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Gene and Protein Expression During Differentiation and Matrix Mineralization in a Chondrocyte Cell Culture System

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Abstract. Endochondral bone formation occurs through a series of developmentally regulated cellular stages, from initial formation of cartilage tissue to calcified cartilage, resorption, and replacement by bone tissue. Nasal cartilage cells isolated by enzymatic digestion from rat fetuses were seeded at a final density of 10^5 cell/cm^2 and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum in the presence of ascorbic acid and β-glycerophosphate. First, cells lost their phenotype but in this condition they rapidly reexpressed the chondrocyte phenotype and were able to form calcified cartilaginous nodules with the morphological appearance of cartilage mineralization that occurs *in vivo* during endochondral ossification. In this mineralizing chondrocyte culture system, we investigated, between day 3 and day 15, the pattern expression of types II and X collagen, proteoglycan core protein, characteristic markers of chondrocyte differentiation, as well as alkaline phosphatase and osteocalcin associated with the mineralization process. Analysis of labeled collagen and immunoblotting revealed type I collagen synthesis associated with the loss of chondrocyte phenotype at the beginning of the culture. However, our culture conditions promoted extracellular matrix mineralization and cell differentiation towards the hypertrophic phenotype. This differentiation process was characterized by the induction of type X collagen mRNA, alkaline phosphatase, and diminished expression of type II collagen and core protein of large proteoglycan after an increase in their mRNA levels before the mineralizing process. These results revealed distinct switches of the specific molecular markers and indicated a similar temporal expression to that observed *in vivo* recapitulating all stages of the differentiation program *in vitro*.

Key words: Chondrocytes — Endochondral bone formation — *In vitro* mineralization — Collagens — Cartilage.

The development and growth of long bones mainly occur by endochondral bone formation. During this process within the fetal growth plate, several studies *in vivo* have documented that cells and matrix of cartilage undergo a series of significant morphological and biochemical changes [1]. In the proliferating zone, bordering the reserve zone, chondrocytes multiply and then produce a hyaline extracellular matrix containing types II, IX, and XI collagen. In the maturation zone, chondrocytes start to enlarge, and the size of the cells is dramatically increased in the hypertrophic zone. Cell enlargement is accompanied by a reduction of matrix volume [2, 3], type X collagen expression, and an increase in alkaline phosphatase (ALP) activity [4]. Within the lower hypertrophic zone, the cartilage matrix calcifies, then partially resorbs and is replaced by bone.

Chondrogenesis, the first step in the endochondral ossification process, has been well described *in vitro*. Cultures of cartilage organ which maintained chondrocytes in their own environment, and chondrocytes from the proliferative zone of epiphyseal growth plate cartilage have been used as experimental models for studies related to cartilage growth [5, 6]. To avoid heterogeneity of the primary cell culture, cell lines isolated from fetal rat calvariae have been used for the study of skeletal development [7, 8]. However, calvariae do not develop via the endochondral pathway but via the intramembranous pathway of bone formation. In addition, clonal cell line, derived from mouse embryonal carcinoma, is used as a model of chondrogenesis [9], but the cartilage nodules formed do not mineralize and cells need dexamethasone in order to express chondrocyte phenotype.

Furthermore, several chondrocyte culture systems have been described recently that support mineral formation *in vitro*. These include chick embryo sternal suspension cultures in agarose [10], micromass cultures of embryonic chick limb buds [11], secondary cultures of either *nonadherent* chick embryo vertebrae [12] or *adherent* chick embryo tibiae, followed by suspension culture [13]. In addition, primary cultures of chick embryo cephalic, caudal sternal chondrocytes [14], cultures of chick embryo mesenchymal limb buds [15], and primary cultures of growth plate chondrocytes from 6 to 8-week-old broiler-strain chicken [16] have been reported. In some mineralizing culture systems, the mineral formed by addition of β-glycerophosphate or increased Pi levels appeared different from the *in vivo* situation [11, 17], resulting in the development of models that formed mineral without these addi-

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tives [16]. Nevertheless, most studies point to the importance of phosphate supplementation, by addition of either β-glycerophosphate or Pi to the culture medium, for proper matrix calcification although the regulation of this process is not well understood.

Moreover, much of our information is derived from the *de novo* differentiation of cartilage cells from culture mesenchyme dissociated from embryonic limb buds. However, *in vitro* systems established from the mandibular condyle, a secondary type of cartilage, relate to mechanisms involved in endochondral ossification. In this cartilage, progenitor cells are biopotential with an ability to differentiate into either cartilage or bone cells, depending upon local environment [18–20].

Nasal septal cartilage is a primary cartilage derived from a primordial cartilaginous mass called the chondrocranium, which is derived from the neural crest. Nasal septum acts as a pacemaker for the growth of the skull and the face. Consequently, nasal cartilage-derived cells provide a model system useful for the study of cranofacial development. However, few studies reported the reappearance of specific functions among chondrocytes cultured from cartilage of mammal tissue and particularly their ability to synthesize a cartilaginous matrix that mineralizes in a similar sequence as *in vivo.*

Chondrocytes isolated from fetal rat nasal septa by enzymatic digestion in the presence of ascorbic acid and β-glycerophosphate formed a matrix which mineralized in less than 2 weeks [21]. The chief prerequisite for an *in vitro* system is that the cultured cells must undergo progressive development and differentiation similar to events known to occur *in vivo.* Furthermore, the deposition of mineral in the extracellular matrix should closely match the normal pattern seen *in vivo.*

In the present study, we explored the expression of types II and X collagen, cartilage proteoglycan core protein characteristic markers of chondrocyte differentiation, as well as ALP, osteocalcin associated with the mineralization process, and type I collagen during the culture period. Our results reveal distinct switches of the specific molecular markers and indicate a temporal expression similar to that observed *in vivo,* recapitulating all stages of the differentiation program *in vitro.*

Material and Methods

Cartilage Cell Cultures

Chondrocytes were isolated from fetal rat nasal septa as previously described [21]. The studies were performed under institutionally approved protocols for the use of animals. Briefly, tissues from 21-day-old fetal Sprague-Dawley rats were used. The fetuses were removed *in utero* and immediately placed in cold phosphate-buffered solution (PBS). Individual fetuses were decapitated and skinned up to the anterior margin of the nasal bone. The frontonasal sutures, central part of internasal sutures, and nasal septum cartilage were cut out under a dissecting microscope, then cleaned of periosteum and connective tissue. Tissue fragments were incubated over a magnetic stirrer for 2 hours at 37°C in an enzymatic mixture of collagenase (0.25%) and hyaluronidase (0.1%). After the incubation period, the cells were then mechanically dissociated by pipetting them through a 10-ml glass pipette. The cell suspension was filtered and centrifuged at 1600 rpm for 5 minutes, washed three times in PBS, counted and resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Biosys, Conpiegne, France), 10 mM β-glycerophosphate (Sigma, St. Louis, MO), 50 mg/ml of ascorbic acid and antibiotics (50 UI/ml penicillin, 50 mg/ml streptomycin). Cells were then seeded at a final density of 10^5 cell/cm^2 in 50 mm diameter culture dishes (Falcon, Polycabo, Strasbourg, France). Cultures were placed in a 5% CO_2 humidified atmosphere at 37°C, and the culture medium was changed every 48 hours for up to 15 days.

Calvaria Cell Cultures

Calvariae bone cells from 21-day-old fetal Sprague Dawley rats were isolated according to the modified procedure outlined elsewhere [22] and used as controls. Briefly, central parts of the parietal and frontal bone with their endosteum and periosteum were incubated for 2 hours at 37°C in PBS containing 0.25% collagenase. Cells released from bone fragments were washed several times in PBS, counted, and seeded at a final density of 2×10^4 cells/cm^2. Then they were cultured under the same conditions as chondrocyte cultures.

Isolation and Biochemical Analysis of Collagens From Cell Culture

Collagen purification was performed from days 3 to 15 of the culture. After washes with PBS, the cell layers were scraped and homogenized in acetic acid 0.5 M containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM N-ethyl maleimide, 2.5 mM EDTA). The cellular extract was digested with pepsin (1 mg/ml) 24 hours at 4°C. The pH of the solution was raised to 8 with 4 M NaOH to inactivate the pepsin and the solution was extensively dialyzed against 0.5 M acetic acid. Types I and II collagen were precipitated by 2.5 M NaCl at acid pH for 24 hours at 4°C. The precipitates were removed by centrifuging, redissolved in acetic acid, dialyzed against 0.5 M acetic acid, and lyophilized. For each sample, the purified protein content was then determined according to the bicinchoninic acid method assay (BCA); bovine serum albumin (BSA) was used as standard (kit, Pierce, Interchim, Monluçon, France). Samples containing 30 μg of purified proteins were dissolved in Laemmli sample buffer, boiled for 5 minutes, and subjected to 6.5% SDS-PAGE under reducing conditions [23]. Gels were stained with Coomassie blue or processed for immunoblotting.

Immunoblotting

Samples were dissolved in Laemmli sample buffer, boiled for 5 minutes, and subjected to 6.5% SDS-PAGE under reducing conditions. Resolved proteins were then electroblotted onto nitrocellulose filters (Schleicher and Schüell BA 85, Seralabo, Ecquevilly, France) for 4 hours at 60 V in buffer containing 25 mM Tris, pH 8.35, 193 mM glycine, 0.1% SDS, and 20% methanol. Blots were probed as previously described [24] using specific anti-rat type II collagen antibodies at a 1/4000 dilution (Hartman, Pasteur, Lyon, lot 2041), and anti-rat type I collagen antibodies at a 1/3000 dilution (Biogenesis, 2150-2304). Horseradish peroxidase-conjugated anti-rabbit IgG was bound to the immunocomplexes, and visualization of immunobands was carried out by addition of chemiluminescence reagent (ECL); Amersham, Lesulis, France).

Assay for Alkaline Phosphatase Activity

ALP was measured in cell layers after lysis with a solution containing 0.1% Nonidet P-40, 1 mM $MgCl_2$, pH 10.2. Enzyme activity was assayed using P-nitrophenyl phosphate as substrate at a final concentration of 10 mM in 2 amino-2 methyl-1-propanol 0.75 M alkaline buffer solution containing $MgCl_2$ (Sigma) at pH 10.3. After 15 minutes of incubation at 37°C, the reaction was stopped with 0.5 N NaOH and the amount of P-nitrophenol released was measured spectrophotometrically at 405 nm. Standards prepared with a P-nitrophenol standard solution (Sigma 104-1) were deter-

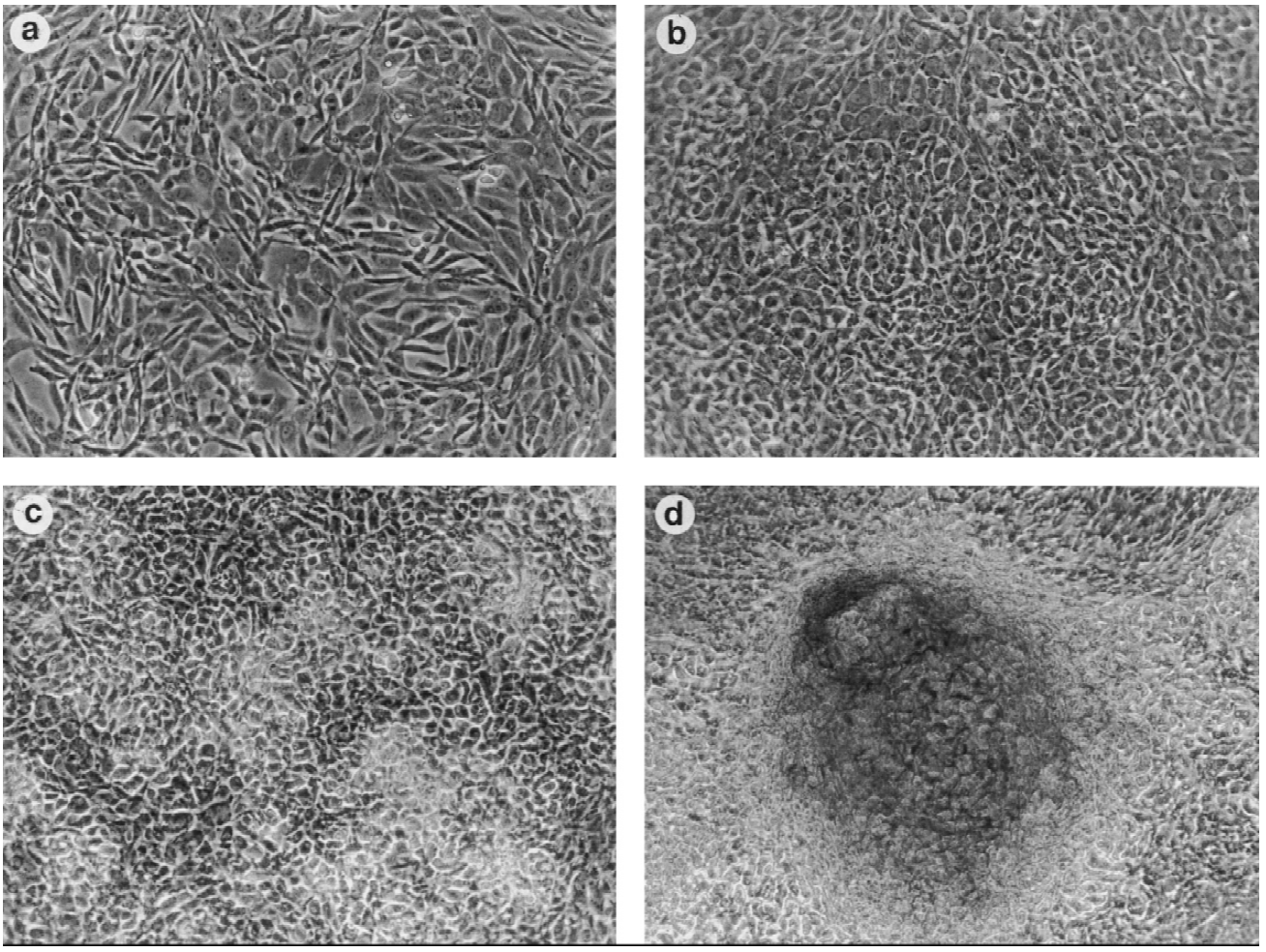

Fig. 1. Phase-contrast microscope study of the different phases of mineralizing rat chondrocytes cell culture. **(a)** First phase: on day 3 of culture, cells form a subconfluent monolayer (×125). **(b)** Second phase; on day 6, several clusters of tight cellular aggregates are visible in the confluent monolayer cell culture (×125). **(c)** Third phase: on day 9 nodule formation as determined by the formation of a matrix seen as a refringent material in phase contrast (×125). **(d)** Fourth phase: on day 15, nodules' mineralization (×125).

mined in parallel. ALP activity was expressed as nmol P-nitrophenol-produced/minute/mg protein. The protein content was determined according to the BCA; BSA was used as standard (kit, Pierce).

Analysis of RNA by Northern Blots

After three washes, the medium was replaced by a serum-free medium and cells were incubated for 20 hours. On different days, total RNAs from cultures were extracted by the acid guanidinium thiocyanate, followed by a phenolchloroform (Sigma) extraction, as previously described [25]. Total RNAs were precipitated with isopropanol, resuspended, and reprecipitated with ethanol 70%. Total RNAs recovered were estimated by spectrophotometry, and 5 μg of RNA from control and test sample was loaded on a formaldehyde agarose gel after denaturation. RNA standards (Gibco, BRL, France) were used to determine transcript size. RNAs were then transferred to a sheet of Hybond-N+ nylon membrane (Amersham). Restriction fragment containing a 405-basepair (bp) rat α_1(II) collagen [26], a 321 bp mouse α_1(I) collagen [27], a 650 bp mouse α_1(X) collagen [28], a 400 bp mouse osteocalcin, a generous gift of Dr. St Arnaud (Hôpital Shriners pour enfant, Montréal, Canada), a 900 bp rat Aggrecan [29], a 2500 bp rat ALP [30], or a 2000 bp human β actine [31] were labeled with [α-^{32}P] deoxy-CTP (3000 Ci/mmol; Amersham) using random priming (Kit Rediprime, Amersham). Hybridizations were carried out at 42°C for 24–96 hours. Posthybridization washes were performed at room temperature in 2 × saline sodium citrate (SSC), 0.5% SDS, and at 55°C or 65°C in 1 × SSC, 0.5% SDS. The blot was stripped and hybridized with a [^{32}P]-labeled human β actin to demonstrate RNA loading of the gel. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 or β-max film (Amersham) using intensifying screens. The Northern analyses shown were representative of experiments performed with three different cell preparations.

Results

Morphological Characteristic of Cartilage Cell Culture

Under our culture conditions, chondrocytes synthesized a matrix that mineralized in less than 2 weeks. The differentiation process of chondrocytes observed during cell culture could be divided into four major steps by phase contrast microscopic observations (Fig. 1). In the course of the first phase (days 1–4), nasal cartilage cells spread to the plastic dishes immediately after seeding and adopted a fibroblastic

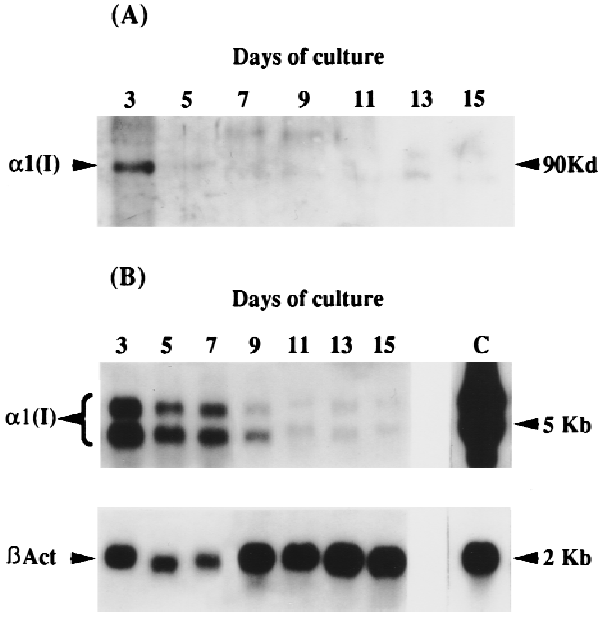

Fig. 2. Expression of type I collagen. **(A)** Western blot analysis of type I collagen isolated from culture at different days of culture after pepsin digestion, precipitation at 2.5 M NaCl, 0.5 M acetic acid, electrophoresis on SDS-PAGE 6%, and transfer onto nitrocellulose membrane. Sizes of pepsin fragments are indicated in kilodaltons to the right. **(B)** Northern blot analysis of total RNA over 15 days of growth in culture hybridized with cDNA probe for α1(I). Hybridization to actin served as internal control of the amount of RNA bound to the filter. Autoradiographic exposure with an intensifying screen: 48 hours. Sizes of the messages are indicated in kilobases to the right.

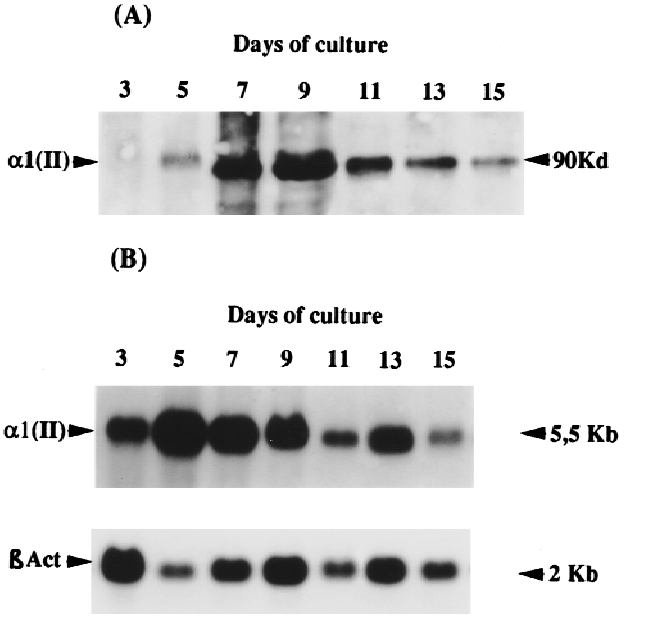

Fig. 3. Expression of type II collagen. **(A)** Western blot analysis of type II collagen isolated from culture at different days of culture after pepsin digestion, precipitation at 2.5 M NaCl, 0.5 M acetic acid, electrophoresis on SDS-PAGE 6% and transfer onto nitrocellulose membrane. Sizes of pepsin fragments are indicated in kilodaltons to the right. **(B)** Northern blot analysis of total RNA over 15 days of growth in culture hybridized with cDNA probe for α1(II). Hybridization to actin served as internal control of the amount of RNA bound to the filter. Autoradiographic exposure with an intensifying screen: 48 hours. Sizes of the messages are indicated in kilobases to the right.

morphology. During their growth phase, cells exhibited either spindle or polygonal shapes and formed a subconfluent monolayer (Fig. 1a) on day 3 of culture. The second phase (Fig. 1b) (days 5–7) was characterized by modifications of the cell shape; under phase contrast microscopy, the cells forming some clusters of functional chondroblastic cells appeared as refractile colonies of round cells compared with the background cell layer where the cells were more elongated. The third phase (days 7–9) was associated with an increase of matrix volume which appeared as refringent material in phase contrast (nodule formation) (Fig. 1c), followed by the last phase (days 9–15) corresponding to matrix mineralization (Fig. 1d).

Collagen Expressions During Chondrocyte Differentiation in Culture

Expression of types I, II, and X collagen was studied during cell cultures by Western and Northern blot. Immunoblotting with specific rate antibody α_1(I) (Fig. 2A) has shown that the major 90 Kd pepsin resistant molecule corresponded to α_1(I) at day 3 but not for the other days of culture. In parallel, expression of collagen α_1(I) mRNA analyzed by northern blot (Fig. 2B), was most prominent during the two first periods of culture corresponding to the loss of chondrocyte phenotype and the proliferation period before the mineralization process. In addition, α_1(I) mRNA type I collagen expression in cartilage cell culture was not as great in comparison with control α_1(I) mRNA type I collagen expression in rat calvaria-osteoblast cell culture. α_1(I) collagen synthesis and α_1(I) mRNA transcription did not seem correlative. The α_1(I) mRNA was mainly detected during the two first phases of chondrocyte culture, but the corresponding protein was detected only on day 3.

Immunoblotting with specific rat antibody α_1(II) has confirmed that the 90 Kd pepsin-resistant molecule expressed from days 5 to 15 corresponded to α_1(II) type II collagen marker of the chondrocyte phenotype (Fig. 3A). It was detectable at day 5 and maximal expression occurred at days 7–9 of the culture, characterized by an active matrix protein synthesis corresponding to cartilagenous nodule formation. After day 9, type II collagen synthesis decreased just before the beginning of the mineralization process but was present during the entire culture period.

In parallel experiments, α_1(II) type II collagen mRNA levels were also determined by Northern blot at different times of culture (Fig. 3B). At days 5 and 7, measurement of mRNA expression revealed a maximal level of α_1(II) transcripts when cells were in differentiation process and appeared as polygonal cells, but decreased with time in culture and therefore cartilage nodule formation. The maximal expression of α_1(II) type II collagen mRNA observed (days 5–7) preceded the increase of type II collagen synthesis illustrated by immunoblotting with rat antibodies α_1(II) (days 7–9) (Fig. 3A), and the expression of α_1(II) transcripts diminished just before the decrease of the synthesis of its associated protein.

SDS page of pepsin-resistant collagen at different days of cell culture did not allow to follow type X collagen synthesis, but Northern blot analysis has revealed that α_1(X)

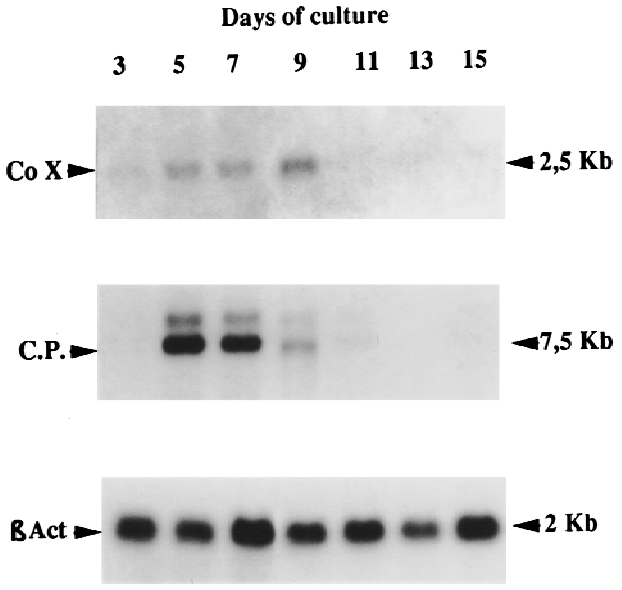

Fig. 4. Enhanced expression of collagen α(1) X and core protein of aggrecan during cell culture. Total RNA was extracted from cells and 5 μg of RNA was applied to each lane and analyzed by Northern blotting. Hybridization to actin served as internal control of the amount of RNA bound to the filter. The mRNA species were analyzed and days on which mRNA was harvested are noted on the figure. Autoradiographic exposure with an intensifying screen varied from 24 to 96 hours depending on the probe use. Sizes of the messages are indicated in kilobases to the right.

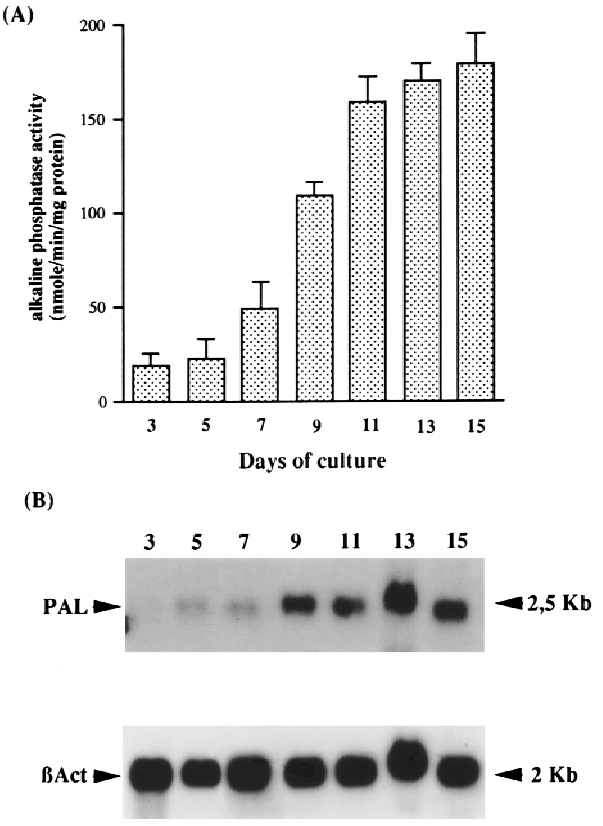

Fig. 5. Alkaline phosphatase enzyme activity and total mRNA expression at different days of culture. **(A)** ALP enzyme activity as a function of time in culture. Triplicate 33-mm culture dishes from three separate experiments were determined for every experimental point. Total nanomolar p-nitrophenol determined in a 30-minute assay was used to determine values depicted in the figure. Error bar denotes the total range of the experimental variation. **(B)** Northern blot analysis of total RNA over 15 days of growth in culture hybridized with cDNA probe for ALP. Five micrograms of total RNA were applied to each lane.

mRNA was synthesized between days 5 and 9 before the beginning of mineralization (Fig. 4). At day 11 and during the mineralization process transcripts were not detected. Parallel experiments have shown that cells isolated from the proliferative zone of epiphyseal growth plate cartilage that were allowed to hypertrophy in culture which did not mineralize *in vitro* and used as control, did not express α_1(X) mRNA collagen (data not shown).

Core Protein mRNA Expression

Expression of proteoglycan core protein mRNA levels appeared to be maximal during the second phase of the culture between days 5 and 7 (Fig. 4). From day 9, aggrecan transcripts declined just before the beginning of calcification. Maximal expressions of proteoglycan core protein mRNA and α_1(II) type II collagen mRNA were in phase during the cell culture.

ALP Expression

Specific activity of ALP was determined in a crude extract prepared from cell culture at various days. At day 9, there was a significant increase of ALP activity which reached a maximum at day 11 and was about 10 times that of day 3 (Fig. 5A). ALP activity increase appeared to be correlated with the beginning of nodule formation. Day 9 corresponded to the end of the third phase culture associated with an active secretion of proteins by chondrocytes forming a matrix. As for ALP activity, the study of ALP mRNAs expression under the same conditions has shown, at day 9, an increase of ALP mRNA level compared with days 3, 5, or 7 (Fig. 5B).

Osteocalcin mRNA Expression

During the chondrocyte culture period, differentiating chondrocytes never express osteocalcin mRNA (data not shown). In contrast, osteocalcin mRNA was detected at day 13 in rat calvaria-osteoblast cell culture treated under the same conditions and used as control. Day 13, in calvaria-osteoblast cell culture, corresponded to the mineralization of the nodule as well as new nodule formation [32].

Discussion

The results of this study have shown that dissociated nasal cartilage cells initially lose their phenotype in culture; this phase was characterized by type I collagen expression. Then during culture, cells reexpressed *in vitro* the chondrocyte phenotype. This was demonstrated by expressions of type II collagen, core protein of aggrecan, and just before miner-

alization of type X collagen which, as for ALP, seemed to be implicated in the mineralization process.

During cell culture, fluorograph of pepsin-resistant, [^{3}H]proline-labeled collagen present in the matrix layer revealed a major 90 kDa band with a maximal expression between days 7 and 9, corresponding to radiolabeled collagen α_1(II) and/or α_1(I) comigrating band (data not shown). Immunoblotting with specific antibody has shown that this band corresponded to α_1(I) type I collagen only at day 3. Whereas α_1(I) mRNA transcripts analyzed by Northern blot were mainly detected during the first phase, they progressively diminished from the second phase to the end of cell culture. In the literature it has been documented that the expression of type I collagen by cartilaginous cell cultures is associated with the loss of chondrocyte phenotype at the beginning of the cell cultures [33, 34]. It has also been shown that type I collagen transcripts, detected by RT-PCR analysis, are present in freshly dissected intact human articular cartilage [35] which signifies that expression of type I collagen mRNA does not necessarily indicate synthesis of type I collagen, despite expression of α_1(I) mRNA, since mechanisms of translational control prevent type I collagen synthesis in chick vertebral chondroblasts [36]. Our results were consistent with these data and indicated the instability of chondrocytes in culture.

In this study we have characterized the pattern of type II collagen synthesis and mRNA expression. Since the differentiation phase, type II collagen was gradually reexpressed with a maximal synthesis between days 7 and 9 preceded by an increase of mRNA type II collagen. Then, levels of type II collagen reduced during hypertrophy, coinciding with the onset of calcification. Similar changes of type II collagen mRNA expression have been observed in mineralizing cell cultures [4]. Otherwise, by *in situ* hybridation [37], it has been suggested that type II collagen mRNA is synthesized primarily by cells of the proliferating zone and by newly formed hypertrophic cells. Moreover, a decreasing proportion of cells stained for type II collagen mRNA is observed for mature hypertrophic chondrocytes, particularly those present in calcifying cartilage. Finally, the decrease of type II collagen expression observed during the mineralizing chondrocyte culture stage was in agreement with other reports in the literature and with the *in vivo* situation.

The expression of α_1(X) mRNA occurred in culture prior to mineralization of the culture, according to a morphological study showing that mineral deposit begins on day 12 [21]. After day 11, type X collagen transcript was not present. Also, cells isolated from the proliferative zone of epiphyseal growth plate cartilage that were allowed to hypertrophy in culture which did not mineralize *in vitro* and used as control, did not express α_1(X) mRNA collagen (data not shown).

In vivo, type X collagen is produced only by chondrocytes undergoing maturation leading to hypertrophy [38]. Biochemical and immunofluorescence studies [39–42] as well as *in situ* hybridization [43] suggest that expression of type X collagen is restricted to cartilage undergoing endochondral ossification. *In vitro,* type X collagen expression does not always spatially correlate with initiation of mineralization, yet, as in our system, cartilage matrix mineralization occurred and followed only type X collagen expression in culture [44]. Our results were in agreement with a prior study which showed that synthesis was rapidly arrested in culture once calcification was established [45]. The function of type X collagen in cartilage is not known. However, it has been suggested that type X collagen may facilitate the removal of cartilage during endochondral ossification [39] or may play a role in calcification [39]. All of these investigations suggest that this collagen may be involved in the mineralization of cartilage. According to these studies, the transitory appearance of type X α collagen transcripts before the beginning of the mineralization observed in our culture system could suggest a role in the mineralizing process.

During nasal septum chondrocyte cell culture, the expression of the proteoglycan core protein mRNA appeared at maximal levels during the second phase of the culture and then declined at the beginning of mineralization. These results are in line with previous studies which show that the amount of [^{35}S]incorporation in total glycosaminoglycans increases rapidly to a maximal level on day 7, and then decreases in our experimental cell culture [46]. However, decreased proteoglycan content and reduced proteoglycan synthesis in zones of cartilage hypertrophy and calcification have previously been reported [47–49]. Our results suggest that the loss of large aggregating proteoglycans in growth plate cartilage might occur once mineralization is initiated, as described in other studies [50, 51].

ALP activity increase seemed to be correlated with the beginning of nodule formation and was maintained at the same level during mineralization. ALP, primarily localized in the plasma membranes of growth plate chondrocytes and matrix vesicles [52, 53], is thought to be involved in the mineralization process [54, 55]; however, its exact function is not clear. Studies using the levamisole drug, an inhibitor of ALP, [56, 57] have demonstrated the inhibition of cartilage mineralization [58, 59] and therefore, its importance in the mineralization process. Enzyme activity is higher in matrix vesicles isolated from cells cultured from the growth region than those derived from resting zone cells of rat costochondral cartilage [60]. Moreover, biochemical analysis showed that in the epiphyseal growth plate, ALP activity is greatest in the hypertrophic zone [55, 61]. The pattern of ALP expression observed in our cell culture system seemed to be correlated with *in vivo* studies.

In the present study, differentiating chondrocytes never expressed osteocalcin mRNA during cell culture but it was detected during the mineralization of the nodule in rat calvaria-osteoblast control cell culture. Osteocalcin has been well documented as a bone-specific protein that does not appear to be necessary for normal mineralization, since administration of warfarin (an inhibitor of osteocalcin synthesis) still results in normal bone formation [63, 64]. Osteocalcin mRNA levels are undetectable in various types of cartilage [65, 66]. Nevertheless, in some systems producing a mineralized matrix *in vitro,* type I collagen reappears with mineral deposition; also expression of osteocalcin was observed [13, 19]. *In situ* hybridization of osteocalcin cDNA to hypertrophic chondrocytes in cultured mouse mandibular condyle has shown that only a subset of cells may produce a low level of osteocalcin and these cells may represent osteoprogenitor cells or a subset of hypertrophic cells which then progress toward a stage in their lineage with specific osteoblastic phenotypic characteristics [19]. On the other hand, studies have suggested that a transdifferentiation could occur between the chondrocyte and osteoblastic lineage [10, 13]. Thus, it is possible that in cell culture, a subset of cells might first dedifferentiate and then be activated to differentiate osteoblasts *in vitro.* In our system, the absence of type I collagen synthesis and osteocalcin transcripts in mineralized culture could suggest that nasal cartilage-derived cell cultures represented a much purer popu-

lation of chondrocytes or phenotypically different chondrocytes compared with other systems used to study chondrocyte mineralization.

In conclusion, our results indicated that type II collagen and core protein mRNA levels were more important before the beginning of mineralization. The time-dependent induction of type X collagen, ALP, and diminished expression of type II collagen and core protein of the large cartilage proteoglycan observed in our system are in agreement with other reports. During normal development, cells in the upper level of the growth plate rapidly divide, mature, differentiate, become hypertrophic, and initiate mineral deposition. With heavy mineral deposition in the extracellular matrix, cells become trapped in their lacunae; however, their fate remains obscure. In addition, chondrogenesis is thought to be controlled by interactions between circulating anabolic hormones and locally produced peptide growth factors, and involves ordered changes in matrix composition which ultimately allow endochondral calcification. Our chondrocyte cell culture appears to be a model for studying growth factors implicated in the differentiation and mineralization process and it could help us to understand *in vivo* chondrocytes differentiation.

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