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Parathyroid Hormone [PTH(1–34)] and Parathyroid Hormone–Related Protein [PTHrP(1–34)] Promote Reversion of Hypertrophic Chondrocytes to a Prehypertrophic Proliferating Phenotype and Prevent Terminal Differentiation of Osteoblast-like Cells

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ABSTRACT

The effects of parathyroid hormone/parathyroid hormone–related protein (PTH/PTHrP) on late events in chondrocyte differentiation were investigated by a dual in vitro model where conditions of suspension versus adhesion culturing are permissive either for apoptosis or for the further differentiation of hypertrophic chondrocytes to osteoblast- like cells. Chick embryo hypertrophic chondrocytes maintained in suspension synthesized type II and type X collagen and organized their extracellular matrix, forming a tissue highly reminiscent of true cartilage, which eventually mineralized. The formation of mineralized cartilage was associated with the expression of alkaline phosphatase (ALP), arrest of cell growth, and apoptosis, as observed in growth plates in vivo. In this system, PTH/PTHrP was found to repress type X collagen synthesis, ALP expression, and cartilage matrix mineralization. Cell proliferation was resumed, whereas apoptosis was blocked. Hypertrophic chondrocytes cultured in adherent conditions in the presence of retinoic acid underwent further differentiation to osteoblast-like cells (i.e., they resumed cell proliferation, switched to type I collagen synthesis, and produced a mineralizing bone-like matrix). In this system, PTH addition to culture completely inhibited the expression of ALP and matrix mineralization, whereas cell proliferation and expression of type I collagen were not affected. These data indicate that PTH/PTHrP inhibit both the mineralization of a cartilage-like matrix and apoptosis (mimicked in the suspension culture) and the production of a mineralizing bone-like matrix, characterizing further differentiation of hypertrophic chondrocytes to osteoblasts like cells (mimicked in adhesion culture). Treatment of chondrocyte cultures with PTH/PTHrP reverts cultured cells in states of differentiation earlier than hypertrophic chondrocytes (suspension), or earlier than mineralizing osteoblast-like cells (adhesion). However, withdrawal of hormonal stimulation redirects cells toward their distinct, microenvironment-dependent, terminal differentiation and fate. (J Bone Miner Res 1999;14:1281–1289)

INTRODUCTION

PARATHYROID HORMONE (PTH), a systemic regulator of skeletal metabolism, and parathyroid hormone–related protein (PTHrP), an autocrine/paracrine regulator of tissue development, exert direct effects on skeletal cells, which express a membrane receptor that both ligands can bind.[1–8] Studies on PTHrP and PTHrP receptor knock-out mice demonstrated the importance of PTHrP in chondrocyte differentiation.[5,6,9,10] In developing bone rudiments, PTHrP regulates both the proliferation and the differentiation of chondrocytes via a negative feedback loop involving

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the soluble factor Indian Hedge-hog (Ihh) and its targets Patched (Ptc) and Gli.[10] In the absence of either the PTHrP or its receptor, these animals undergo an aberrant and apparently unmodulated endochondral bone formation. Interestingly, a reciprocal alteration in bone formation was observed in a constitutively active PTH/PTHrP receptor mutant in Jansen-Type Metaphyseal Chondrodysplasia[11] and in mice constitutively expressing the active receptor.[12]

The Indian Hedge-hog/PTHrP signaling system plays a major role in controlling the differentiation of and the interaction between hypertrophic chondrocytes and osteogenic perichondrial cells. We have proposed that all hypertrophic chondrocytes have the capability to differentiate to osteoblast-like cells, but that only chondrocytes at the "borderland" between cartilage and noncartilage osteogenic tissue ("borderline chondrocytes") undergo further differentiation to osteoblast-like cells and participate in the initial bone formation. Other hypertrophic chondrocytes in other cartilage areas are exposed to an inappropriate microenvironment, cannot differentiate to osteoblast-like cells, and undergo apoptosis.[13]

Over the past years, we have developed a culture system, starting from dedifferentiated chondrocytes obtained from chicken embryo tibia, which allowed us to investigate the role of hypertrophic chondrocytes in bone formation.[14] In the presence of ascorbic acid, hypertrophic chondrocytes in suspension culture organize an extracellular matrix highly reminiscent of hypertrophic cartilage "in vivo," express alkaline phosphatase (ALP) and undergo mineralization.[15] When cultured in adhesion culture in the presence of ascorbic acid, hypertrophic chondrocytes progressively decrease synthesis of type II and type X collagen, start synthesizing type I collagen and organize a different extracellular matrix. Under these conditions, cells resume proliferation, change their morphology from a polygonal to an elongated shape, and further differentiate to osteoblast-like cells. ALP is expressed and mineralization occurs on a bone-like matrix. Retinoic acid (RA) can speed up this process in culture.[16,17] Therefore, also "in vitro" different microenvironments can promote different hypertrophic chondrocyte differentiation pathways. Suspension culture favors the formation of a tissue similar to a true hypertrophic cartilage, whereas adhesion culture reproduces "in vitro" the capability of the hypertrophic chondrocytes at the boundary between cartilage and noncartilage tissue of taking part in the initial stage of bone formation and of producing a bone matrix. The aim of our study was to clarify a possible role of PTH/PTHrP on the modulation of hypertrophic chondrocyte differentiation in different culture microenvironments. The amino-terminal(1–34) fragment of PTH/PTHrP is the ligand for the specific receptor and induces "in vivo" the same calcemic effect of the whole hormone(s). When the cells in suspension culture were treated with (1–34) PTH/PTHrP, a very compact extracellular matrix was organized. Cells resumed proliferation and stopped type X collagen synthesis, keeping the synthesis of type II collagen. ALP was not expressed and mineralization did not occur. Control cultures organized a correct extracellular matrix and expressed type II and type X collagen. Eventually cells expressed characteristic apoptotic traits. When hypertrophic chondrocytes were cultured in adhesion in the presence of RA, both PTH-treated cells and controls resumed cell proliferation and further differentiated to osteoblast-like cells; however, PTH-treated cells did not express ALP, and mineralization did not occur.

These data are consistent with the notion that PTH/PTHrP in both culture conditions (adhesion and suspension culture) keep cells in a proliferating status and impair terminal cell differentiation.

MATERIALS AND METHODS

Cell cultures

Dedifferentiated and hypertrophic chondrocytes were obtained from 6-day chick embryo tibiae according to published procedures.[14] Briefly, cells derived from cartilaginous bone were expanded as adherent dedifferentiated cells for 2 weeks and then transferred into suspension culture for 3–4 weeks until a homogeneous population of single isolated hypertrophic chondrocytes was obtained. Hypertrophic chondrocytes were filtered through a nylon filter Nitex 42 μm mesh, to avoid any contamination of still aggregated cells, digested with hyaluronidase (1 mg/ml), and plated in suspension culture on 1% agarose at a concentration of 4×10^5/3-cm dish and as adherent cells at a concentration of $2–3 \times 10^5$ cells/3-cm dish in Coon's modified F12 culture medium containing 10% fetal calf serum. Medium was supplemented with 100 μg/ml ascorbate and 10 mM β-glycerophosphate. When indicated, RA was added to the culture medium at a concentration of 1 × 10–7 M. PTH(1–34) and PTHrP(1–34) were added at a concentration of 5 × 10–8 M. Medium was changed every day for cells cultured in adhesion and every 2 days for cells cultured in suspension, where factors were added every day.

Cell labeling and protein analysis

Cells were labeled with [^{35}S]-methionine as described.[18] Aliquots of culture media were run for protein analysis on SDS-PAGE in unreducing conditions as described.[18] Except otherwise indicated, polyacrylamide gel concentration was 15%.

Histochemistry

ALP activity was determined by using the Histolocal kit 86 from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and mineralization was detected by von Kossa staining.[16] Apoptotic cells in the cartilage formed "in vitro" either in the presence or absence of PTH were detected by TUNEL, ApopTag Plus in Situ Apoptosis Detection Kit from ONCOR (Gaithersburg, MD, U.S.A.).

Immunohistochemistry

Monoclonal antibodies against type X collagen were a generous gift from Dr. Thomas Lynsenmayer (Tufts University, Boston, MA, U.S.A.). Rabbit polyclonal antiserarecognizing chick bone sialoprotein (BSP) (LF-119[19])

were a generous gift from Dr. Larry W. Fisher (CSDB/NIDR, National Institutes of Health, Bethesda, MD, U.S.A.). Rabbit polyclonal antiserum recognizing chicken osteocalcin was a generous gift from Dr. Simon Robins (The Rowett Research Institute, Aberdeen, U.K.).

Deparaffinized sections of "in vitro" formed cartilage both in the presence and absence of PTH were treated with methanol/H_2O_2 (49:1) for 30 minutes to inhibit endogenous peroxidases. Sections were then digested with 1 mg/ml of hyaluronidase for 15 minutes in phosphate-buffered saline (PBS), pH 6, at 37°C, incubated for 20 minutes at room temperature with goat serum and exposed to the specific antiserum for 1 h at room temperature. After two additional washings with PBS, the secondary antibody was added and incubation was continued for 30 minutes at room temperature. For BSP and osteocalcin immunolocalization, a biotinylated-goat anti-rabbit immunoglobulin G (diluted 1:200 in PBS containing goat serum 1:500) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, U.S.A.) was used. For type X collagen immunolocalization, a biotinylated-goat anti-mouse immunoglobulin G (diluted 1:500) was used.

To detect sites of antibody binding, sections were challenged with streptoavidine peroxidase for 30 minutes at room temperature (Jackson Immunoresearch Laboratories, Inc.; diluted 1:500 in PBS containing goat serum 1:500). Sections were then washed with PBS and 50 mM Na acetate, pH 5, and the peroxidase activity was visualized during an incubation in the dark at room temperature by the enzymatic modification of the 3-amino-9-ethylcarbazole substratum according to manufacturer's instructions. Some sections were counterstained with Harris' hematoxylin and mounted with Glycergel, from DAKO Corporation (Carpinteria, CA, U.S.A.). Sections were observed and photographed in a Zeiss Axiophot (Oberkochen, Germany).

DNA measurement

Cell layers were scraped in 0.01% SDS in PBS and digested overnight at 50°C with proteinase K (50–150 μg/ml in 10 mM Tris HCl, pH 7.8, 5 mM EDTA). Alternatively, cells in suspension culture were collected by centrifugation and digested as above. DNA content was determined in a DNA fluorometer from Hoefer (San Francisco, CA, U.S.A.) on a 0.1 ml sample added to 2 ml of dye solution containing 10 mM Tris HCl, pH 7.6, 1 mM EDTA, 0.1 M NaCl, 0.1 μg/ml Hoechst 33258 from Sigma Immunochemicals (St. Louis, MO, U.S.A.).

RESULTS

PTH/PTHrP prevents terminal differentiation and apoptosis of hypertrophic chondrocytes in suspension culture

Single hypertrophic chondrocytes, obtained from dedifferentiated chick embryo chondrocytes as described in the Materials and Methods, were filtered and cultured in the presence of ascorbic acid in suspension on agar for 18 days until mineralization occurred. A parallel culture was additionally supplemented with 5×10^{-8} M PTH(1–34). When filtered and transferred into the ascorbic acid–containing culture, all cells expressed type X collagen by immunohistochemistry criterion (data not shown). In both cultures, cells aggregated and organized their extracellular matrix, although in PTH-treated cultures cell aggregates were much more compact than in control cultures, not PTH supplemented. Cells were labeled with [^{35}S]-methionine at different culture days, cell proliferation determined by DNA measurement, ALP expression by histochemistry, and matrix mineralization by Von Kossa staining. Figure 1 shows the metabolically labeled proteins secreted into the culture medium. PTH repressed type X collagen synthesis starting from day 1; on the contrary, type II collagen expression was not impaired. Interestingly, Ex-FABP, a developmentally regulated lipocalin expressed both "in vitro" and "in vivo" by hypertrophic chondrocytes at the time type X collagen is expressed, was not repressed in PTH-treated culture. Transferrin, another factor expressed by hypertrophic chondrocytes both "in vitro" and "in vivo,"[18] was expressed in control culture at 14 days, but was not detectable, at the same time, in PTH-treated culture. Immunohistological staining of 14-day aggregate sections with antibodies against type X collagen showed that both extracellular matrix and cells were highly positive in control culture aggregates, while aggregates in PTH-treated cultures were negative (Figs. 2A and 2B). In the PTH-treated aggregates, cells were embedded in a more compact extracel-

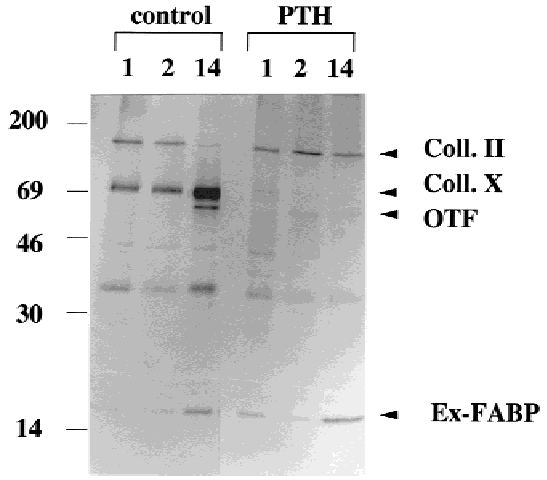

FIG. 1. Proteins released by hypertrophic chondrocytes cultured in suspension. Hypertrophic chondrocytes were cultured in the presence of ascorbic acid in suspension on agar for 18 days until mineralization occurred. Culture medium was either supplemented with 5×10–8 M PTH(1–34) or not supplemented (control). [^{35}S]-methione–labeled proteins secreted by the cells at different times from the beginning of the culture (from 1 to 14 days) were analyzed on SDS-PAGE. Numbers on the left refer to migration of molecular weight markers. Coll II, Type II collagen; coll X, Type X collagen; OTF, ovotransferrin; Ex-FABP, extracellular fatty acid binding protein. The proteins have been identified by immunoprecipitation with specific antibodies.

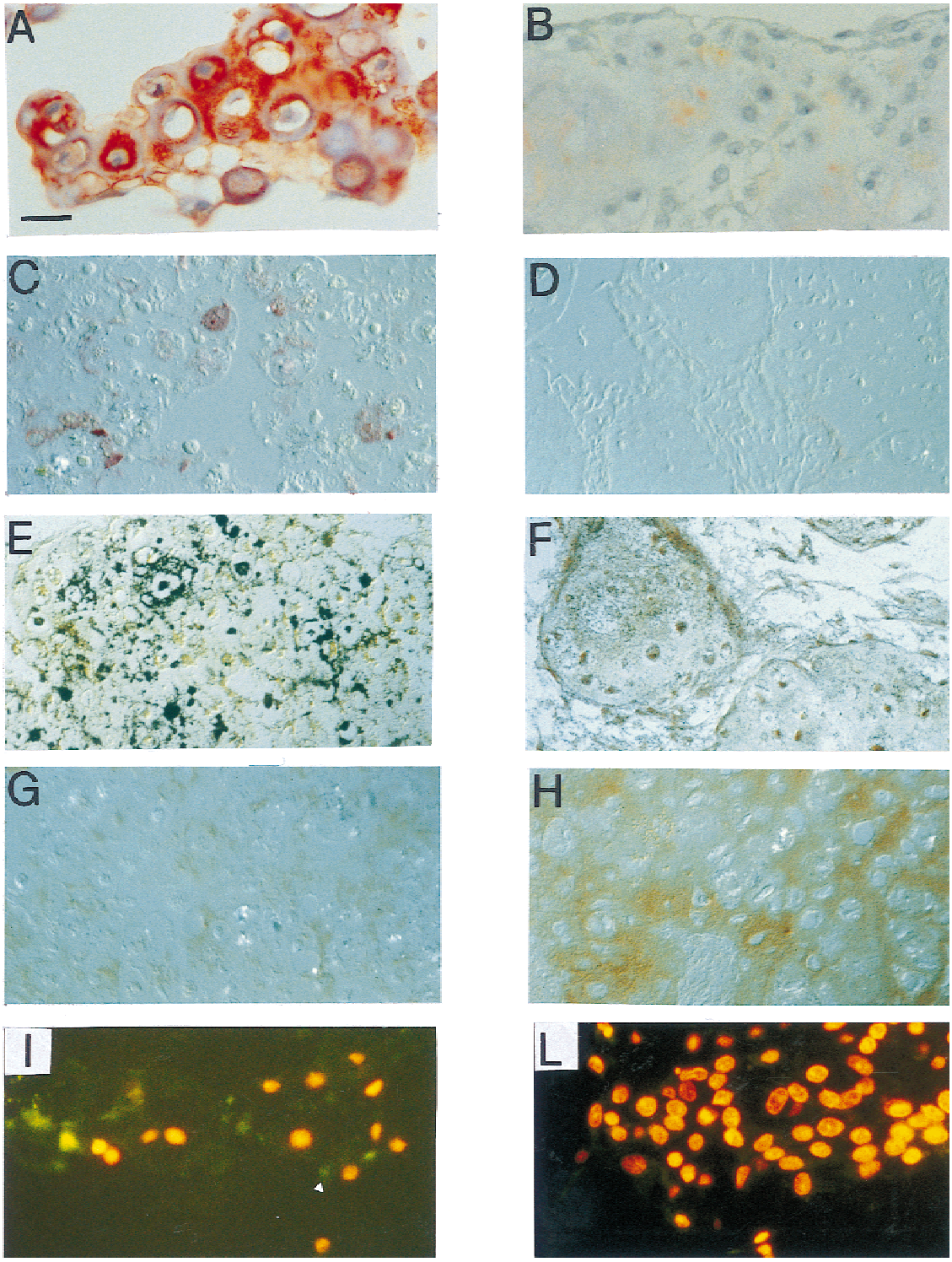

FIG. 2. Histochemistry and immuno-histochemistry of the "in vitro" formed cartilage in suspension culture supplemented with PTH(1–34) (on the right) or not supplemented (on the left) at day 15 of culture, except for BSP (G, H) at day 18 of culture. Immunolocalization of Type X collagen (A, B), the sections were counterstained with hematoxylin. ALP staining (C, D), Von Kossa staining (E, F), and immunolocalization of BSP (G, H). Apoptotic cells stained with TUNEL (I, L), arrows indicate some apoptotic cells in control. Bar = 5 μm.

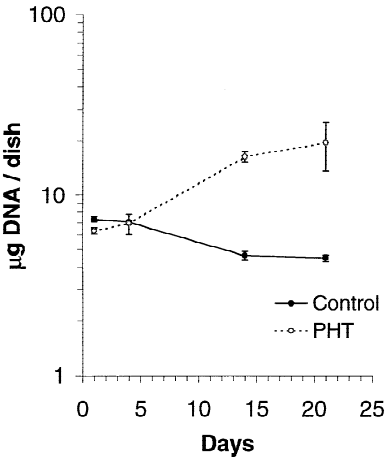

FIG. 3. Growth rate of hypertrophic chondrocytes cultured in suspension. Hypertrophic cells were cultured on 1% agarose as described in the Materials and Methods. Half of the culture dishes were supplemented with 5×10^{-8} M PTH(1–34). At different culture times, DNA content was determined for culture dishes supplemented and not supplemented with PTH. Each time point was tested in duplicate. Different cultures were tested with similar results. The values represent the average of two independent experiments. Bars indicate SD.

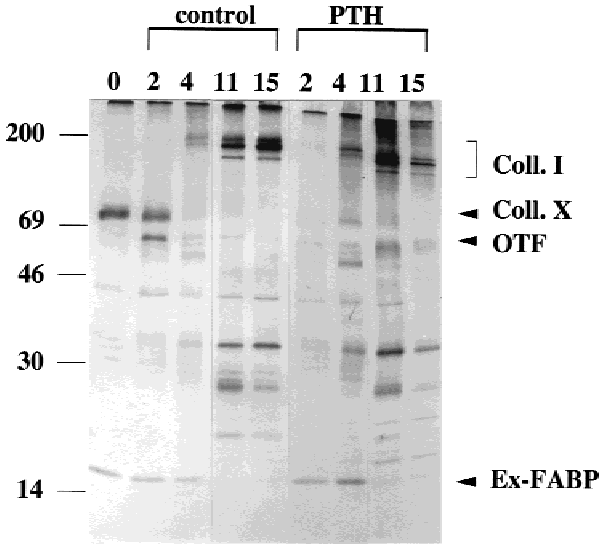

FIG. 4. Proteins released by hypertrophic chondrocytes cultured in adhesion and further differentiating to osteoblast-like cells. Hypertrophic chondrocytes were cultured in the presence of RA and ascorbic acid in adhesion for 15 days. Culture medium was either supplemented with 5×10^{-8} M PTH(1–34) or not supplemented (control). [^{35}S]-methione–labeled proteins secreted by the cells at different times from the beginning of the culture (from 2 to 15 days) were analyzed on SDS-PAGE. Numbers on top refer to days in culture. Lane 0 refers to the beginning of the culture. Numbers on the left refer to migration of molecular weight markers. Coll I, Type I collagen; coll X, Type X collagen; OTF, Ovotransferrin; Ex-FABP, extracellular fatty acid binding protein. The proteins have been identified by immunoprecipitation with specific antibodies.

lular matrix, and the tissue presented a higher cellularity, suggesting an enhanced cell proliferation. The enhancement of cell proliferation in PTH-treated cultures was confirmed by the determination of the cell growth rate based on DNA measurement at different culture times (Fig. 3). Figure 2 also shows that ALP was not expressed in PTH-treated cultures (Fig. 2D) and that mineralization did not occur (Fig. 2F). This was at variance with control culture (Figs. 2C and 2E). Interestingly, BSP, a putative inducer/controller of bone mineralization, was expressed in both culture conditions, but at day 18, its expression was highly enhanced in the culture performed in the presence of PTH (Figs. 2G and 2H). Apoptotic cells were detected in control culture at day 14, while PTH-treated cells occasionally presented few apoptotic cells (Figs. 2I and 2L).

Experiments were performed also by supplementing the culture medium with PTHrP(1–34) fragment. Similar results were obtained (data not shown).

PTH reversibly prevents terminal differentiation of osteoblast-like cells derived from adherent hypertrophic chondrocytes.

Hypertrophic cells were cultured as adherent cells in several culture dishes in the presence of both ascorbic acid and 10^{-7} M RA as described[16,17] and cultured for 15 additional days. Half of the culture dishes was supplemented with 5×10^{-8} M PTH(1–34). As in the case of suspension cultures, cell protein metabolic labeling was performed at different culture days, cell proliferation was determined by DNA measurement, ALP activity by histochemistry, and matrix mineralization by Von Kossa staining. The analysis by PAGE of the metabolically labeled secreted proteins confirmed the further differentiation of hypertrophic chondrocytes to osteoblast-like cells (Fig. 4). In agreement with previously published data,[16] at day 4 type X collagen synthesis had already stopped, and at day 11 type I collagen was clearly the major collagen synthesized. PTH-treated culture behaved similarly, but type X collagen synthesis was already repressed at day 2. Figures 5C and 5D shows that, both in PTH supplemented (Fig. 5D) and non-PTH supplemented cultures (Fig. 5C), at day 7 cells exhibited the elongated morphology characteristic of osteoblast-like cells.[16,17] ALP was expressed by osteoblast-like cells at day 7 in culture and was clearly higher at later times (Fig. 5A). ALP was completely repressed in PTH-treated culture (Fig. 5B). When PTH treatment was stopped at day 7, ALP expression was observed at later time (day 15) (Fig. 5E). A different culture carried out for 21 days (Fig. 6) showed that mineral deposition was already present at day 11 in control culture and was very evident at day 18 (Fig. 6A), while in PTH-treated culture mineral deposition did not occur (Fig. 6B). When PTH treatment was stopped at day 11, cells were able to mineralize at a later time (day 18; Fig. 6C). In both culture conditions, cells proliferated at a similar rate until day 7 (Fig. 7). PTH-treated cells exhibited a higher proliferation rate from day 7 to day 21, possibly because of the mineralization of untreated cells, normally accompanied by an arrest in cell proliferation. Immunolocalization

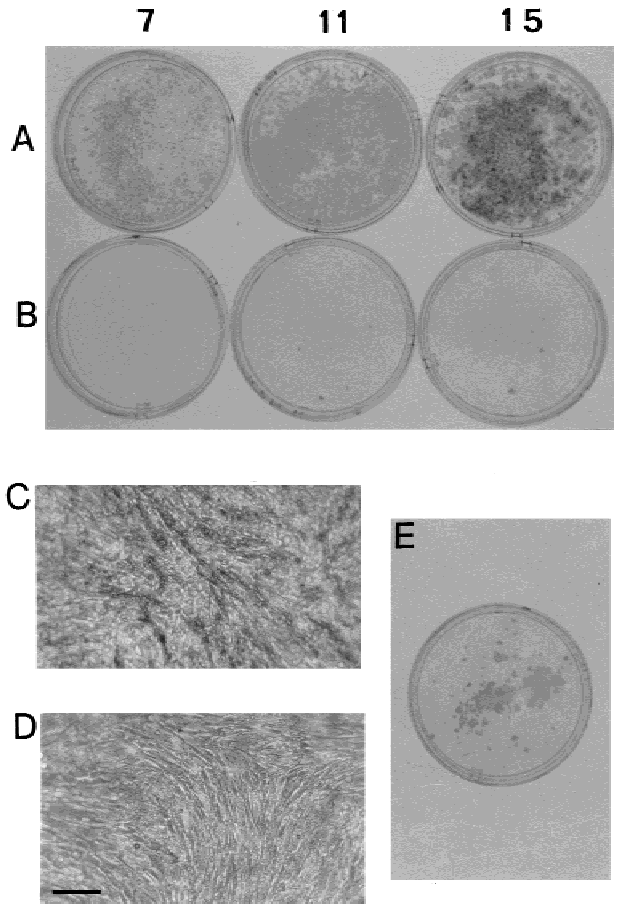

FIG. 5. Further differentiations to osteoblast-like cells of hypertrophic chondrocytes in culture supplemented or not supplemented with PTH. ALP staining of control chondrocytes (A) and PTH-treated cells (B) at 7, 11, and 15 days of culture. Mineralization is evident as opaque precipitate in control cells at 15 days even without Von Kossa staining. Morphology of control (C) and treated (D) cells at 7 days of culture, ALP staining. (E) PTH-treated cells deprived of PTH at day 7 of culture and stained with ALP at 15 days. Bar = 100 μm.

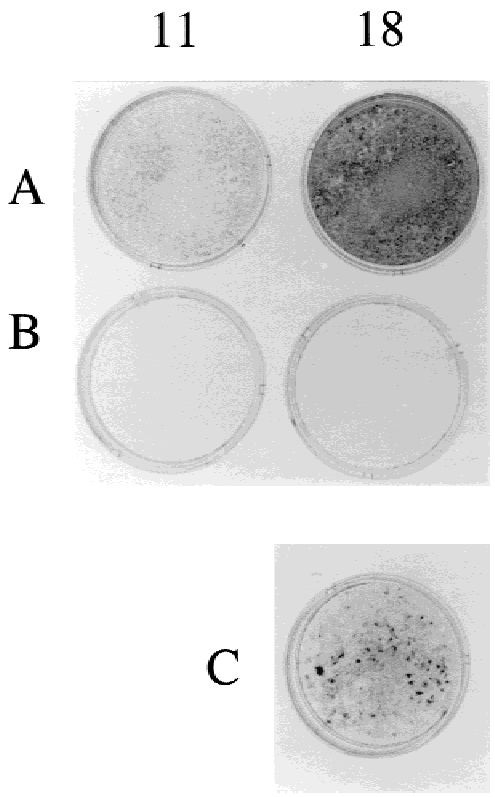

FIG. 6. Mineral deposition in osteoblast-like cells. Von Kossa staining of control chondrocytes (A) and PTH-treated cells (B) at 11 and 18 days. (C) PTH-treated cells from day 0 to day 11 of culture, deprived of PTH at day 11, and stained with Von Kossa at day 18. Numbers refer to days in culture.

of mineralizing cells with anti-osteocalcin antibodies confirmed the osteoblastic nature of these cells (Fig. 8). These data show that PTH keeps osteoblast-like cells in proliferating conditions. Removal of PTH allows cells to reach full differentiation, express ALP, and undergo mineralization. We also performed cultures of hypertrophic chondrocytes plated as adherent cells in the absence of RA. These cultures were treated with PTH up to day 21. Both PTH-treated cells and control untreated cells underwent further differentiation also in the absence of RA, although at a lower rate. At day 21, type I collagen was the only major collagen expressed (data not shown).

DISCUSSION

Hypertrophic chondrocytes play a key role in the autocrine/paracrine PTHrP/Ihh loop controlling cartilage growth and differentiation and the subsequent onset of bone formation.[9,10] Both PTHrP and the PTH/PTHrP receptor (I) are expressed in cartilage in a spatially and temporally defined fashion, at the interface between proliferating and early hypertrophic chondrocytes. It has been proposed that, acting principally as a paracrine factor, PTHrP delays "in vivo" terminal differentiation of endochondral chondrocytes, i.e., cell hypertrophy, matrix mineralization, and apoptosis.[20] According to this observation, PTHrP increases the expression of the apoptosis-controlling protein, Bcl-2 in chondrocytes, both "in vivo" and "in vitro."[21] Apoptosis of hypertrophic chondrocytes certainly takes place in the growth plates,[22–24] and hypertrophic chondrocytes can also differentiate to osteoblast-like cells. Differentiation of hypertrophic chondrocytes to osteoblast-like cells has been unequivocally shown in organ cultures[25–29] and in cell cultures.[16,17] The culture system developed in our laboratory allows us to obtain single hypertrophic cells capable of assembling and generating a tissue strongly resembling hypertrophic cartilage, when kept in suspension cultures supplied with ascorbic acid.[15] Cells grown under these conditions produce and secrete large amounts of transferrin, a molecule chemotactic for endothelial cells, involved in cartilage neoangiogenesis, a key process in new bone formation.[18,30] Upon replating as ad-

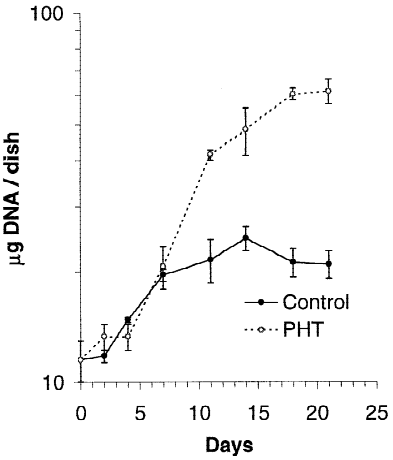

FIG. 7. Growth rate of hypertrophic chondrocytes cultured in adhesion and further differentiating to osteoblast-like cells. Hypertrophic cells were cultured as adherent cells in several culture dishes in the presence of RA as described in the Materials and Methods. Half of the culture dishes were supplemented with 5×10^{-8} M PTH(1–34). At different culture times, DNA content was determined for culture dishes supplemented and not supplemented with PTH. Each time point represents the average of two different dishes, each tested in duplicate. Different cultures were tested with similar results. A representative experiment is shown. Bars indicate SD.

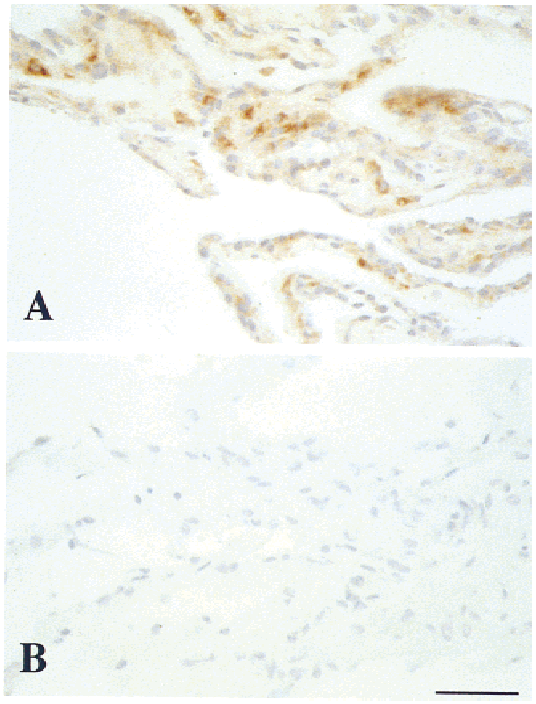

FIG. 8. Immunolocalization of osteocalcin in osteoblast-like cells. Osteoblast-like cells were detached from cell culture dish by scraping the cell layer and included in wax. (A) Antiosteocalcin antibody. (B) Negative control staining (without specific antibody). Bar = 6 µm.

herent cells, single hypertrophic chondrocytes obtained in suspension culture further differentiate into osteoblast-like cells.[16,17] Culture conditions that either prevent or permit cell adhesion thus translate into different microenvironments, which determine alternative "in vitro" differentiation pathways for the same hypertrophic chondrocytes. We have proposed that also "in vivo," the fate of hypertrophic chondrocytes is in fact determined by the different microenvironment.[13] Hypertrophic chondrocytes located in the inner part of cartilage undergo apoptosis in conjunction with cartilage mineralization; early hypertrophic chondrocytes located at the lateral edges of the uninvaded cartilage anlage (borderline chondrocytes), expressing many osteoblastic phenotypic properties, do contribute to the formation of the early bony collar. We believe this function is mirrored "in vitro" as the differentiation of adherent cultured hypertrophic chondrocytes into osteoblast-like cells.

The role of PTH/PTHrP on chondrocyte maturation has also been the object of extensive studies in culture.[31–35] It has been shown that the hormone(s) inhibit(s) chondrocyte differentiation to hypertrophy. When treating hypertrophic chondrocytes with PTH/PTHrP in both conditions, we observed that in suspension culture type X collagen expression is repressed and cells organize a tissue-mimicking proliferating cartilage and are unable to express ALP and mineralize. In adhesion condition in the presence of RA, PTH-treated cells differentiate to osteoblast-like cells that keep proliferating without expression of ALP and without mineralization. It should be noted that cells are still able to express ALP and to mineralize when PTH addition to the culture medium is stopped. Our data demonstrate that PTH/PTHrP can revert hypertrophic chondrocytes to a prehypertrophic proliferating stage, and that terminal differentiation of osteoblast-like cells is also reversibly inhibited in the presence of PTH that keeps osteoblast-like cells in proliferating conditions. It should be noted that our investigation was performed on embryonic cells. Koike et al.[36] have reported that PTH is a potent mitogen for embryonic chondrocytes and that its mitogenic effect disappears after birth. It will be of interest in the future to investigate whether PTH has the same effect on embryo-derived and postnatal hypertrophic chondrocytes.

Several studies have been performed to investigate the mechanism of PTH action on proliferation and differentiation of cultured chondro-/osteogenic cells. So far the studies were very contradictory, thus suggesting that the PTH action varies depending on cell type and experiment conditions.[37–43] PTH markedly inhibited ALP activity and calcification in a dose-dependent manner in case of cultures of the carcinoma-derived chondrogenic cell line ATDC5; these can form cartilage nodules where centrally located cells become hypertrophic chondrocytes expressing type X collagen and eventually mineralize.[43] C3H10T1/2 cells correspond to a comparatively early stage of mesenchymal cell determination and can differentiate toward several connective tissue lineages, including chondrocytes and osteoblasts.[44] Hollnagel et al.[35] have reported that, in C3H10T1/2 cells constitutively expressing a bone morpho-

genetic protein (BMP2) and the PTH/PTHrP receptor, PTH(1–34) stimulates early stages and suppresses late stages of chondro-/osteogenic development. This finding is in agreement with the idea that the opposite effects observed of the PTH treatment are depending on the different developmental stages of the target cells. Although interesting, these data suffer from the fact that they were obtained by investigating the effect of PTH in transformed cell lines. Isogai et al.[42] have shown that PTH positive or negative regulation of primary osteoblast differentiation depends on differentiation stages. In a more recent study, the same group reported that when osteoblastic cells were intermittently exposed to PTH their differentiation to bone-forming ALP positive cells was either inhibited or stimulated, depending on the exposure time. When osteoblasts were instead continuously exposed to the hormone, their differentiation was in all cases inhibited.[45]

The abnormalities in endochondral bone formation observed in mice in which either the PTHrP or the PTH/PTHrP receptor gene were ablated[5,6] represent an extremely interesting key to the function and fate of hypertrophic chondrocytes. These animals exhibit a premature or aberrant perichondral osteogenesis not paralleled by an equally premature or aberrant membranous ossification, presumably due to an accelerated differentiation of chondrocytes in bone. Changes in skeletal cartilages in PTH-depleted mice can be explained as an effect of the removal of a brake to chondrocyte late differentiation events. Since PTHrP delays apoptosis of hypertrophic chondrocytes by regulating the expression of bcl-2, accelerated apoptosis of central growth plate hypertrophic chondrocytes may be invoked as an explanation for the growth plate abnormalities and shortening of long bones in these mice. We have shown here that PTH/PTHrP also exert a negative regulatory effect on the further differentiation of hypertrophic chondrocytes to osteoblast-like cells in culture. We have proposed that subperichondral hypertrophic chondrocytes might directly participate in the formation of the bony collar by converting "in vivo" to an osteoblast-like phenotype.[13] The bony collar is a peculiar variety of membranous ossification and is also remarkably accelerated in PTHrP-depleted mice. It is conceivable that enhanced differentiation into osteoblast-like cells upon removal of the PTHrP regulatory influence may be reflected into the enhanced formation of subperichondral bone observed in these mice.

In a recent review,[13] we discussed the biological significance of hypertrophic cartilage and of the associated expression of osteoblast-like phenotypic traits and tried to reconcile the described properties of hypertrophic chondrocytes with the processes of tissue formation and tissue removal occurring during bone organogenesis. Hypertrophic chondrocytes allow cartilage removal by apoptosing in the growth plate; they promote bone formation at the bony collar. Our data on the effect of PTH on hypertrophic chondrocytes and on hypertrophic chondrocyte-derived osteoblast-like cells suggest that PTH/PTHrP can interfere with both cartilage removal and initial perichondral bone formation. The hormone has in fact the capability of preventing terminal differentiation of hypertrophic chondrocytes and osteoblast-like cells and of keeping both cell types in a proliferating status.

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