 minutes to block any endogenous peroxidase activity. The cells were rinsed in distilled water and then in 0.05 M TBS before incubation with normal rabbit serum (1:5 dilution in 0.05 M TBS; DAKO, Buckinghamshire, UK) for 20 minutes. The cells were incubated with a 1:100 dilution of the primary antibody against bovine type I collagen (Biogenesis Ltd, Dorset, UK) for 30 minutes at room temperature. TBS was used instead of the antibody as a negative control. After rinsing with 0.05 M TBS, the cells were incubated with a 1:100 dilution of peroxidase-conjugated rabbit anti-guinea pig secondary antibody for 1 h at room temperature. The cells were then washed in 0.05 M TBS and incubated with 3,3′-diaminobenzidine (DAB)/H_2O_2 solution (SIGMA *FAST;* Sigma Chemicals Co.) for 10 minutes. The cells were counterstained in Mayer's hematoxylin and mounted with Loctite adhesive. Skin fibroblasts were used as a positive control for the type I collagen antibody.

Collagen types II and VI: Cut paraffin wax sections of cell layers were dewaxed and processed as described previously. Some sections were incubated with 1.5 mg/ml hyaluronidase type I-S at 37°C for 1 h. They were then washed three times in 0.05 M TBS. All sections were incubated with normal swine serum (1:5 dilution in 0.05 M TBS; DAKO) for 15 minutes at room temperature before the addition of the primary antibody for 1 h. For the detection of collagen types II and VI, the sections were incubated with a 1:200 dilution of a polyclonal antibody against bovine type II collagen (Biogenesis Ltd) and a 1:500 dilution of a primary antibody against bovine type VI collagen,[20] respectively. The sections were rinsed three times in 0.05 M TBS and then incubated with a 1:300 dilution of biotinylated swine anti-rabbit secondary antibody for 45 minutes at room temperature. After rinsing three times in 0.05 M TBS, the sections were incubated with streptavidin-peroxidase (1:500 dilution with 0.05 M TBS) for 30 minutes at room temperature. They were then washed in 0.05 M TBS before incubating with DAB/H_2O_2 solution. Cells were counterstained in Mayer's hematoxylin and mounted as described previously.

Histological studies

Hematoxylin and eosin staining: Paraffin wax sections were dewaxed and rehydrated. Sections were stained in Mayer's hematoxylin for 5 minutes, rinsed in water, and then stained in slightly acidified eosin for 30 s. Sections were rinsed and left to air dry before being mounted with Loctite adhesive.

Alcian blue staining: Cultures were washed three times with PBS. The cells were then fixed in ice-cold methanol for 10 minutes, rinsed in 0.05 M TBS, and then stained with alcian blue (pH 2.5) for 15 minutes. The cells were counterstained with 1% aqueous neutral red and mounted with Loctite adhesive.

RNA extraction: Chondrocytes released by trypsinization were pelleted by centrifugation and resuspended in RNAzol B (Biogenesis) and 1/10 volume of chloroform. Total RNA was extracted according to the manufacturer's instructions. The resulting RNA pellet was washed with ice-cold 70% ethanol, dried, and resuspended in 0.5% NP-40 containing 1:100 dilution of RNAse inhibitor (Boehringer Mannheim, Lewes, UK).

Reverse-transcription polymerase chain reaction: Total RNA (1.0 μg) was denatured by heating at 90°C for 5 minutes and then cooled on ice for 5 minutes. RNA was incubated with freshly prepared complementary DNA (cDNA) first-strand buffer (100 mM Tris, pH 8.3, 100 mM KCl, 100 mM $MgCl_2$, 1 mg/ml bovine serum albumin [BSA], 1 mM deoxynucleoside triphosphate [dNTP] 5 μM Random Hexamers, and 1 μl AMV reverse transcriptase [Boehringer Mannheim, Mannheim, Germany]) in a total volume of 20 μl at room temperature for 10 minutes. The mixture was transferred to 42°C for 30–60 minutes followed by 5 minutes at 95°C and then cooled on ice. Type X collagen-polymerase chain reactions (PCRs) were carried out in a total volume of 100 μl containing 1 μl of cDNA, 10 μl 10× buffer (15 mM $MgCl_2$, 0.5 M KCl, 0.1 M Tris, pH 8.3), 100 pmol of forward primer (5′GAA ACA TTC GGG AGA TGT CAT3′), 100 pmol of reverse primer (5[prime]TTT CAT GAG GCA CAG CTT AAG3′), 10 μl of dNTP mix (20 mM each), and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim). All PCRs were performed for 35 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C, in a Omnigene PCR machine (Hubaid, Ashford, Middlesex, UK).

In situ hybridization: Suspensions of chondrocytes obtained by trypsinization and collagenase digestion were centrifuged in a cytocentrifuge onto glass slides and fixed with paraformaldehyde. cDNA probes were used to identify the messenger RNA (mRNA) expression of type X collagen, PTHrP, PTHrP receptor, alkaline phosphatase, and α-enolase on cytospins from aza-C–treated and –untreated chondrocytes by in situ hybridization as previously described.[3]

Image analysis: In situ hybridization slides were viewed using a Leica DB research microscope (Leica Ltd., Milton Keynes, UK) to which was attached a CF8/1 Kappa monochrome camera for image capture. All images were downloaded in to a Quantimet 600S image analysis package (Leica Ltd.). Hybridization signals were seen as black grains and the level of mRNA expression was represented by the concentration of black grains obtained. Results were quantified as described previously.[21–23] Data from 10 ex-

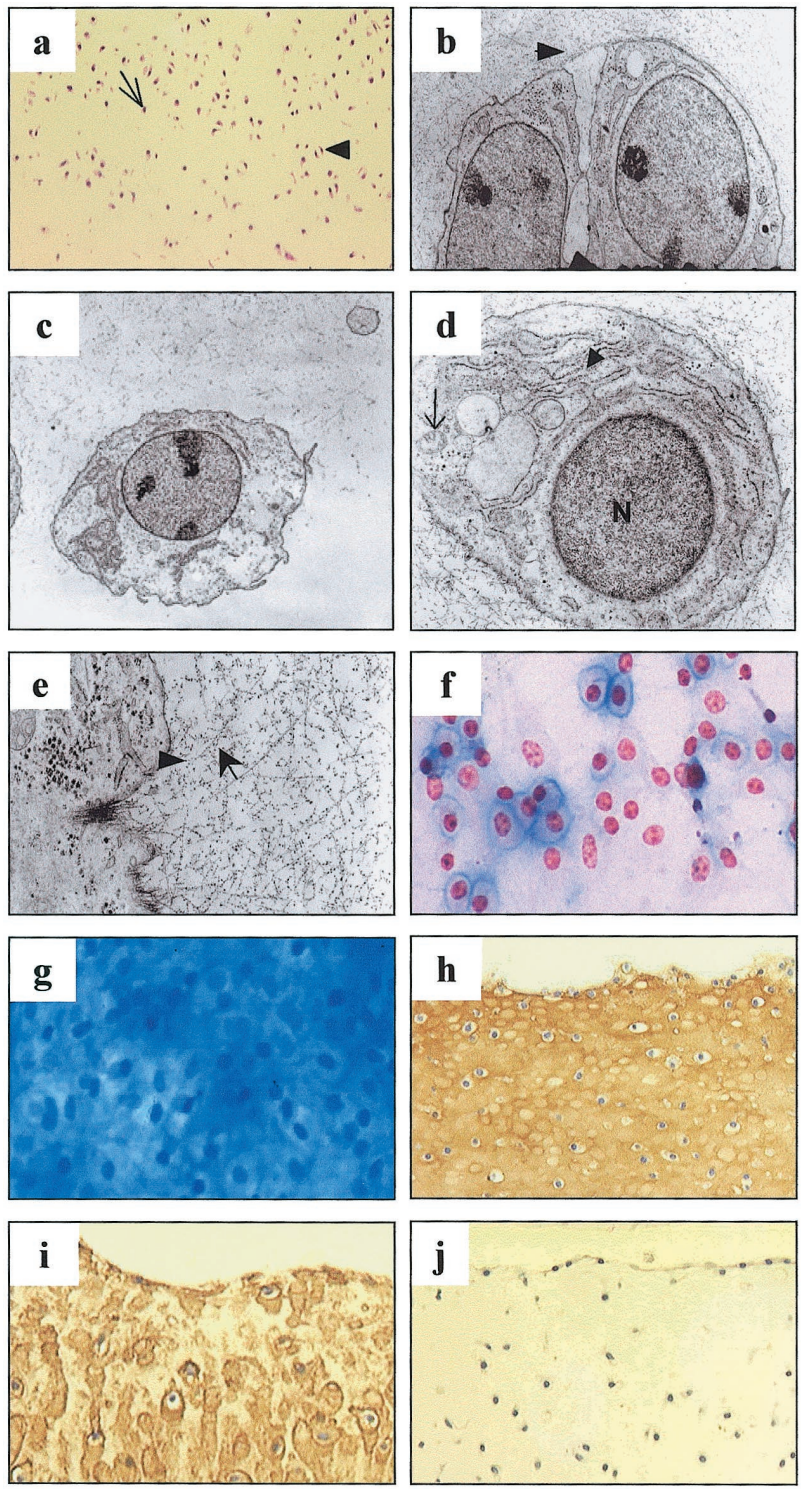

FIG. 1. Cell morphology and extracellular matrix expression. (a) Hematoxylin and eosin staining of an aza-C–treated culture. Both aza-C–treated and –untreated cultures showed a structure similar to cartilage in vivo. Note the presence of round chondrocytes either singly (open arrow) or in pairs (arrowhead; magnification ×242). (b) Electron micrograph of a pair of chondrocytes located in lacunae. Chondrocytes found in the deeper areas of the cell layer are round in shape. Note the separation of the territorial extracellular matrix from the interterritorial matrix by a capsular structure consisting of fine fibrils (arrow; magnification ×7915). (c) Electron micrograph of a fetal bovine epiphyseal chondrocyte grown for 20 days in the absence of aza-C treatment. The chondrocyte has relatively few cytoplasmic organelles and its nucleus contains condensed chromatin indicating that the cell is in a "resting" stage (magnification ×18,750). (d) Electron micrograph showing the ultrastructural characteristics of a hypertrophic chondrocyte 20 days after aza-C treatment. Note the presence of abundant rough endoplasmic reticulum (arrowhead), mitochondria (arrow), and a euchromatic nucleus with dispersed chromatin (N; magnification ×18,750). (e) Electron micrograph

periments were pooled. For each time point studied, 10 fields were chosen at random and the number of grains and cells were counted in the areas selected. The analysis program counted the grains automatically, but a person counted the cells in each field in a blinded manner. The level of mRNA expression was expressed as the mean grain density per cell.

RESULTS

Effects of aza-C treatment on chondrocyte phenotype in culture

When fetal bovine epiphyseal chondrocytes were isolated and plated out, they appeared spherical and were dispersed uniformly on culture dishes. After approximately 4–5 days, cells were surrounded by an extracellular matrix, leading to the formation of a 3D structure several cell layers thick. Chondrocytes were released from the cell layers by trypsinization and collagenase digestion and cell counts were carried out using a Coulter counter. Both treated and untreated cultures had similar cell counts. There was an initial decrease in the cell number immediately after plating out followed by an increase in cell number by day 3. By day 11 in culture, the cell number had returned to the original plating density of 0.5×10^6 cells/cm^2 and remained unchanged at subsequent time points.

Paraffin wax sections of chondrocyte cell layers were cut and used for histological examination under the light microscope. Hematoxylin and eosin staining of the cut sections revealed a cartilage-like structure in which chondrocytes were surrounded by an extracellular matrix (Fig. 1a). Transmission electron microscopy showed the chondrocytes had a rounded morphology and were present either singly or in pairs in separate "lacunae" surrounded by a distinct pericellular and fine fibrillar territorial matrix (Figs. 1b and 1e). The interterritorial matrix was dense and consisted of randomly arranged collagen fibrils. Proteoglycan granules were attached to the collagen fibrils at regular intervals (Fig. 1e). In all cases, cells were healthy and viable as shown by their maintenance of an intact plasma membrane and cytoplasmic organelles and a prominent nucleus and nucleoli.

When epiphyseal chondrocytes were treated with aza-C, they exhibited changes in morphology after a few days in culture. From day 6 onward, treated cells were larger in size than their untreated counterparts (Figs. 1c and 1d). To quantify the relative increase in cell volume during the culture period, cells were released from the extracellular matrix by treatment with trypsin and collagenase, as for cell counts, resuspended in culture media and viewed under phase contrast on a hemocytometer. The diameters of both untreated and treated cells were measured directly from the photographs taken at regular time points. Assuming the released chondrocytes have a spherical shape,[23,24] cell volumes were calculated from cell diameters. A graph of cell volume (μm^3) against days in culture is presented in Fig. 2. The small SEM shows that the treated cells were all at the same stage of differentiation. A steady increase in the cell volume of treated chondrocytes was observed compared with untreated cultures, reaching a 14-fold increase by day 15 in culture. This degree of increase in cell size is consistent with the size increase from resting to hypertrophic chondrocytes seen in vivo.[23,24]

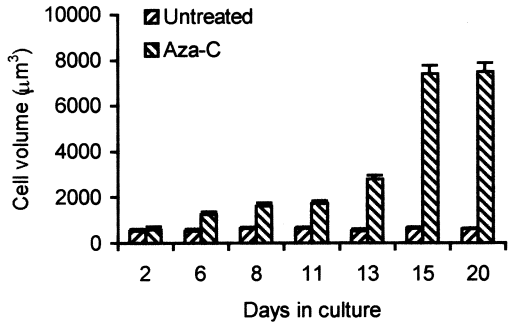

FIG. 2. Effect of aza-C on cell size with time in culture. The diameters of aza-C–treated and −untreated chondrocytes were measured at each indicated time point. Their cell volumes were calculated on the assumption that chondrocytes were spherical in shape. One hundred cells were measured at each time point in each culture. This was repeated in 10 cultures. Values represent mean ± SEM (100 cells were measured for each time point in 10 experiments).

The effects of aza-C on extracellular matrix composition

The expression of type I collagen, a marker of a fibroblastic phenotype, was investigated by immunohistochemistry. Type I collagen was not detected in any of the treated or untreated cultures at any time point (results not shown),

(Fig. 1 legend cont.) showing the extracellular matrix produced by chondrocytes with time in culture. The extracellular matrix is dense and consists of randomly organized collagen fibrils (arrow) in close association with smaller structures resembling proteoglycans (arrowhead; magnification ×11,875). All electron micrographs were taken after 5 days in culture and are typical representations of both aza-C–treated and control chondrocytes throughout the culture period. Proteoglycans were detected by alcian blue staining. Cell nuclei were stained red with aqueous neutral red. The increase in staining intensity corresponding to an increase in matrix synthesis as the cells proliferated with time in culture at (f) 2 days and (g) 8 days. All results here are typical representations of the presence of proteoglycans in the extracellular matrix in aza-C–treated and −untreated chondrocyte cultures throughout the culture period (magnification ×90). Collagen expression was detected on wax sections using rabbit antibovine (h) type II and (i) type VI collagen antibody with hyaluronidase treatment. (j) TBS buffer was used instead of the antibody in the negative control. Results shown are typical representations of type II and VI collagen expression in aza-C–treated (h and i) cultures at all time points throughout the 3- to 4-week culture period. Both collagens were expressed at all time points and type VI collagen's pericellular localization in the matrix was clearly apparent. (h) Magnification ×80. (i) and (j) magnification ×103.

showing that the cells had not dedifferentiated. To confirm that the extracellular matrix produced by the cultures was consistent with a chondrocytic phenotype, the presence of proteoglycan, type II collagen and type VI collagen was confirmed by alcian blue staining (Figs. 1f and 1g) and immunolocalization (Figs. 1h–1j), respectively. Alcian blue staining initially was restricted to lacunae, but with time the chondrocytes surrounded themselves with a proteoglycan-rich matrix (Fig. 1g). Immunolocalization showed type II collagen to be present at high levels throughout the interterritorial matrix surrounding the chondrocytes (Fig. 1h) whereas type VI collagen revealed high expression in the pericellular environment of chondrocytes and was only faintly present in the interterritorial matrix (Fig. 1i). Both aza-C–treated and −untreated cultures showed similar expression of these three matrix constituents throughout the culture period showing the chondrocytic phenotype of the cultured cells.

The expression of type X collagen, a marker of chondrocyte hypertrophy in vivo, was investigated by RT-PCR. In the aza-C–treated cultures, type X collagen expression was detectable on day 11 and at all subsequent time points (Fig. 3a), which was confirmed by SDS-PAGE (results not shown). In situ hybridization studies confirmed these findings (Figs. 3b and 3c) and showed that from day 11 onward all cells expressed type X collagen. However, no expression of type X collagen mRNA was detected in the untreated cultures throughout the culture period (Fig. 3c).

Effects of aza-C on nonextracellular matrix gene expression

The pattern of expression of three genes known to be expressed at discrete stages of the chondrocyte differentiation pathway in the growth plate in vivo was determined in the aza-C–treated and −untreated cultures by in situ hybridization. The three genes chosen for this part of the study were PTHrP and PTH/PTHrP receptor, two genes expressed during chondrocyte proliferation,[25–27] and alkaline phosphatase, which is expressed during hypertrophy.[28,29] In addition, a gene recently identified as being up-regulated in the mammalian growth plate, α-enolase, also was studied.[30]

In aza-C–treated cultures, PTHrP mRNA expression was detected on day 5, peaked at day 7, and decreased at subsequent time points (Fig. 4a). PTH/PTHrP receptor mRNA expression was up-regulated on day 9 when cell proliferation was maximal and then decreased at subsequent time points (Fig. 4b). Spectrophotometric assay showed alkaline phosphatase was not produced until day 14 (results not shown); therefore, alkaline phosphatase mRNA expression was only measured from day 14 onward, showing levels increased during the hypertrophic period of culture when type X collagen was expressed (Fig. 4c). α-Enolase mRNA expression increased markedly on day 9 and decreased by day 12 (Fig. 4 days). In untreated cultures, low levels of each gene were present throughout the culture period.

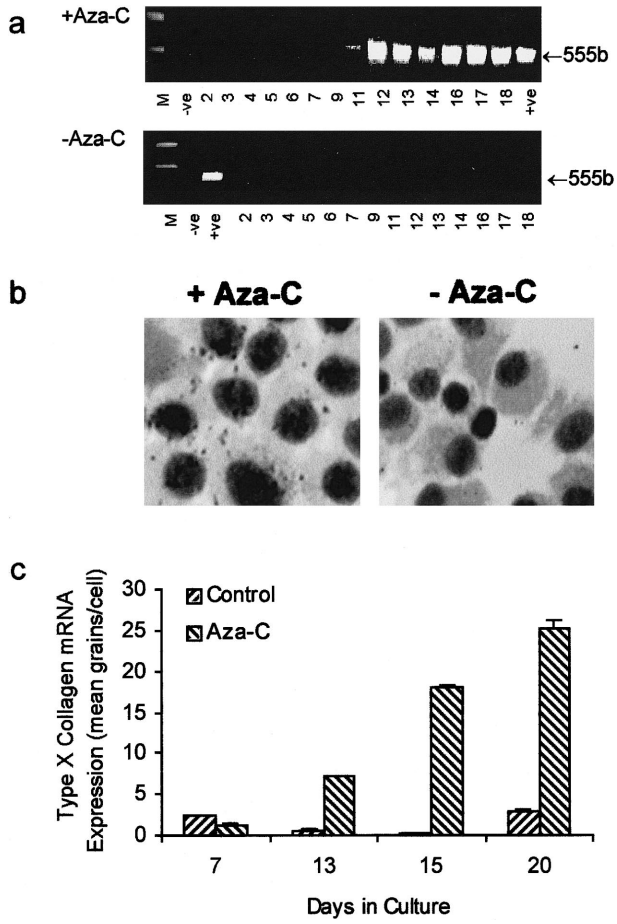

FIG. 3. Expression of type X collagen in aza-C–treated and −untreated cultures. (a) RT-PCR showed the expression of type X collagen was evident from day 11. No expression of type X collagen was observed when RT-PCR was performed under the same experimental conditions using total RNA extracted from untreated chondrocyte cultures. (M, 1 kb ladder; −ve, negative control; +ve, positive control [mRNA extracted from the hypertrophic region of a bovine growth plate]; 1–18, days in culture). (b) In situ hybridization using a human cDNA probe specific for type X collagen against aza-C–treated and −untreated chondrocytes. An RNAse control was included where the chondrocytes were treated with RNAse to eliminate any hybridization signal (magnification ×582). (c) All ISH results were quantified using a Quantimet 600S image analysis system. Values represent mean ± SEM (10 counts were made for each of 10 experiments).

Effects of PTHrP on aza-C–induced chondrocyte differentiation

In vivo, PTHrP expression must be down-regulated in order for type X collagen to be expressed.[31] In the aza-C–treated cultures we observed that type X collagen is only expressed once PTHrP is down-regulated. To determine whether the expression of type X collagen is regulated negatively by the expression of PTHrP in the aza-C cultures, as has been shown in vivo, aza-C–treated cultures were supplemented with PTHrP (10^{-7} M) for various lengths of time (0, 6, 24, and 48 h) on day 14 (the time point where a decrease in PTHrP was first observed). We found that

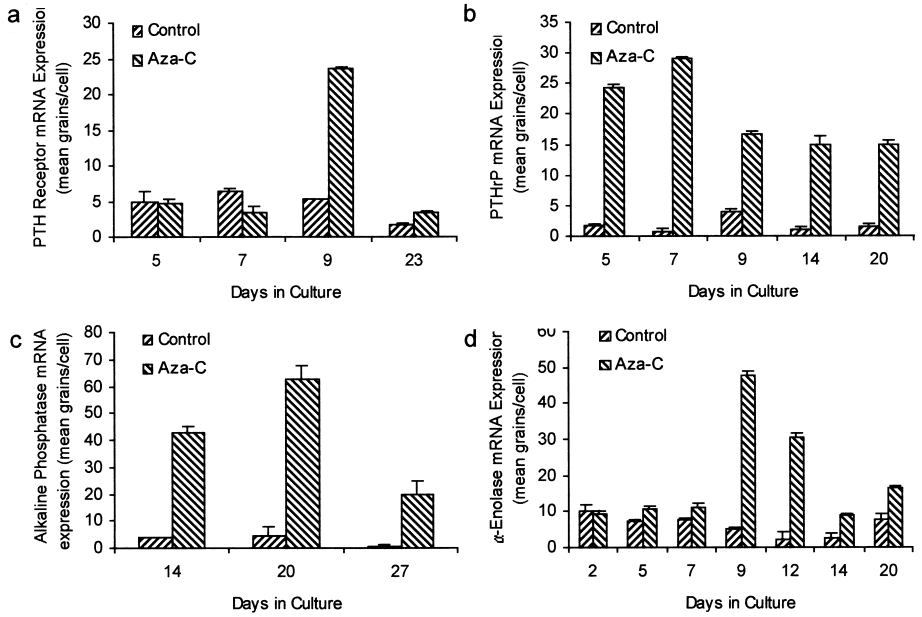

FIG. 4. Expression of markers of chondrocyte differentiation in aza-C–treated and –untreated cultures. In situ hybridization analyses of chondrocyte cultures at selected time points, using human cDNA probes for (a) PTHrP, (b) PTHrP receptor, (c) alkaline phosphatase, and (d) α-enolase. RNAse control represents chondrocytes treated with RNAse to eliminate any hybridization signal. All results were quantified using a Quantimet 600S image analysis system. Values represent mean ± SEM (10 counts were made for each of 10 experiments).

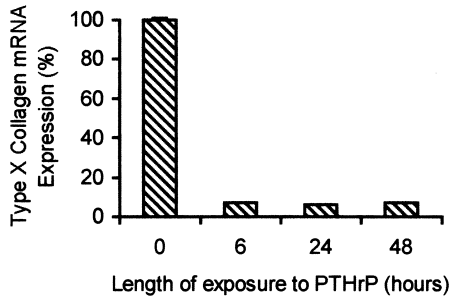

FIG. 5. Effect of PTHrP on type X collagen expression in aza-C–treated cultures. Aza-C–treated chondrocyte cultures were exposed to PTHrP (10^{-7} M) for 6, 24, and 48 h on day 14 and processed for in situ hybridization analyses using a human cDNA probe specific for type X collagen. RNAse control represents chondrocytes treated with RNAse to eliminate any hybridization signal. A positive control also was included where no PTHrP was added to the aza-C–treated cultures. All results were quantified using a Quantimet 600S image analysis system. Values represent mean ± SEM (10 counts were made for each of 10 experiments).

treatment of aza-C cultures for 6, 24, and 48 h resulted in an at least a 90% decrease in the level of expression of type X collagen, as determined by in situ hybridization (Fig. 5).

DISCUSSION

We have investigated the effects of the treatment of fetal bovine epiphyseal chondrocytes with aza-C in terms of their morphology, extracellular matrix formation, and gene expression. We have found that aza-C–treated cultures differentiate and become hypertrophic in vitro in a manner similar to that which occurs in the mammalian growth plate in vivo. In contrast, untreated chondrocyte cultures maintained a resting phenotype. This mechanism of inducing chondrocyte differentiation therefore provides a potential in vitro model for the study of chondrocyte differentiation during EO.

Because DNA methylation is known to play an important role in the control of eukaryotic gene expression, there has been considerable interest in cytosine analogues such as aza-C and their effects on the methylation state of genes and their expression. Aza-C is an antileukemia drug, known to be a powerful inhibitor of DNA methylation.[32] It is still obscure how aza-C exerts its demethylating effect.[33] Aza-C has been shown to induce novel gene expression both in vitro and in vivo.[19] aza-C converts cultures of the mouse embryo cell line C3H10T1/2 into three functionally and biochemically differentiated cell types: myoblasts, chondrocytes, and adipocytes. aza-C also converts teratocarcinoma-derived mesenchymal cells into epithelial cells[34] and induces erythroid differentiation in treated Friend erythroleukemia cells.[33]

In the characterization of the effects of aza-C on chondrocytes in culture it did not appear that aza-C had a generalized toxic effect on the chondrocytes because both the aza-C–treated and the control cultures had similar growth profiles. Histological staining and electron microscopical studies of both treated and untreated cultures revealed the formation of a cartilage-like structure in which chondrocytes were present in lacunae and surrounded by an extracellular matrix.[35] Therefore, it appears that culturing fetal bovine epiphyseal chondrocytes under conditions described in this study allows the chondrocytes to grow and establish their own environment by elaborating an extracellular matrix similar to that found in cartilage in vivo. The intensity of the alcian blue reaction increased gradually with time, indicating an increase in the amount of matrix produced by the chondrocytes with time in culture. Morphologically, in the treated and the untreated cultures the chondrocytes appeared similar up to day 11 when the treated

cells increased in size. Estimates of cell volume of the aza-C–treated chondrocytes showed that from day 1 to day 20 there was a 14-fold increase in cell volume. Such an increase is characteristic of the hypertrophic stage of chondrocyte differentiation and is consistent with previous studies that have indicated a steady increase in cellular volume as chondrocytes progressed down the maturation pathway and became hypertrophic.[1,23,36]

During EO, the differentiation of growth plate chondrocytes is accompanied by the synthesis of several collagen types, which are incorporated into the extracellular matrix of growth plate cartilage. The composition of the extracellular matrix produced by the cells in culture was studied for two reasons. First, to confirm that the cells had maintained a chondrocytic phenotype and not dedifferentiated to fibroblasts as is often seen in chondrocyte cell culture.[37] Second, to confirm that the large cells seen in the treated cultures expressed type X collagen, a marker of hypertrophy in EO. No type I collagen was detected, confirming cells had not dedifferentiated to fibroblasts. Immunohistochemical analyses revealed the presence of type II and VI collagens throughout the extracellular matrix in all cultures, confirming the chondrocytic nature of the cell cultures. RT-PCR and in situ hybridization studies showed that type X collagen was expressed from day 11 onward in aza-C–treated cultures but not in the untreated cultures. These findings confirm that the aza-C–treated chondrocytes had attained a hypertrophic phenotype, as had been indicated by their morphological status. The hypertrophic phenotype was further confirmed by the expression of alkaline phosphatase in the later stages of the aza-C–treated cultures, which is consistent with hypertrophy in vivo.[29]

EO occurs under strict spatial and temporal control during the embryonic development of the axial and appendicular skeletal system. The control of EO is achieved by a variety of regulatory molecules, which are expressed by chondrocytes at specific stages of cell differentiation in the growth plate. PTHrP and PTH receptor are key regulatory factors known to play a crucial role in the chondrocyte differentiation pathway.[31,38] They are expressed specifically in the proliferative and maturation zones of the growth plate before hypertrophy.[25,39] In the present study, results obtained by in situ hybridization revealed a high expression of PTHrP and PTH receptor in aza-C–treated cultures at early time points in culture with a maximum level of expression reached on day 7 and day 9, respectively. It is interesting to note that the high expression of PTHrP and PTH receptor was obtained before the expression of type X collagen. Consequently, it is possible to define the culture period "days 5–9" as the maturation stage of chondrocyte differentiation in vitro.

In vivo PTHrP blocks the expression of type X collagen.[25] To verify that type X collagen expression is controlled directly by the down-regulation of PTHrP in the aza-C–treated cultures, aza-C–treated chondrocytes were exposed to PTHrP on day 14, which was soon after they had entered hypertrophy and synthesized type X collagen in culture. Results showed that exogenous PTHrP at a concentration of 10^{-7} M suppressed type X collagen expression by over 90% in hypertrophic chondrocytes that had been treated previously with aza-C. Other studies also have obtained a similar level of suppression of type X collagen in short-term cultures of chick growth plate chondrocytes at the same concentration and for the same length of exposure to PTHrP.[40] The present findings show that the hypertrophic stage of differentiation induced by aza-C treatment is regulated, at least in part, by PTHrP, as is the case in the growth plate in vivo.

The mechanism by which aza-C induces the chondrocytes to differentiate in culture is not known. It is possible that aza-C switches on the expression of EO genes individually by its demethylating action, as shown in previous studies.[41,42] It is also possible that aza-C acts on a single gene involved in the early stages of the differentiation pathway, which in turn triggers the whole differentiation process. Despite the mechanism of action of aza-C being uncertain, the fact that the hypertrophic stage of differentiation in this cell culture model can be influenced by the presence of exogenous PTHrP in a fashion similar to that in the growth plate in vivo suggests that the stage of hypertrophy has not been induced directly by aza-C. Rather, the hypertrophic stage is part of the differentiation pathway that has been induced by aza-C and is influenced by stage-specific regulatory molecules involved in the EO pathway.

In addition to the many well-characterized differentiation-associated molecules involved in the chondrocyte differentiation pathway in the growth plate, a number of novel genes expressed in specific regions of the bovine growth plate during EO recently have been identified by an RT-PCR–based subtractive hybridization technique.[43,44] One of the cDNAs identified as being significantly up-regulated in the growth plate represents the gene for α-enolase.[30] In situ hybridization studies revealed high expression of α-enolase on day 9 and day 12 in aza-C–treated cultures, showing that α-enolase is expressed in both maturing and hypertrophic chondrocytes. The significance of the abundant presence of α-enolase in growth plate chondrocytes is yet to be determined. The aza-C–based system provides an opportunity for further characterization of the role of α-enolase and other newly identified genes in specific zones of the growth plate.

The molecular mechanisms that initiate the entry of epiphyseal chondrocytes into the differentiation pathway still remain obscure. In addition, the role(s) of regulatory factors involved in the differentiation pathway and their intricate interplay in the control of the developmental program of growth plate chondrocytes is not fully understood. Understanding the precise mechanisms regulating EO is crucial because abnormalities in this process lead to severe, and often lethal, skeletal disorders. The cell culture system described here provides an excellent tool for determining the factors controlling mammalian chondrocyte differentiation.

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