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BMP-6 Is an Autocrine Stimulator of Chondrocyte Differentiation

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ABSTRACT

While parathyroid hormone-related protein (PTHrP) has been characterized as an important negative regulator of chondrocyte maturation in the growth plate, the autocrine or paracrine factors that stimulate chondrocyte maturation are not well characterized. Cephalic sternal chondrocytes were isolated from 13-day embryos, and the role of bone morphogenetic protein-6 (BMP-6) as a positive regulator of chondrocyte maturation was examined in monolayer cultures. Progressive maturation, which was accelerated in the presence of ascorbate, occurred in the cultures. During maturation, the cultures expressed high levels of BMP-6 mRNA which preceded the induction of type X collagen mRNA. Treatment of the cultures with PTHrP (10^{-7} M) at the time of plating completely abolished BMP-6 and type X collagen mRNA expression. Removal of PTHrP after 6 days was followed by the rapid (within 24 h) expression of BMP-6 and type X collagen mRNA, with BMP-6 again preceding type X collagen expression. The addition of exogenous BMP-6 (100 ng/ml) to the cultures accelerated the maturation process both in the presence and absence of ascorbate and resulted in the highest levels of type X collagen. When exogenous BMP-6 was added to PTHrP containing cultures, maturation occurred with the expression of high levels of type X collagen, despite the presence of PTHrP in the cultures. Furthermore, BMP-6 did not stimulate expression of its own mRNA in the PTHrP treated cultures, but it did stimulate the expression of Indian hedgehog (Ihh) mRNA. These latter findings suggest that while PTHrP directly inhibits BMP-6, it indirectly regulates Ihh expression through BMP-6. Other phenotypic changes associated with chondrocyte differentiation were also stimulated by BMP-6, including increased alkaline phosphatase activity and decreased proliferation. The results suggest that BMP-6 is an autocrine factor that initiates chondrocyte maturation and that PTHrP may prevent maturation by inhibiting the expression of BMP-6. (J Bone Miner Res 1999;14:475–482)

INTRODUCTION

THE FORMATION AND GROWTH of long bones during vertebrate development depends upon a remarkable series of biochemical and morphological changes that are controlled by secreted paracrine and autocrine factors. This process of endochondral ossification requires the formation and maturation of a cartilage template which develops from condensations of mesenchymal cells that differentiate into chondrocytes.[1] Newly formed chondrocytes undergo a period of proliferation during which they secrete a matrix rich in proteoglycans and type II collagen.[1,2] After proliferating, the chondrocytes begin a phase of maturation marked by an increase in cellular volume and the expression of phenotypic markers of hypertrophy, including alkaline phosphatase (ALP) activity and type X collagen.[3] Finally, the mature cartilage matrix surrounding the hypertrophic chondrocytes undergoes calcification, resorption, and replacement with bone.[4–6] These phases of endochondral ossification can be mimicked in the postnatal animal by implanting bone morphogenetic proteins (BMPs) along with a collagen carrier in an ectopic site.[7–9] This remarkable ability of the BMPs to induce ectopic bone has led to investigations of their role in regulating normal bone formation.

The transition of chondrocytes from a state of proliferation to maturation is a critical step during endochondral ossification. Chondrocyte proliferation supplies the carti-

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laginous template with additional cells as the template enlarges during normal growth. Several important mitogens, including transforming growth factor-β and insulin-like growth factor-I promote cell division in the proliferative phase of chondrocyte differentiation.[10] Parathyroid hormone-related protein (PTHrP) acts as a mitogen in chondrocytes[11] but also controls the transition from proliferation to maturation. PTHrP is a potent inhibitor of chondrocyte maturation[12–14] and is indirectly stimulated by Indian hedgehog (Ihh), a protein secreted by hypertrophic chondrocytes.[15] PTHrP reciprocally inhibits Ihh expression, and together PTHrP and Ihh form a negative feedback loop that controls the number of chondrocytes entering the hypertrophic phase.[15]

In mice with homozygous disruptions of the PTHrP gene or disruption of the PTHrP receptor, chondrocytes undergo accelerated maturation, leading to disorganization of the cytoarchitecture of the growth plate and skeletal malformation.[12,13] While these findings suggest that chondrocytes within the growth plate are primed to complete the maturation process, the factors that commit chondrocytes to undergo this rapid differentiation are unknown. Thyroid hormone, ascorbic acid, vitamin D, and retinoids are extrinsic agents that can stimulate chondrocyte differentiation.[16–20] However, the mechanism through which these agents regulate chondrocyte phenotype are unknown. Our laboratory has recently demonstrated that BMP-7 is expressed at low levels in growth plate chondrocytes and that its synthesis is stimulated by retinoic acid in these cells.[21] Since other studies have demonstrated that BMPs are potent stimulators chondrocyte maturation, endogenous BMPs may be the factors that commit and drive chondrocytes in the growth plate toward completion of the maturation process.

Prior studies have demonstrated high levels of expression of BMP-6 in the developing growth plate.[15,22] This is in contrast to BMP-2, BMP-4, and BMP-7, which are expressed at lower levels or are absent in the growth plate.[9,21–23] BMP-6 expression is highest in prehypertrophic and hypertrophic chondrocytes.[15,22] However, the role of BMP-6 in the growth plate is unknown. To gain insight into the role that BMP-6 plays in chondrocyte maturation, we have utilized chondrocytes isolated from day 12–13 chicken embryo sterna. The chondrocytes slowly mature over a 6–8 day culture period. This model allows us to examine the events that trigger chondrocyte maturation. Our results suggest that BMP-6 is an autocrine factor that initiates chondrocyte maturation and may be an important mechanistic component of the PTHrP mediated inhibition of maturation.

MATERIALS AND METHODS

Chondrocyte cell culture

Embryonic cephalic sternal chondrocytes (day 13) were prepared and cultured as described.[16] After isolation and primary culture for 5–7 days, floating cells were plated in secondary cultures at 2.5×10^5 cells/cm^2 in Dulbecco's modified Eagle's medium containing 10% NuSerum IV (Collaborative Biomedical, Bedford, MA, U.S.A.), 4 U/ml of hyaluronidase (Sigma Chemical, St. Louis, MO, U.S.A.), and 2 mM L-glutamate (Sigma). Chondrocytes were cultured in the presence or absence of ascorbate (increasing doses 10–50 μg/ml as described[16]). PTHrP (10^{-7} M; Bachem AG, Bubendorf, Switzerland) was added to the cultures in some experiments at the time of plating in serum-containing medium in the presence or absence of BMP-6 (100 ng/ml; Genetics Institute, Cambridge, MA, U.S.A.).

Analysis of mRNA levels

mRNA was isolated from cultures using RNAzol (Teltest, Friendswood, TX, U.S.A.). Northern analysis was performed using denaturing formaldehyde/agarose gels as described[24] with the modification that ethidium bromide (40 mg final concentration) was added to the RNA samples before loading. This step facilitates the visualization of the ribosomal bands and does not interfere with the transfer or hybridization protocols.

Double-stranded cDNA fragments encoding chick Ihh and BMP-6 were generously provided by C. Tabin[15] and B. Houston,[25] respectively. ^{32}P-labeled cDNA probes were synthesized using random priming (Gibco/BRL, Grand Island, NY, U.S.A.). A synthetic type X oligonucleotide was end-labeled as described.[24] The Ihh and BMP-6 probes were hybridized at 59°C and 52°C, respectively, in solutions containing 2× SSC (3 M NaCl, 0.15 M Citrate) and 25% formamide. The type X oligonucleotide probe was hybridized at 44°C in a solution containing 2× SSC without formamide. The washes for all probes were performed in solutions composed of 0.1× SSC and 0.1% SDS at 60°C for Ihh and BMP-6 and 42°C for type X. Probed membranes were exposed to Kodak XAR film (Rochester, NY, U.S.A.).

ALP activity

ALP activity was measured as previously described.[26] Culture medium was aspirated from chondrocytes cultured in 24-well plates, which then were rinsed with 150 mM NaCl. One milliliter of reaction buffer containing 0.25 M 2-methyl-2-amino propanol, 1 mM magnesium chloride, and 2.5 mg/ml of p-nitrophenyl phosphate (Sigma) at pH 10.3 was added to the wells at 37°C. The reaction was stopped after 5 minutes by the addition of 0.5 ml. of 0.3 M Na_3PO_4 (pH 12.3). The ALP activity was determined spectrophotometrically at 410 nm by comparison with standard solutions of p-nitrophenol and an appropriate blank.

[^{3}H]thymidine incorporation

[^{3}H]thymidine incorporation was determined as previously described.[10] Briefly, the sternal chondrocyte cultures in 24-well plates were labeled with 8 μCi of [^{3}H]thymidine (40 Ci/mmol) (New England Nuclear, Boston, MA, U.S.A.) in the presence of 5 μM unlabeled thymidine added to the existing culture medium. After 4 h of incubation, the radioactive medium was discarded, the cells were removed,

and the DNA was precipitated by the addition of perchloric acid. Following centrifugation, the supernatant was discarded and the pellet was redissolved in NaOH. Acid insoluble radioactivity was determined by liquid scintillation spectrometry. Standards of the radiolabeled medium were prepared for the direct estimation of the specific activity of the labeling solution.

Crystal violet assay

During the culture period, the cells proliferated at different rates according to the factors that were added. Since ALP activity and [^{3}H]thymidine activity were measured in 2- to 6-day-old cultures, we controlled for changes in total cell number by staining parallel cultures with crystal violet as described.[27] Briefly, chondrocytes were plated in 24-well plates and treated and harvested in parallel with the ALP of [^{3}H]thymidine cultures. At the termination of the culture period the cells were fixed in 1% glutaraldehyde and air dried. 0.02% crystal violet (Sigma) was added to the wells for 30 minutes, the wells were rinsed thoroughly with water to remove excess crystal violet and air dried. Seventy percent ethanol was added to the wells and allowed to incubate for 20 minutes at room temperature to release the crystal violet, and the absorbance was read at 578 nm.

Volume regulation experiments

Secondary cultures of sternal chondrocytes were prepared as described above. Cells were plated at 50,000 cells per well in plastic multichamber microscope slides. To control for changes in cell shape when the cells were in monolayer culture, measurement of cell diameter was performed in suspension and monolayer cultures. For monolayer culture the cells were plated with hyaluronidase to promote attachment as described above. In cultures in which hyaluronidase was omitted, the cells remained in suspension throughout the culture period. The chondrocytes were cultured for 3 days the presence or absence of PTHrP (10^{-7} M) or BMP-6 (100 ng/ml). At the end of the culture period, multiple random fields of the chondrocyte cultures were digitally photographed at ×200 magnification using an American Optical Biostar phase contrast microscope with an attached JVC GR-DV1 U digital video camera. A calibrated reticle was used during the digital photography to allow accurate cell diameter quantitation. The digitized photographs were analyzed using a Power Mac 8100 computer, with 20–60 cells being analyzed for each measurement.

Statistical analysis

Results from ALP, [^{3}H]thymidine, and volume regulation experiments were analyzed using a paired analysis of variance (StatView 4.5; Abacus, Berkeley, CA, U.S.A.). Experimental treatments were compared with the ascorbate treated control. p values are described in the figures.

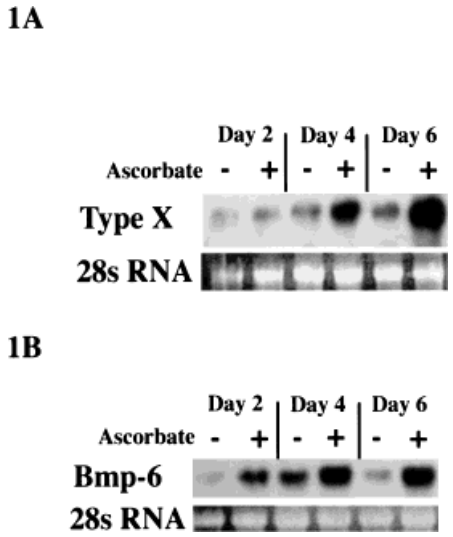

FIG. 1. Northern blots of RNA isolated from sternal chondrocytes treated with or without ascorbate were probed for the expression of type X collagen (A), or BMP-6 (B). Ethidium bromide staining of the 28s ribosomal band was used to control for differences in loading between lanes.

RESULTS

The molecular and cellular events that occur during chondrocyte differentiation were examined using an in vitro embryonic sternal chondrocyte model. Chondrocytes isolated from the cephalic portion of 13-day chick sternum and placed in secondary monolayer culture slowly undergo maturation. mRNA expression at various time points was investigated by Northern blotting. As previously reported,[17,28] the expression of type X collagen was increased by the addition of ascorbate (Fig. 1A), while the levels of type II collagen were relatively constant over time, but slightly decreased in the absence of ascorbate (data not shown). BMP-6 mRNA was expressed early in the culture period, gradually increased over time, and preceded the appearance of type X collagen. BMP-6 expression appeared to reach a peak after 4 days in ascorbate-treated cultures, while type X collagen mRNA expression peaked at 6 days (Fig. 1B). These findings are consistent with the previously reported expression of BMP-6 mRNA in situ,[15,22] and they suggest that BMP-6 may be involved in the process of chondrocyte differentiation.

To investigate a possible role for endogenous BMP-6 in chondrocyte differentiation, cultures were treated with PTHrP at the time of plating and the expression of BMP-6 and type X collagen was examined (Fig. 2). As expected, PTHrP (10^{-7} M) suppressed chondrocyte maturation and prevented the appearance of type X collagen expression. PTHrP also completely prevented BMP-6 expression in chondrocyte cultures. To examine the temporal sequence of BMP-6 and type X collagen expression, we treated ascorbate-containing chondrocyte cultures with PTHrP for 6 days, and then removed PTHrP from the culture medium

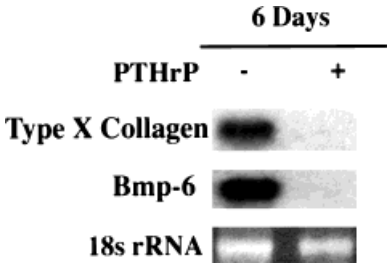

FIG. 2. Northern blots of RNA isolated from sternal chondrocytes treated with ascorbate with or without PTHrP for 6 days. Blots were probed for type X collagen or BMP-6 expression. Ethidium bromide staining of the 18s ribosomal band was used to control for differences in loading between lanes.

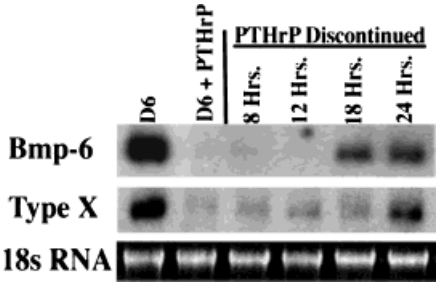

FIG. 3. Sternal chondrocytes were cultured with ascorbate with or without PTHrP for 6 days. After 6 days of PTHrP treatment, PTHrP was discontinued and RNA was collected at 8, 12, 18, and 24 h. RNA was transferred to Northern blots and probed for BMP-6 and type X collagen. Ethidium bromide staining of the 18s ribosomal band was used to control for differences in loading between lanes.

allowing chondrocyte maturation to proceed (Fig. 3). Eighteen hours following PTHrP removal, there is a sharp rise in BMP-6 expression and the level is similar to that observed in 6-day cultures without prior exposure to PTHrP. In contrast, an increase in type X collagen expression begins at 24 h and the levels are still much lower than present in the cultures without prior exposure to PTHrP. BMP-6 expression thus precedes the onset of type X expression, suggesting that BMP-6 may be important during the early events of chondrocyte maturation.

To determine whether BMP-6 plays an active role in driving the maturation process or is simply a marker of the differentiated chondrocyte, BMP-6 was added to chondrocyte cultures in the presence and absence of ascorbate (Fig. 4). BMP-6 accelerated the expression of type X collagen in both ascorbate- and non–ascorbate-treated cultures, and the highest levels of type X collagen expression were observed in cultures treated with BMP-6 and ascorbate. These findings demonstrate that BMP-6 stimulates the differentiation process in the sternal chondrocyte cultures.

The effect of BMP-6 on other aspects of chondrocyte differentiation, including the expression of AlP activity, cellular size, and cellular proliferation were investigated. The addition of 100 ng/ml of BMP-6 increased ALP activity in

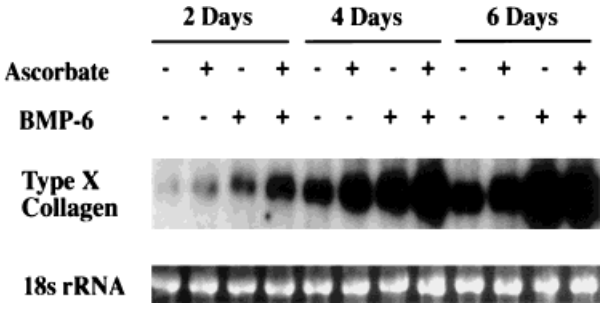

FIG. 4. Northern blots of RNA isolated from sternal chondrocytes treated with (+), or without (–), ascorbate and BMP-6. The blot was probed with type X collagen, and ethidium bromide staining of the 18s ribosomal band was used to control for differences in loading between lanes.

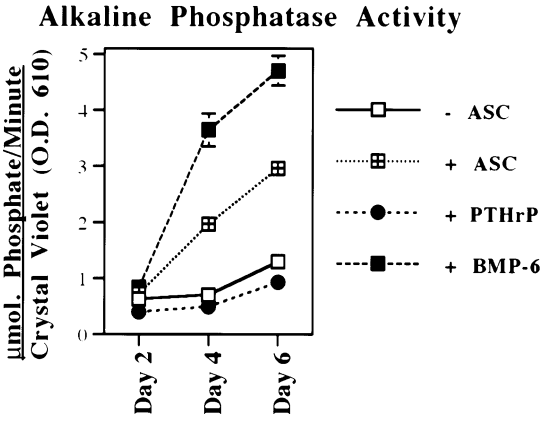

FIG. 5. Sternal chondrocytes were plated in 24-well plates and treated without ascorbate (– Asc), with ascorbate (+ Asc), with ascorbate and PTHrP (+ Asc + PTHrP), or with ascorbate and BMP-6 (+ Asc + BMP-6). Duplicate cultures were assayed for ALP activity or crystal violet staining. Results are plotted as a ratio of ALP to crystal violet staining. All values were statistically significant. Day 2 ($p < 0.05$), day 4 ($p < 0.005$), and day 6 ($p < 0.025$).

the cultures as compared with ascorbate-treated controls (Fig. 5). In contrast, PTHrP suppressed ALP activity in these cell cultures. Similarly, BMP-6 increased, while PTHrP decreased, the mean cellular diameter (Fig. 6), with similar changes in both monolayer and suspension cultures. In addition to stimulating the markers of hypertrophy, BMP-6 suppressed the rate of proliferation in the chondrocytes indicating that addition of BMP-6 shifted the cultures toward a more differentiated state (Fig. 7).

Since BMP-6, expressed at high levels, temporally precedes type X collagen expression and actively stimulates a differentiated phenotype, experiments were performed to investigate if PTHrP may exert its inhibitory effects on the maturation process through a suppressive effect on BMP-6 synthesis. Chondrocytes were plated in the presence of PTHrP to suppress endogenous BMP-6 production and completely prevent maturation. However, when exogenous

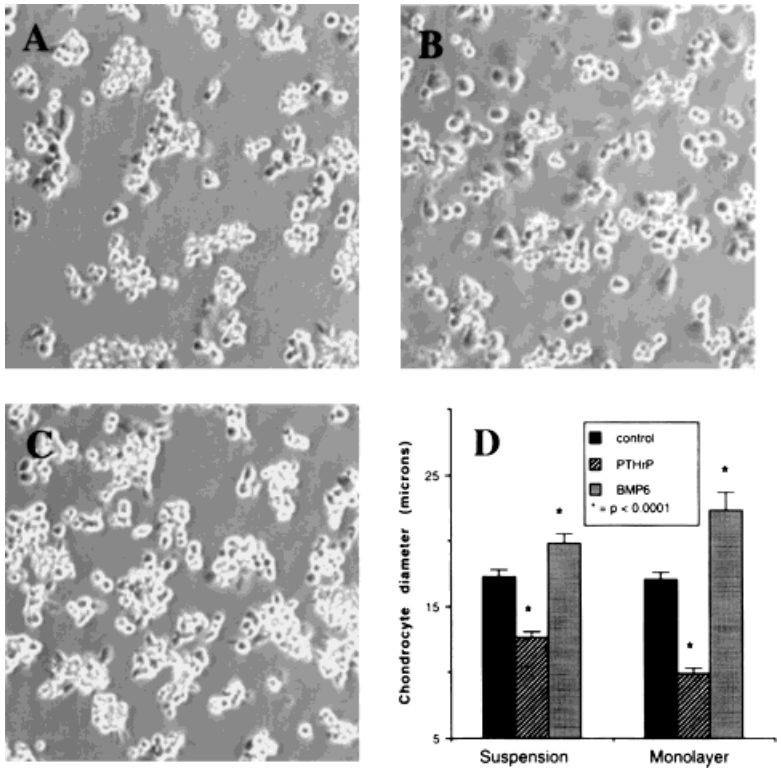

FIG. 6. (A–C) Phase contrast photomicrographs (×100) of sternal chondrocytes cultured for 3 days in monolayer culture and treated with ascorbate alone (A), or ascorbate plus BMP-6 (B), or ascorbate plus PTHrP (C). Quantitation of differences in cell size were quantitated and are displayed in (D). Quantitation was performed in both monolayer culture (with hyaluronidase) and suspension cultures (without hyaluronidase) by analyzing the cellular diameters of digitized images of 20–60 cells per measurement.

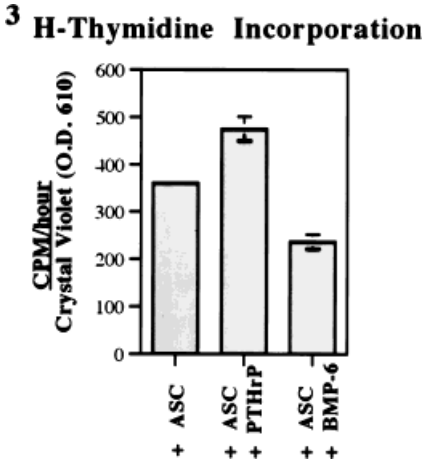

FIG. 7. [^{3}H]thymidine incorporation was assayed in sternal chondrocytes cultured for 3 days in 24-well plates. The chondrocytes were treated with ascorbate (+ Asc), with ascorbate and PTHrP (+ Asc + PTHrP), or with ascorbate and BMP-6 (+ Asc + BMP-6). Duplicate cultures were assayed for [^{3}H]thymidine incorporation and crystal violet staining to control for differences in cell number between samples. All values were statistically significant when compared with the ascorbate control. PTHrP + ascorbate ($p < 0.05$): BMP-6 + ascorbate ($p < 0.005$).

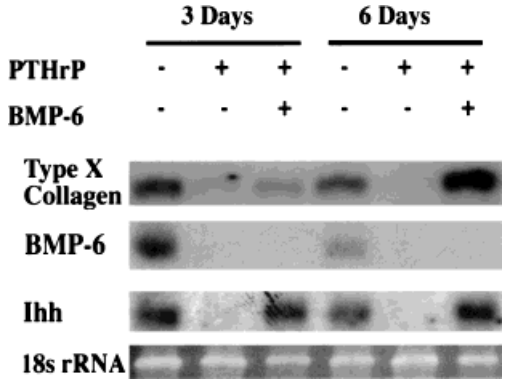

FIG. 8. Northern blots of RNA isolated from sternal chondrocytes treated with ascorbate with (+), or without (–), PTHrP and/or BMP-6. Blots were probed for the expression of type X collagen, BMP-6 and Indian hedgehog (Ihh). Ethidium bromide staining of the 18s ribosomal band was used to control for differences in loading between lanes.

BMP-6 (100 ng/ml) was added to PTHrP containing cultures the chondrocytes matured as evidenced by the expression of type X collagen in 3-day cultures and high levels of type X expression in 6-day cultures (Fig. 8). BMP-6 is thus able to override the suppressive effects of PTHrP on chondrocyte maturation. These findings confirm the potent stimulatory effects of BMP-6 on sternal chondrocyte maturation and suggest that PTHrP may mediate its effects on chondrocyte differentiation, in part, through suppression of BMP-6 expression.

The addition of BMP-6 to PTHrP-treated cultures did not alter the suppression of BMP-6 mRNA, suggesting that BMP-6 expression is independent of the maturational state

of the cells and may be regulated directly by PTHrP (Fig. 8). In contrast, the addition of exogenous BMP-6 to PTHrP-treated cultures resulted in a high level of Ihh mRNA expression (Fig. 8). These latter findings suggest that Ihh is not directly regulated by PTHrP, but instead is either under the direct regulation of BMP-6 or is dependent upon the maturational state of the cells. Since BMP-6 regulates the maturational state of these cells and also is associated with an increase in Ihh mRNA expression, BMP-6 appears to be an intermediary factor in the PTHrP/Ihh regulatory loop which has been shown to regulate chondrocyte maturation.[15]

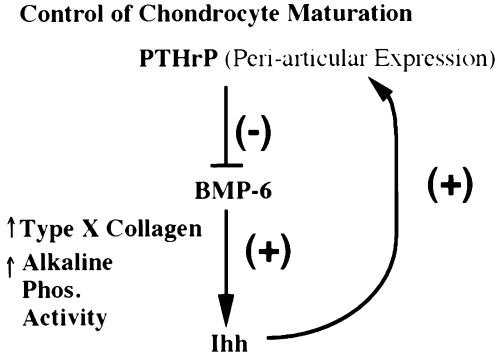

FIG. 9. BMP-6 is an intermediary in the PTHrP/Ihh signaling pathway. In the absence of PTHrP, BMP-6 is expressed by growth plate chondrocytes, inducing chondrocyte terminal differentiation and the expression of Ihh. Ihh stimulates PTHrP expression in the periarticular region leading to a reduction in BMP-6 and an inhibition of terminal differentiation. With a decrease in terminal differentiation, Ihh levels fall, accompanied by a decline in PTHrP expression and a new round of terminal differentiation stimulated by increased expression of BMP-6 in the growth plate.

DISCUSSION

Although high levels of BMP-6 have been previously identified in the growth plate, the role that this factor plays in the events of endochondral ossification has not been elucidated. The present study demonstrates that BMP-6 expression precedes the onset of maturation and that the expression of BMP-6 is inhibited by PTHrP, an agent that blocks maturation. In addition, exogenous BMP-6 accelerated the process of maturation, and these changes occurred even in the presence of PTHrP. Our results suggest that BMP-6 induces chondrocyte hypertrophy during endochondral ossification. This pattern of early expression of BMP-6 fits with a role as an initiator of chondrocyte differentiation in the growth plate.

The expression of BMP-6 prior to the onset of type X collagen expression suggests that BMP-6 may regulate the onset of chondrocyte maturation, and its expression throughout chondrocyte hypertrophy indicates that its expression may be necessary to sustain the hypertrophic phenotype. Similar temporal and spatial patterns of BMP-6 expression have been shown by in situ hybridization of chick and mouse growth plates.[15,22] BMP-6 enhanced the expression of all markers of maturation examined, including type X collagen, ALP activity, and cell size. Furthermore, BMP-6 decreased the rate of proliferation, also consistent with a more differentiated phenotype.

Prior work has demonstrated a central role for PTHrP in the regulation of maturation.[15] According to the current model, PTHrP is stimulated by Ihh and prevents chondrocyte maturation. Since Ihh is produced by prehypertrophic cells, and its expression is dependent upon chondrocyte maturation, PTHrP causes a decrease in Ihh expressing cells. This ultimately results in a decrease in PTHrP levels. Chondrocytes then escape the inhibitory influence of PTHrP and complete another round of maturation, thereby causing a subsequent increase in Ihh which again functions in a feedback loop to dampen chondrocyte maturation.

Although PTHrP blocks chondrocyte maturation at a stage prior to Ihh expression, the mechanism of the block on maturation is unknown. The current data suggest that BMP-6 is a positive regulator of chondrocyte differentiation in the growth plate and is an important intermediary in the PTHrP/Ihh signaling pathway. This is supported by the findings that: (1) PTHrP is a potent inhibitor of BMP-6; (2) exogenous BMP-6 over-rides the inhibitory effect of PTHrP on chondrocyte maturation; (3) exogenous BMP-6 does not up-regulate its own mRNA levels in PTHrP treated cultures, demonstrating direct inhibition of BMP-6 gene expression by PTHrP; and (4) BMP-6 up-regulates Ihh mRNA levels, despite PTHrP treatment, demonstrating that the PTHrP-mediated inhibition of Ihh is indirect.

In this revised model based upon these observations, PTHrP is produced in response to Ihh and blocks the synthesis of BMP-6. In the absence of BMP-6, cells do not undergo maturation and thus do not develop a Ihh expressing phenotype. With a decrease in Ihh levels, PTHrP expression also falls with a subsequent increase in BMP-6 levels. In the presence of BMP-6, chondrocytes complete the maturation process with the development of Ihh producing cells which signal another round of PTHrP synthesis (Fig. 9). The influence of BMP-6 on Ihh is either direct or is associated with the development of a more mature phenotype. Although the role of BMP-6 requires further investigation, the model accounts for a positive regulatory influence on chondrocyte maturation and is consistent with the findings of the present investigation.

The signaling mechanisms by which BMP-6 induces chondrocyte maturation are unclear. The BMPs are known to signal through a heterodimeric receptor complex containing type I and type II receptors, with the specificity of interaction mediated by the type I receptor. While BMP-2, BMP-4, and BMP-7 each bind to both type IA and IB BMP receptors and BMP-7 also binds to the activin receptor, the receptor binding specificity of BMP-6 is unknown. Zou et al. recently published a report demonstrating expression of the BMP type IA receptor in prehypertrophic chondrocytes and show that activation of the IA receptor induces PTHrP expression in periarticular cells.[1] They propose that BMP-2, BMP-4, and BMP-7, from perichondrial cells,[29–31] inhibit maturation through activation of the type IA receptor and a subsequent increase in PTHrP levels.

In contrast, our results suggest that BMP-6 triggers a signaling pathway that leads to chondrocyte maturation. These findings are consistent with prior studies demonstrating that BMPs stimulate maturational changes in cultured chondrocytes. BMP-7 has been found to stimulate type X collagen in embryonic chick caudal chondrocytes and in rat calvarial cell cultures.[32,33] Similarly, BMP-2 has recently been shown to stimulate type X collagen and ALP activity in chicken cephalic sternal chondrocytes.[17] In rabbit costal chondrocytes, BMP-2 and BMP-3 stimulate ALP activity, another marker of chondrocyte differentiation.[34] Enomoto-Iwamoto et al. have recently shown that sternal chondrocyte maturation is prevented in cultures expressing a dominant negative type II BMP receptors.[35] Collectively, the findings suggest that BMP-6 is a member of a family of proteins that stimulate chondrocyte maturation.

Our studies elucidate a mechanism by which chondrocytes may progress through their developmental program. We demonstrate that BMP-6 is expressed by maturing chondrocytes and acts in an autocrine manner to accelerate chondrocyte maturation. During development, other factors, including PTHrP, slow the rate of chondrocyte maturation. Our results further suggest that PTHrP inhibits maturation by suppressing the expression of BMP-6. Further studies will be necessary to clarify the interaction between Ihh and BMP-6 and understand the BMP-6 signaling pathway.

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REFERENCES

1. Zou H, Wieser R, Massague J, Niswander L 1997 Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. Genes Dev **11:**2191–2203.
2. Oshima O, Leboy PS, McDonald SA, Tuan RS, Shapiro IM 1989 Developmental expression of genes in chick growth cartilage detected by in situ hybridization. Calcif Tissue Int **45:**182–192.
3. Buckwalter JA, Mower D, Ungar R, Schaeffer J, Ginsberg B 1986 Morphometric analysis of chondrocyte hypertrophy. J Bone Joint Surg **68A:**243–255.
4. Kirch T, Nah H-D, Shapiro IM, Pacifici M 1997 Regulation of mineralization-competent matrix vesicle production in hypertrophic chondrocytes. Trans Orthop Res Soc **22:**131.
5. Hatori M, Klatte KJ, Teixeira CC, Shapiro IM 1995 End labeling studies of fragmented DNA in the avian growth plate: Evidence of apoptosis in terminally differentiated chondrocytes. J Bone Miner Res **10:**1960–1968.
6. Lewinson D, Silbermann M 1992 Chondroclasts and endothelial cells collaborate in the process of cartilage resorption. Anat Rec **233:**504–514.
7. Gitelman S, Kobrin M, Ye J, Lopez A, Lee A, Derynck R 1994 Recombinant Vgr-1/BMP-6-expressing tumors induce fibrosis and endochondral bone formation in vivo. J Cell Biol **126:**1595–1609.
8. Campbell J, Kaplan F 1992 The role of morphogens in endochondral ossification. Calcif Tissue Int **50:**283–289.
9. Hogan BL 1996 Bone mophogenetic proteins: Multifunctional regulators of vertebrate development. Genes Dev **10:**1580–1594.
10. O'Keefe RJ, Crabb ID, Puzas JE, Rosier RN 1994 Effects of transforming growth factor-beta 1 and fibroblast growth factor on DNA synthesis in growth plate chondrocytes are enhanced by insulin-like growth factor-1. J Bone Miner Res **9:**1713–1722.
11. Crabb ID, O'Keefe RJ, Puzas JE, Rosier RN 1992 Differential effects of parathyroid hormone on chick growth plate and articular chondrocytes. Calcif Tissue Int **50:**61–66.
12. Amizuka N, Warshawsky H, Henderson JE, Goltzman D, Karaplis AC 1994 Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. J Cell Biol **126:**1611–1623.
13. Lanske B, Karaplis AC, Lee K, Lutz A, Vortkamp A, Pirro A, Karperien M, Defize LHK, Ho C, Mulligan R, Abou-Samra AB, Juppner H, Segre GV, Kronenberg HM 1996 PTH/PTHrp receptor in early development and indian hedgehog-regulated bone growth. Science **273:**663–666.
14. O'Keefe RJ, Loveys L, Hicks DG, Reynolds PR, Crabb ID, Puzas JE, Rosier RN 1997 Differential regulation of type II and type X collagen synthesis by PTHrP in chick growth plate chondrocytes. J Orthop Res **15:**162–174.
15. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ 1996 Regulation of rate of cartilage differentiation by indian hedgehog and PTH-related protein. Science **273:**613–622.
16. Leboy PS, Vaias L, Uschmann B, Golub E, Adams SL, Pacifici M 1989 Ascorbic acid induces alkaline phosphatase, type X collagen, and calcium deposition in cultured chick chondrocytes. J Biol Chem **264:**17281–17286.
17. Leboy PS, Sullivan TA, Nooreyazdan M, Venezian RA 1997 Rapid chondrocyte maturation in serum-free culture with BMP-2 and ascorbic acid. J Cell Biochem **66:**394–403.
18. Iwamoto M, Shapiro IM, Yagami K, Boskey AL, Leboy PS, Adams SL, Pacifici M 1993 Retinoic acid induces rapid mineralization and expression of mineralization-related genes in chondrocytes. Exp Cell Res **207:**413–420.
19. Berry JL, CF.Whitehead CC, Mawer EB 1996 Growth plate chondrocyte vitamin D receptor number and affinity are reduced in avian tibial dyschondroplastic lesions. Bone **19:**197–203.
20. Ballock RT, Reddi AH 1994 Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. J Cell Biol **126:**1311–1318.
21. Grimsrud CD, Rosier RN, Puzas JE, Reynold PR, Reynolds SD, Hicks DG, O'Keefe RJ 1998 BMP-7 in growth plate chondrocytes: Regulation by retinoic acid is dependent upon the stage of chondrocyte maturation. J Orthop Res **16:**247–255.
22. Lyons KM, Pelton RW, Hogan B 1989 Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor-beta-like genes coordinately regulate aspects of embryonic development. Genes Dev **3:**1657–1668.
23. Francis PH, Richardson MK, Brickell PM, Tickle C 1994 Bone morphogenetic proteins and a signaling pathway that controls patterning in the developing chick limb. Development **120:**209–218.
24. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K 1990 Current Protocols in Molecular Biology, Vol. 2. Wiley Interscience Press, New York, NY, U.S.A.
25. Houston B, Thorp BH, Burt DW 1994 Molecular cloning and expression of bone morphogenetic protein-7 in the chick epiphyseal growth plate. J Mol Endocrinol **13:**289–301.
26. O'Keefe RJ, Crabb ID, Puzas JE, Rosier RN 1989 Countercurrent centrifugal elutriation: high-resolution method for the separation of growth plate chondrocytes. J Bone Joint Surg **71A:**607–620.
27. Hicks DG, O'Keefe RJ, Reynolds PR, Cory-Slechta DA, Puzas JE, Rosier RN 1996 Effects of lead on growth plate chondrocyte phenotype. Toxicol App Pharmacol **140:**164–172.
28. Romano P, Wang J, O'Keefe RJ, Puzas JE, Rosier RN, Reynolds PR 1998 Hiper 1, a phosphatase of the endoplasmic re-

ticulum with a role in chondrocyte maturation. J Cell Sci **111:**803–813.
29. Lyons KM, Hogan BL, Robertson EJ 1995 Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. Mech Dev **50:**71–83.
30. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz R, Hewick R, Wang E 1988 Novel regulators of bone formation: Molecular clones and activities. Science **242:**1528–1534.
31. Jones WK, Richmond EA, White K, Sasak H, Kusmik W, Smart J, Opperman H, Rueger DC, Tucker RF 1994 Osteogenic protein-1 (OP-1) expression and processing in chinese hamster ovary cells: Isolation of a soluble complex containing the mature and pro-domains of OP-1. Growth Factors **11:**215–225.
32. Asahina I, Sampath TK, Hauschka P 1996 Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or adipocytic differentiation of clonal murine target cells. Exp Cell Res **222:**38–47.
33. Chen P, Vukicevic S, Sampath TK, Luyten FP 1995 Osteogenic protein-1 promotes growth and maturation of chick sternal chondrocytes in serum-free cultures. J Cell Sci **108:**105–114.
34. Suzuki F 1992 Effects of various growth factors on a chondrocyte differentiation model. Adv Exp Med Biol **12:**101–106.
35. Enomoto-Iwamoto M, Iwamoto M, Mukudai Y, Kawadami Y, Nohno T, Higuchi Y, Takmoto S, Ohuchi H, Noji S, Kurisu K 1998 Bone morphogenetic protein signaling is required for maintenance of differentiated phenotype, control of proliferation, and hypertrophy in chondrocytes. J Cell Biol **140:**409–418.

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