Hypertrophic Chondrocytes Undergo Further Differentiation in Culture

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Abstract. Conditions have been defined for promoting growth and differentiation of hypertrophic chondrocytes obtained in culture starting from chick embryo tibiae. Hypertrophic chondrocytes, grown in suspension culture as described (Castagnola P., G. Moro, F. Descalzi Cancedda, and R. Cancedda. 1986. *J. Cell Biol.* 102:2310–2317), when they reached the stage of single cells, were transferred to substrate-dependent culture conditions in the presence of ascorbic acid. Cells showed a change in morphology, became more elongated and flattened, expressed alkaline phosphatase, and eventually mineralized. Type II and X collagen synthesis was halted and replaced by type I collagen synthesis. In addition the cells started to produce and to secrete in large amount a protein with an apparent molecular mass of 82 KD in reducing conditions and 63 KD in unreducing conditions. This protein is soluble in acidic solutions, does not contain collagenous domains, and is glycosylated. The Ch21 protein, a marker of hypertrophic chondrocytes and bone cells, was synthesized throughout the culture. We have defined this additional differentiation stage as an osteoblast-like stage. Calcium deposition in the extracellular matrix occurred regardless of the addition of β glycerophosphate to the culture medium. Comparable results were obtained both when the cells were plated at low density and when they were already at confluence and maintained in culture without passaging up to 50 d. When retinoic acid was added to the hypertrophic chondrocyte culture between day 1 and day 5 the maturation of the cells to the osteoblast-like stage was highly accelerated. The switch in the collagen secretion was already observed after 2 d and the production of the 63-kD protein after 3 d. Mineralization was observed after 15–20 d.

LONG bone organogenesis occurs in the embryo by endochondral ossification from undifferentiated mesenchyme. During the early stages of development, mesenchymal cells in the limb buds condense to form a core of differentiated chondrocytes; osteogenesis starts at the periphery of the cartilage core, which is subsequently invaded by blood vessels and replaced by bone marrow and trabecular bone. After birth, similar events take place in the long bone growth plate and at the bone fracture sites. Bone formation and remodeling have been extensively investigated, starting from pioneering work describing the morphological and biochemical changes occurring during early bone formation to more recent studies aimed at the elucidations of the cellular and molecular mechanisms involved (7, 22, 34). It is widely agreed that cells present in a continuous collar surrounding, but separated from the cartilage rudiment, give rise to osteoblasts, i.e., cells responsible for the synthesis and mineralization of the osteoid extracellular matrix. In the past, occasionally and recently more frequently, it has been postulated that growth plate hypertrophic chondrocytes might also contribute to the formation of a bone matrix, since in some organ cultures these cells start to express bone markers. During culture of mouse mandibular condyles, the expression of type I collagen, osteonectin, alkaline phosphatase, osteopontin, and osteocalcin by mature chondrocytes was detected by in situ hybridization (38). A morphological study has been recently published consistent with the idea that in condylar cartilage of rat mandible hypertrophic chondrocytes can become bone (48). Conversion of mature chondrocytes into osteogenic cells was also observed in Meckel's cartilage from rat in ocular culture (30).

We have described that hypertrophic chondrocytes in suspension culture undergo terminal differentiation and express large amount of type X collagen (8), a marker specific for hypertrophic cartilage (6, 31, 32), and Ch21, a protein belonging to the superfamily of proteins binding small hydrophobic molecules that is expressed both by cartilage and bone (11, 12, 24).

In the present investigation we show that when hypertrophic chondrocytes isolated as single cells after 3 wk in suspension culture are transferred to anchorage-dependent culture conditions in the presence of ascorbic acid, they undergo profound changes in their morphology and biosynthetic activity suggesting a further maturation to an osteoblast-like stage. Cells acquire an elongated or star shaped morphology, start to express alkaline phosphatase, and to reorganize their extracellular matrix by switching from the synthesis of the cartilage specific type II and type X collagens to the synthesis of type I collagen. At the same time, cells express and secrete a large quantity of a glycoprotein with an apparent molecular mass of 63 kD in unreducing conditions. Eventually, on the newly formed matrix calcium minerals are deposited.

In addition, we report that when retinoic acid, a compound well known for its capability to interfere with skeletal development in vivo and with cartilage and bone cells differentiation in vitro, is added to the hypertrophic chondrocyte culture between day 1 and day 5, the conversion of chondrocytes from stage II to the osteoblast-like stage is highly accelerated. The switch in the collagen secretion is already observed after 2 d and the production of the 63k protein after 3 d. Mineralization is observed after 15–20 d.

Materials and Methods

Cell Cultures

Dedifferentiated and hypertrophic chondrocytes were obtained from 6-d chick embryo tibiae. Cells derived from the cartilaginous bone were expanded as adherent dedifferentiated cells for 2 wk and then transferred in suspension culture for 3–4 wk until a homogeneous population of single isolated hypertrophic chondrocytes was obtained (8). Hypertrophic chondrocytes were filtered through a nylon filter Nitex 42 μm mesh in order to avoid any contamination of cells still aggregated, digested with hyaluronidase (1 mg/ml), and plated either at low density (5 × 10^4 cells in 100-mm dish) or at confluence (2 × 10^5 in a 35-mm dish) in Coon's modified F12 culture medium (1) containing 10% FCS. After 3 d the medium was supplemented with 100 μg/ml ascorbate and 10 mM β-glycerophosphate (complete medium). The medium was changed every other day without cell passaging. Parallel cultures grown without ascorbic acid were used as control. When indicated, retinoic acid was added to the culture medium at 1 μM concentration. Retinoic acid from a freshly prepared solution was daily added to the medium from day 1 to day 5 of the experiment and afterwards cultures were maintained in complete medium.

Cell Labeling and Protein Analysis

Cells were labeled with ^{35}S methionine as described (11). When indicated, tunicamycin (2 μg/ml) was added to the culture medium during methionine starvation and the 2-h labeling period. Aliquots of culture media or cell lysates were run for protein analysis on SDS-PAGE in unreducing and reducing conditions as described (3). Except when indicated polyacrylamide gel concentration was 12.5%. When indicated, samples were digested with bacterial collagenase (20 μg/ml) in 0.05 M Tris-HCl, pH 7.6, 0.01 M CaCl$_2$ at 37°C for 90 min before electrophoresis. In some experiments, the samples were dialyzed for 16 h at 4°C in 0.5 N acetic acid, digested with pepsin (100 μg/ml) overnight and lyophilized before analysis. Immunoprecipitation of specific proteins was performed as previously described (11).

Histochemistry

Alcian blue staining (Chroma, Stuttgart, Germany) specific for cartilage proteoglycans was performed at pH 1 (4). Alkaline phosphatase activity was
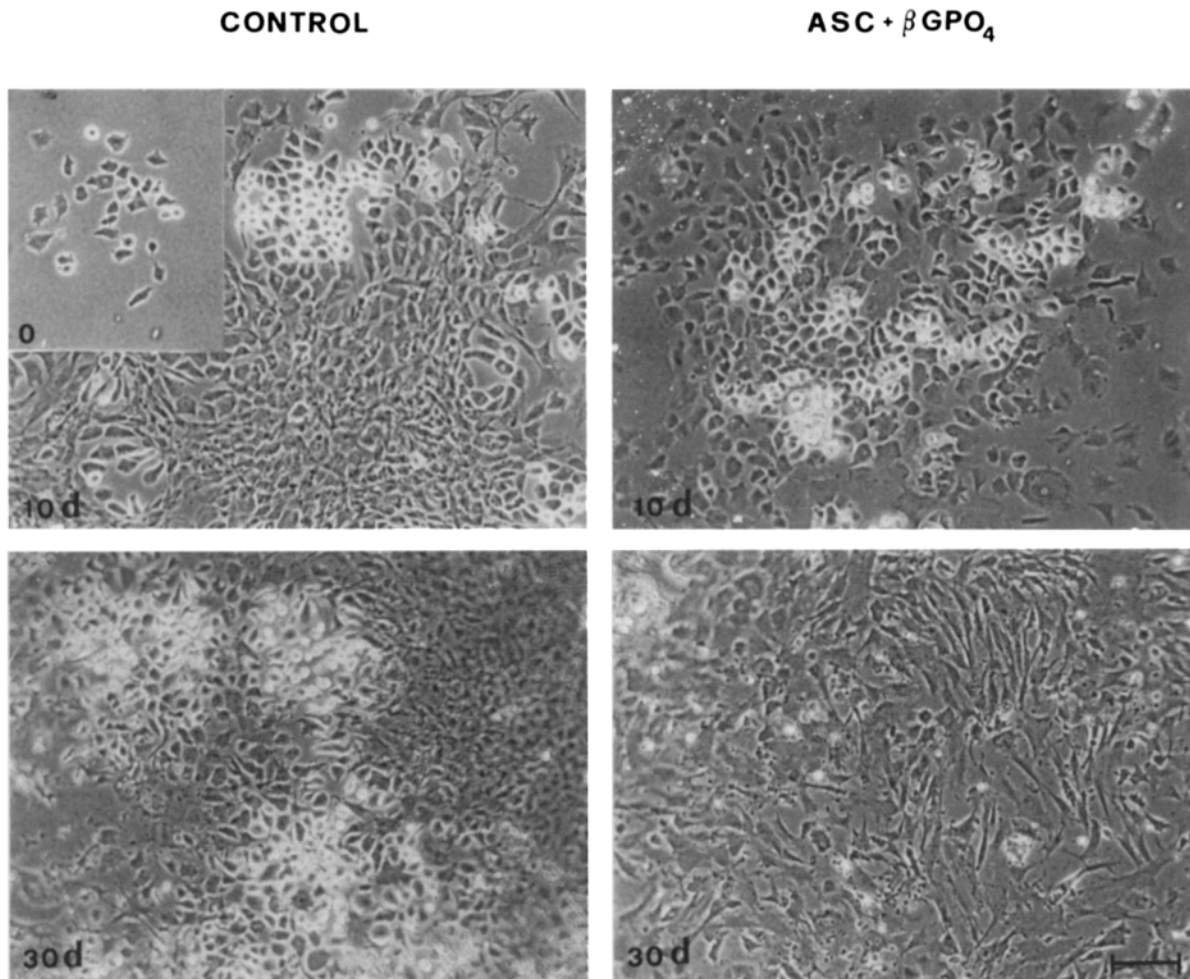
CONTROL   **ASC + βGPO$_4$**

Figure 1. Cell morphology of hypertrophic chondrocytes plated at low density. Cells were maintained in Coon's modified F12 medium (*control*) or in the same medium supplemented with 100 μg/ml ascorbic acid and 10 mM β glycerophosphate. Numbers refer to days in culture. The insert "0" refers to the starting cell population. Bar, 100 μm.
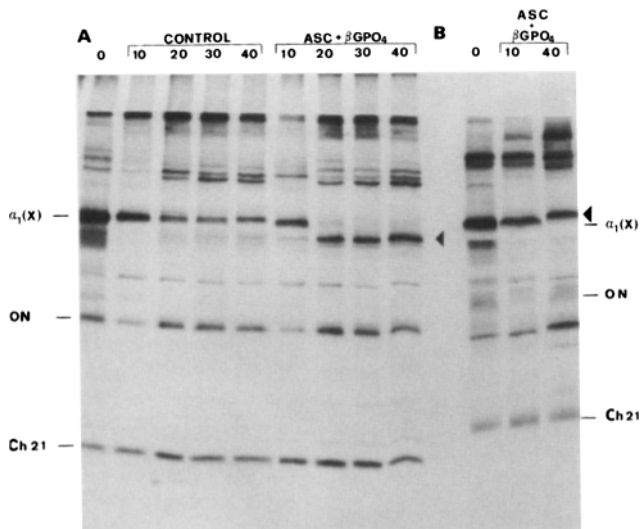
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Figure 2. Proteins secreted by cells plated at low density. Cells were maintained in Coon's modified F12 medium (*control*) or in the same medium supplemented with 100 μg/ml ascorbic acid and 10 mM β glycerophosphate. Numbers refer to days in culture; "0" refers to suspension culture of hypertrophic chondrocytes at the time they were plated. Proteins were analyzed in unreducing (*A*) and reducing (*B*) conditions. Arrowheads refer to the protein of 63 KD in unreducing conditions and 82 KD in reducing conditions described in the text. Other differentiation markers: type X collagen, osteonectin (ON), and Ch21 were identified based on their electrophoretic migration and their immunoprecipitation by specific antibodies (not shown).

determined using the histological kit 86 by Sigma Chemical Co. (St. Louis, MO) and mineralization was detected by von Kossa staining (4).

Results

Protein Synthesis by Plated Hypertrophic Chondrocytes Maintained in Supplemented and Unsupplemented Control Medium

Hypertrophic chondrocytes were recovered from a 3–4-wk suspension culture, filtered through a Nitex filter in order to eliminate any possible residual cell aggregate, digested with hyaluronidase, and plated at a low density both in the presence and in the absence of ascorbic acid and β glycerophosphate. In these conditions, hypertrophic chondrocytes remained replicative, although at a low rate, and reached confluence in 10–15 d. Fig. 1 shows the cell morphology at different time of culture in both conditions. We constantly observed that after 4–5 wk of culture the cells maintained in the presence of ascorbic acid and β glycerophosphate, but not the control cells, presented a frank fibroblastic morphology. The different morphology was even more evident when the hypertrophic chondrocytes were plated at confluence (not shown).

The pattern of proteins secreted by the cells during culture is shown in Fig. 2. It is evident from Fig. 2 *A* that major differences exist between proteins secreted by cells maintained in the presence of ascorbic acid and β glycerophosphate and proteins secreted by control cells. In particular, the cells maintained in the supplemented medium stopped releasing type X collagen in the medium between day 10 and day 20

of culture, while control cells continued to secrete the same molecule, although at a lower level, until at least the 40th day of culture. The disappearance of type X collagen from the culture medium of the cells maintained in the supplemented medium was contemporary to the appearance of a new protein with an apparent molecular mass of 63 kD (Fig. 2, *arrowhead* in *A*). The same protein was barely detectable in the culture medium of control cells only at late times. It must be noted that when polyacrylamide gel electrophoresis was performed in reducing conditions (Fig. 2 *B*) this protein had an electrophoretic mobility very similar to the mobility of type X collagen making it difficult to discriminate between the two proteins. When the production of collagens was specifically investigated by the analysis of the pepsin resistent domains and their immunoprecipitation by specific antibodies, we observed that the arrest in type X collagen synthesis was contemporary to the switch from the synthesis of type II collagen to the synthesis of type I collagen (Fig. 3). The analysis of the collagens that remained associated with the cell layers confirmed the observations made by analyzing the proteins released into the media (not shown).

In some experiments, hypertrophic chondrocytes were plated directly at confluence. In the presence of ascorbic acid the production of the 63k protein and the expected changes in the collagen secretion occurred regardless of the different concentration at which the cells were plated (see Fig. 8 *A*).

A preliminary characterization of the 63k protein secreted by the cells maintained in supplemented medium revealed that this protein is not a collagen, since it was completely digested by pepsin treatment (Fig. 3, *A* and *B*) and its electrophoretic migration was not affected by collagenase digestion (Fig. 4 *A*), but it is glycosylated (Fig. 4 *B*). The 63k protein was not synthesized by the starting population of dedifferentiated cells, from which the hypertrophic chondrocytes were obtained, both in supplemented and control medium (Fig. 4 *C*). It must be noted that the dedifferentiated cells were only labeled 5 d after reaching confluence; in fact, the dedifferentiated cells, at variance with the cells derived from the hypertrophic chondrocytes, started to detach from the culture dish a few days after becoming confluent.

Extracellular Matrix Mineralization

Cells maintained in culture in supplemented and in control medium were Alcian stained at low pH in order to search for the presence of cartilage specific proteoglycans in the extracellular matrix. We observed a progressive decrease of Alcian positive extracellular matrix in cultures made in the presence of ascorbic acid and β glycerophosphate. By the fourth week in culture except for a few spotted areas all the matrix was negative; at the same time the cell extracellular matrix of control cultures was still highly positive (Fig. 5).

Cultures maintained in supplemented medium were also examined for alkaline phosphatase activity and deposition of calcium minerals by histological staining. Cells with alkaline phosphatase activity were already present at 10 d. The cells with maximal level of activity had both a stellate and a fibroblastic morphology (Fig. 6 *A*); in the same dish and in cultures maintained for the same time in control medium, some cells presenting the characteristic chondrocyte polygonal morphology also had detectable level of alkaline phosphatase activity. The first histologically detectable calcification was observed in the culture maintained in the presence
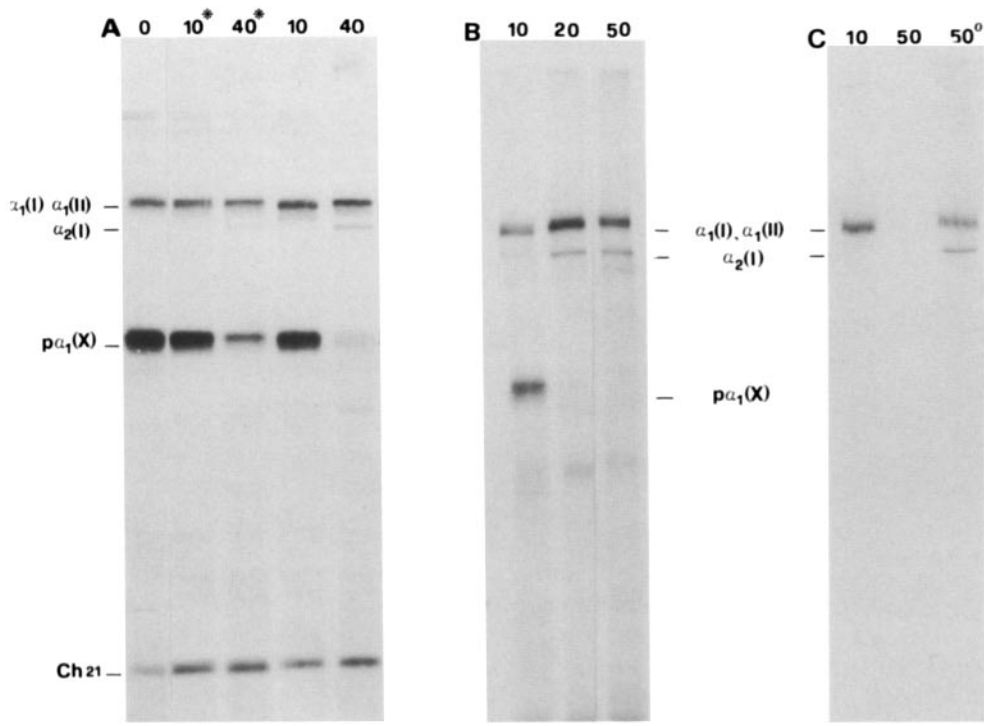
Figure 3. Collagens secreted by cells plated at low density. Aliquots of media from the same experiment of Fig. 2 were pepsin digested and analyzed on 10% polyacrylamide gel. Numbers refer to days in culture. All media were from cells maintained in the presence of ascorbic acid and β glycerophosphate, but the ones marked with asterisks were from cells maintained in F12 without additions (*control*). $p(\alpha_1)X$ indicates the collagenous domain of pepsinized type X collagen. Samples in *C* are the same samples as in *B* after immunoprecipitation with specific antibodies against type II collagen (first two lanes) and against type I collagen (symbol ° in third lane).
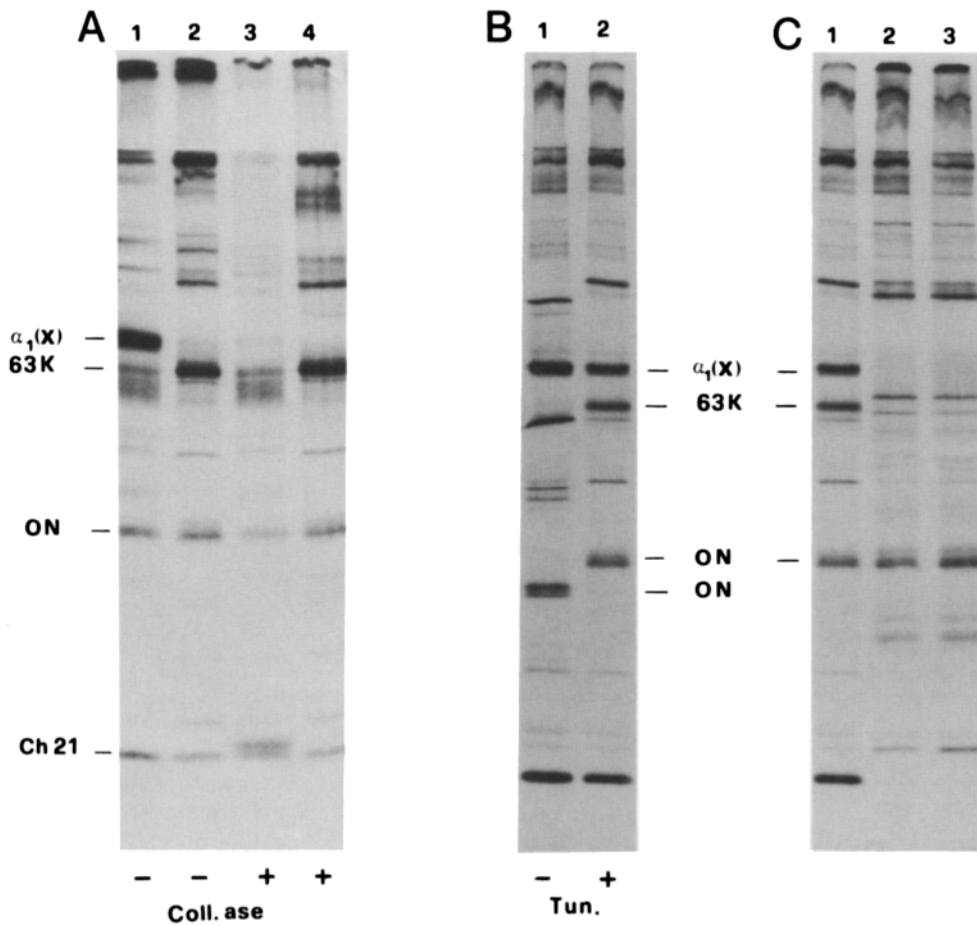

Figure 4. Characterization of the 63-KD protein. (*A*) Proteins secreted by control hypertrophic chondrocytes in suspension (lanes *1* and *3*) and by hypertrophic chondrocytes plated and cultured in the presence of ascorbic acid and β glycerophosphate (lanes *2* and *4*) were analyzed before and after collagenase digestion. (*B*) Proteins secreted by hypertrophic chondrocytes grown as adherent cells in supplemented medium and labeled in the presence or in the absence of tunicamycin. (*C*) Proteins secreted by hypertrophic chondrocytes grown as adherent cells in supplemented medium for 5 d (lane *1*) were analyzed in parallel with proteins secreted by dedifferentiated chondrocytes grown for 5 d in supplemented (lane *2*) and nonsupplemented (lane *3*) medium. ON indicates osteonectin.
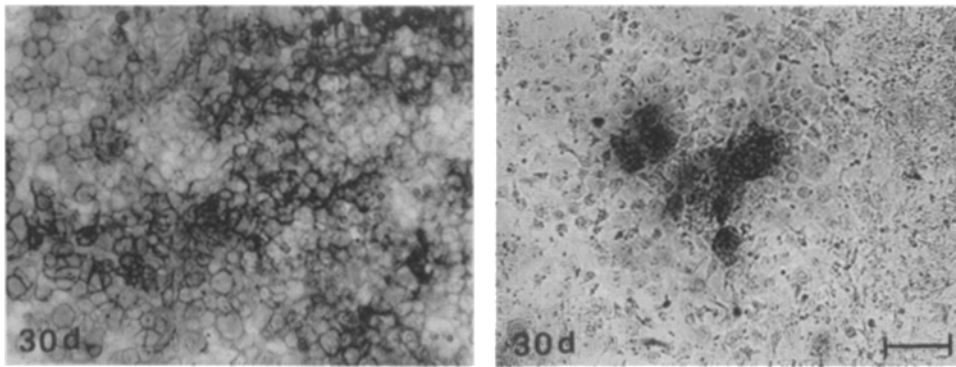
CONTROL   ASC+βGPO$_4$

Figure 5. Alcian staining of cultured cells. Hypertrophic chondrocytes were stained after 30 d from plating in supplemented and not supplemented medium. Bar, 100 μm.

of ascorbic acid and β glycerophosphate after $\sim$40–50 d. The mineralization extended throughout the culture and it reached its highest peak after $\sim$7–8 wk (Fig. 6 *B*). The deposition of calcium mineral was enhanced by the high cell density.

It has been suggested that the presence of mM concentrations of β glycerophosphate in the culture medium may promote calcium mineral deposition per se independently of the differentiation stage of the cells in culture. To rule out a direct role of the glycerophosphate on the mineralization occurring in our cell system, some cultures were performed in the presence of ascorbic acid, but omitting the addition of β glycerophosphate to the medium. No major differences were observed in the timing and nature of stellate and fibroblastic morphology development. No major differences were observed when proteins secreted during culture were analyzed and alkaline phosphatase activity and mineralization were determined by histological staining (not shown).

Retinoic Acid Plays a Major Role in Promoting Maturation of Hypertrophic Chondrocytes to Osteoblast-like Cells

Hypertrophic chondrocytes were plated at confluence and maintained in culture for $\sim$4 wk in the presence of ascorbic acid. Phase-contrast micrographs were taken both of cultures that were supplemented with 1 μM retinoic acid during the first 5 d and of control cultures (Fig. 7, *A* and *B*). In the

cultures supplemented with retinoic acid, the cells acquired a fibroblastic morphology already during retinoic acid treatment. Histochemical stainings revealed absence of cartilage-specific proteoglycans after 5 d only in retinoic acid–treated cultures (Fig. 7 *C*). Alkaline phosphatase–positive cells were observed after 15 d in both cultures, although the morphology of positive cells varied. In retinoic acid–treated cultures, positive cells always had a fibroblastic or stellate shape, while in control cultures there were also positive cells presenting a more regular polygonal morphology (not shown). Calcium mineral deposition was observed after 20 d in the retinoic acid–treated cultures, but not in control cultures (Fig. 7 *D*).

Proteins secreted by the cells were analyzed from day 1 to day 20. Fig. 8 shows the proteins secreted by hypertrophic chondrocytes cultured in: (*a*) Coon's modified F12 medium supplemented with FCS and containing also ascorbic acid and β glycerophosphate (Fig. 8 *A*) (*b*) the same medium with the further addition of retinoic acid during the first 5 d of culture (Fig. 8 *B*). Disappearance of type X collagen and production of the 63k glycoprotein was observed in retinoic acid–treated cultures between the first and the fifth day (see also Fig 9 *B*), while in control cultures not supplemented with retinoic acid, type X collagen was still present, although at a low level, after 20 d and the 63k protein continuously increased during culture. The high molecular weight interstitial collagens were better analyzed on pepsin digested
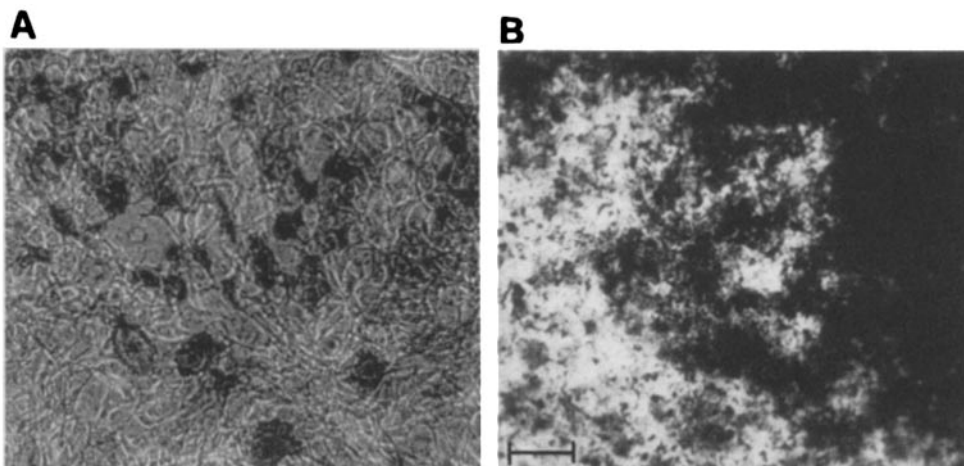
A   **B**

Figure 6. Histochemical staining of cultured cells. Hypertrophic chondrocytes maintained as adherent cells in supplemented medium were stained for alkaline phosphatase after 30 d (*A*) and for mineral deposition by von Kossa staining after 50 d (*B*). Bar, 100 μm.
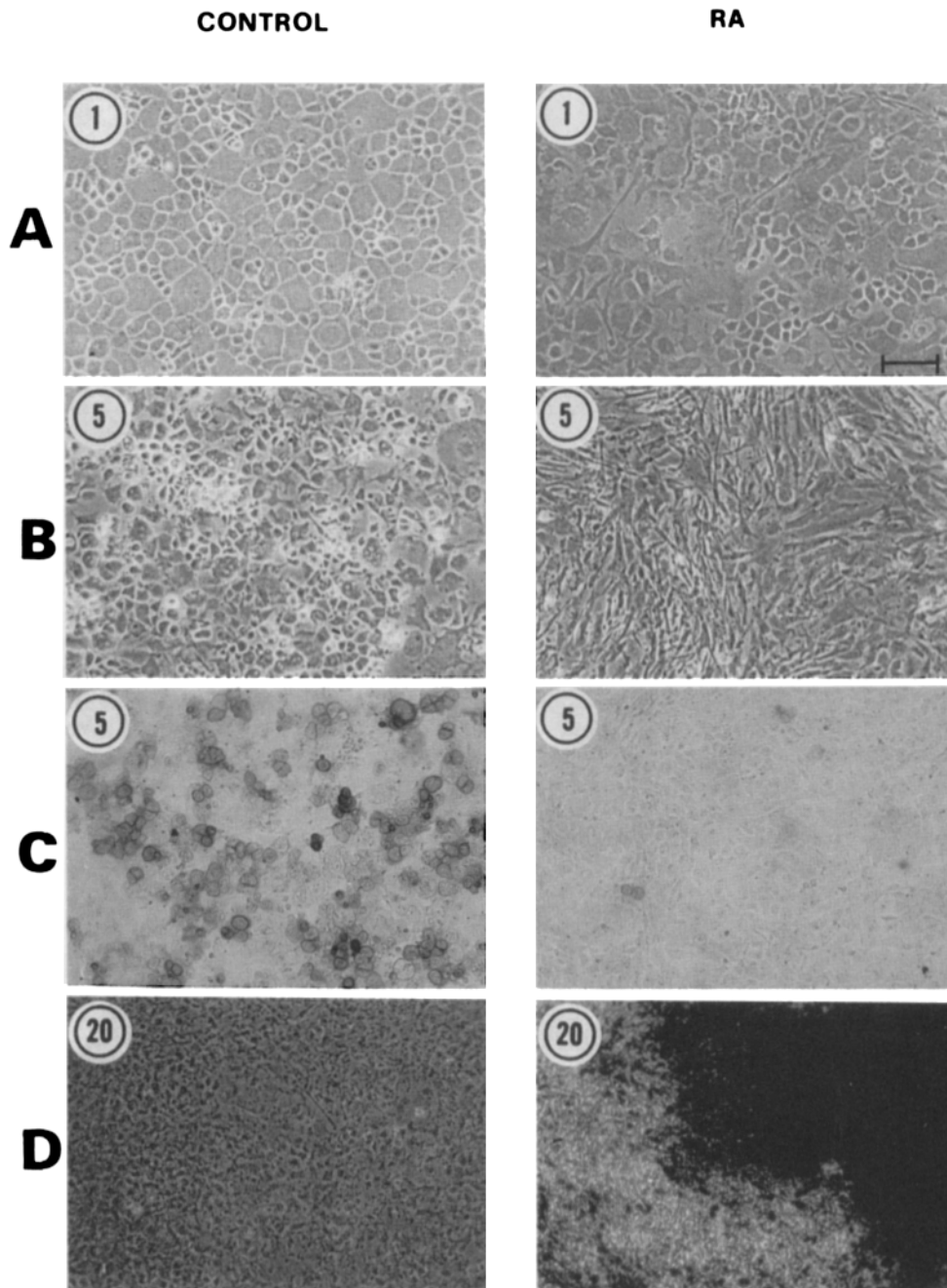
Figure 7. Effect of retinoic acid on cell morphology and histochemical staining of plated hypertrophic chondrocytes. Hypertrophic chondrocytes were plated at confluence and maintained in supplemented medium. Retinoic acid (*RA*) was added during the initial 5 d of culture. In control cultures the addition of retinoic acid was omitted. Phase-contrast pictures were taken 1 and 5 d after the addition of retinoic acid (*A* and *B*). Parallel cultures were Alcian stained at day 5 (*C*) and von Kossa stained after 20 d (*D*). Bar, 100 μm.

samples (Fig. 9). Switch from type II to type I collagen was investigated daily from day 1 to day 5 and it was observed that the collagen switch occurred in retinoic acid–treated cells between day 2 and day 3 (*B*). Type I collagen was the only collagen detectable between day 15 and day 25, when mineralization occurred.

Discussion

The relevance of the extracellular matrix in determining the cell microenvironment and the importance of the cell–extracellular matrix interactions in promoting and maintaining a differentiated phenotype is widely acknowledged. Several authors, including us, have shown that environmental conditions strongly influence chondrocyte development in cell cul-

ture systems and may promote cell hypertrophy starting from cartilage cells of different origin (5, 8–10, 14, 16, 36, 37, 39, 40, 45). Cultures within collagen gels, long-term secondary cultures on plastic, and suspension culture on agarose have been used to obtain chondrocytes at different development stages. In particular, by culturing dedifferentiated chondrocytes on agarose we were able to obtain a homogeneous population of single cells with hypertrophic chondrocyte traits with regard to hypertrophy, type X collagen production (9), and loss of proliferation capacity (15). Here we reported that when these hypertrophic chondrocytes are kept as adherent cells in the presence of ascorbic acid, a condition that allows a correct stable assembly of the collagen molecules, they become elongated or star shaped, express alkaline phosphatase, and modify their extracellular matrix by stopping synthesis
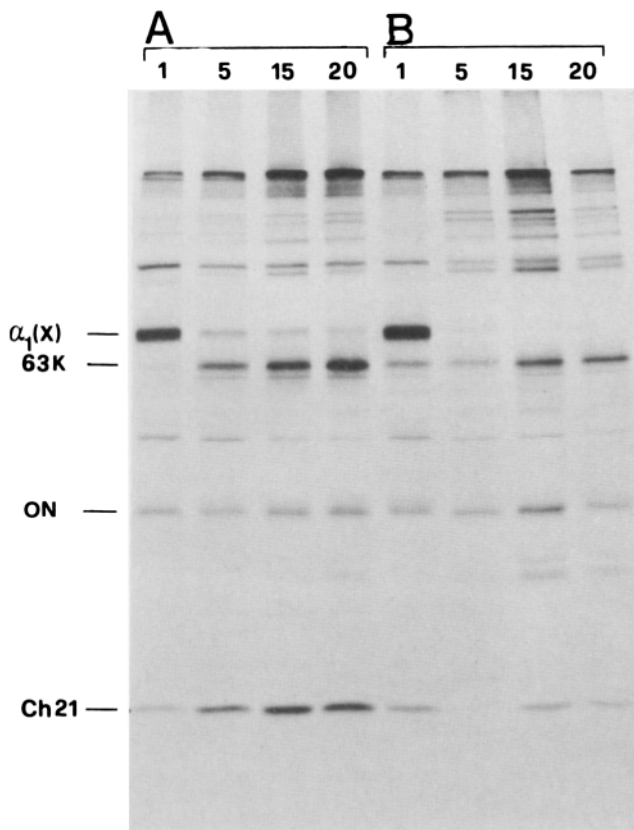
Figure 9. Collagens secreted by cells treated with retinoic acid. Cells were maintained in Coon's modified F12 medium supplemented with 100 μg/ml ascorbic acid and 10 mM β glycerophosphate (*A*) or in the same medium with the further addition of retinoic acid from day 1 to day 5 (*B*). Numbers refer to days in culture. Lanes A_{25} and B_{25} were from the same cultures but were analyzed on a separated gel. At 25 d the only collagen synthesized was type 1 collagen. Aliquots of media were pepsin digested and analyzed on 10% polyacrylamide gel in unreducing conditions.

Figure 8. Proteins secreted by cells treated with retinoic acid. Cells were maintained in Coon's modified F12 medium supplemented with 100 μg/ml ascorbic acid and 10 mM β glycerophosphate (*A*) or in the supplemented medium with the further addition of retinoic acid from day 1 to day 5 (*B*). Numbers refer to days in culture. Proteins were analyzed in unreducing conditions. ON indicates osteonectin.

of cartilage-specific proteoglycans and type II and type X collagens and by initiating synthesis of type I collagen. The Ch21 protein is produced by these cells at all differentiation stages, while the synthesis and secretion of a 63k glycoprotein initiates during the culture and is highly expressed by the osteoblast-like cells. Studies to further characterize this 63-k glycoprotein are in progress in our laboratory. Eventually in the modified matrix mineralization occurs. The addition of β glycerophosphate results in a more rapid mineral deposition but is not an absolute requirement for mineralization. Certainly other factors modulate extracellular matrix mineralization. We observed that the same cells maintained in culture media containing different batches of FCS underwent mineralization at significantly different time after plating (our unpublished results). When the chondrocytes were kept in suspension culture in the absence of ascorbic acid we did not observe the phenotypic transition (not shown).

It is interesting to note that hypertrophic chondrocytes plated in culture dishes after enzymatic removal of their extracellular matrix attach to the dishes and resume proliferation. Cells plated at very low density reach confluence in 10–15 d. Nevertheless, differentiation of hypertrophic chondrocytes to osteoblast-like cells occurs also when cells are directly plated at confluence, a condition where cell multipli-
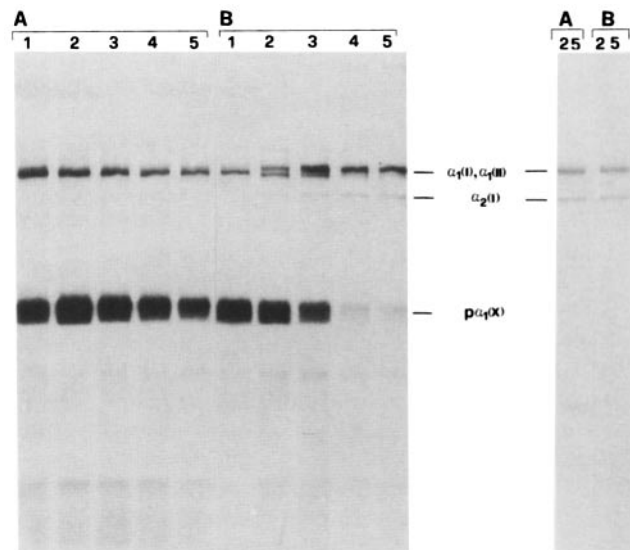
cation is impaired. In agreement with the knowledge that an inverse relationship exists between cell proliferation and expression of differentiation markers, maximum of differentiation was observed in confluent cultures.

In vivo the expression of genes characteristic of osteogenic differentiation have been reported in cells in the zone of hypertrophic chondrocytes and the possibility that mature chondrocytes start to sequentially activate osteogenic marker genes has been considered (30, 38, 48). In this report we have shown that the possibility exists that hypertrophic chondrocytes, depending on their microenvironment, undergo further differentiation and express markers common to bone cells. It is worth noting that the boundary between cartilage and bone in the growth plate of the long bones is characterized by profound rearrangement and modification of the extracellular matrix. Based on our in vitro findings, it is tempting to suggest an active role of hypertrophic chondrocytes during in vivo remodeling of hypertrophic cartilage into bone, but further studies are necessary to prove this hypothesis.

The vitamin A derivative retinoic acid is widely known to affect the differentiation state of a variety of tissues including cartilage. In early studies it has been shown that this compound induces facial and cranial malformations in developing embryos (21, 41). More recent investigations suggest that retinoic acid is actually involved in normal cartilage development. It has been reported that chick embryo limb buds contain endogenous retinoic acid forming a concentration gradient from the anterior to the posterior region of the limb bud (13, 42). Retinoic acid–soaked beads induce striking digit pattern duplication when locally applied to the developing limb bud and mimic the action of a graft of the posterior margin region, named zone of polarizing activity, where a group of mesenchymal cells are localized which specify the

pattern of mesenchymal tissues on the antero-posterior axis (25, 27, 43, 46).

The effect of retinoic acid on the chondrocytes "in vitro" has been extensively studied. Retinoids affect specific characteristics of cultured chondrocytes including cell shape and glycosaminoglycan and protein synthesis. Its mechanism of action is unknown but there are evidences that retinoids cause changes in the synthesis of the extracellular matrix components without any indication of toxic effects (2, 18, 19, 20). In the early phases of chondrocyte differentiation, as it has been reported by several authors (17, 23, 28, 33, 35, 44, 47), retinoic acid either inhibits or induces chondrogenesis. At later stages of chondrocyte differentiation, retinoic acid stimulates chondrogenesis and promotes maturation of chondrocytes to stage II (hypertrophic, type X producing) (26–29) and osteoblast-like cells (type I producing, mineralizing) (this report). It is therefore evident that differentiating chondrocytes exhibit different responses to exogenous retinoic acid depending upon their differentiation stage.

Studies aimed at further investigating the organization of the extracellular matrix and the nature of mineralization in osteoblast-like cell culture both in the presence and in the absence of retinoic acid are currently under investigation.

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