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Direct Inhibition of Indian Hedgehog Expression by Parathyroid Hormone (PTH)/PTH-Related Peptide and Up-regulation by Retinoic Acid in Growth Plate Chondrocyte Cultures

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Running title: regulation of Ihh mRNA by PTH and RA in chondrocytes

Abstract

Indian hedgehog (Ihh) is highly expressed in prehypertrophic chondrocytes *in vivo* and has been proposed to regulate the proliferation and maturation of chondrocytes and bone collar formation in the growth plate. In high density cultures of rabbit growth plate chondrocytes, Ihh mRNA was also expressed at the highest level in the prehypertrophic stage. To explore endogenous factors that regulate Ihh expression in chondrocytes, we examined the effect of various growth factors on Ihh mRNA expression in this system. Retinoic acid (RA) and bone morphogenetic protein-2 (BMP-2) enhanced Ihh mRNA expression, whereas PTH/PTH-related peptide (PTHrP) markedly suppressed Ihh expression. RA at more than 10^{-8} M induced the expression of Ihh and Patched 1 (Ptc1) within 3 h before it increased the type X collagen mRNA level at 6-24 h. Cycloheximide blocked the up-regulation of Ihh by RA, indicating the requirement of *de novo* protein synthesis for this stimulation. These findings suggest that RA is involved in the up-regulation of Ihh during endochondral bone formation. In contrast to RA, PTH (1-84) at 10^{-7} M abolished the mRNA expression of Ihh and Ptc1 within 2-4 h before it suppressed the expression of type X collagen at 12-24 h. The inhibition of Ihh expression by PTH (1-84) did not require *de novo* protein synthesis. PTH (1-34), PTHrP (1-34), and $(Bu)_2$cAMP also suppressed Ihh expression. On the other hand, Ihh has been reported to induce PTHrP synthesis in the perichondrium. Consequently, the direct inhibitory action of PTH/PTHrP on Ihh appears to be a negative feedback mechanism that prevents excess PTHrP accumulation in cartilage.

Key words: PTH; PTHrP; RA; Ihh; chondrocyte; growth plate

INTRODUCTION

During endochondral bone formation, chondrocytes undergo a sequential process of proliferation, matrix synthesis, hypertrophy, and calcification. Hypertrophic and calcificated cartilage is susceptible to invasion by blood vessels and is eventually replaced by new bone. Several hormones and growth factors including parathyroid hormone (PTH) and PTH-related peptide (PTHrP) have been reported to be involved in this process [1-12]. Using chondrocyte cultures, we have shown that PTH/PTHrP increases the synthesis of DNA, aggrecan, and matrix

metalloproteases, and suppresses alkaline phosphatase activity, vitamin D receptor synthesis, type X collagen synthesis, increase in the cell size (hypertrophy), and matrix calcification [1-4]. *In vivo* studies have shown that PTHrP- or the PTH/PTHrP receptor-deficient mice show accelerated hypertrophy and mineralization in cartilage, and that overexpression of PTHrP or expression of a constitutively active PTH/PTHrP receptor delays chondrocyte hypertrophy [6-12]. These *in vitro* and *in vivo* findings emphasized the important role of PTHrP in the control of chondrocyte matrix synthesis and hypertrophy.

In contrast, retinoic acid (RA), a physiological metabolite of vitamin A, decreases aggrecan synthesis, and induces mineralization and the expression of the hypertrophy-related genes including alkaline phosphatase, type X collagen, and osteopontin in chondrocyte cultures [13-17]. Cartilaginous elements in chick embryo limbs contain endogenous retinoids, and retinoic acid receptor g gene expression is selectively up-regulated in the hypertrophic zone of the growth plate [18]. Implantation of beads filled with retinoid antagonists in the chick embryo limbs suppressed chondrocyte hypertrophy [18]. In addition, numerous studies of both dietary hypo- and hypervitaminosis A showed disturbance of endochondral bone formation [19-22]. These findings suggested that RA is also critical for chondrocyte matrix synthesis and hypertrophy.

Indian hedgehog (Ihh) is another critical secreted factor by chondrocytes and its expression is restricted to the prehypertrophic and upper hypertrophic zones. Vortkamp *et al.* [10] have shown that Ihh up-regulates PTHrP expression in the periarticular perichondrium and that the newly synthesized PTHrP is involved in the decrease in the rate of hypertrophy. Ihh secreted by prehypertrophic chondrocytes transmits signals to the adjacent perichondrium and chondrocytes by inducing the Ihh target genes Patched (Ptc) and Gli, and then the signals from Ptc/Gli appears to indirectly cause up-regulation of PTHrP in the periarticular perichondrium. Accordingly, Ihh-deficient mice showed the absence of periarticular expression of PTHrP and disturbance of endochondral bone formation [23]. These findings have established the physiological importance of Ihh in endochondral bone formation. However, autocrine, paracrine or systemic factors that regulate Ihh expression in growth plate chondrocytes remain to be determined, except that bone morphogenetic proteins (BMPs) enhanced Ihh expression in chick embryo chondrocyte cultures [24, 25].

The purpose of the present study was to analyse the direct effects of PTH/PTHrP and RA on Ihh expression in growth plate chondrocytes. We show that 1) PTH/PTHrP/cAMP suppresses Ihh mRNA expression within 2-4 h without *de novo* synthesis of other proteins; 2) PTH/PTHrP suppresses Ihh mRNA expression within 2-4 h before it inhibits the expression of the hypertrophy-related genes; and 3) RA induces Ihh mRNA expression within 3 h before it stimulates the expression of the hypertrophy-related genes. The physiological significance of these actions of PTH and RA on Ihh expression will be discussed.

MATERIALS AND METHODS

Materials. Human recombinant PTH (1-84), PTH (1-34), PTH (35-84), and PTHrP (1-34) were supplied by Dr. Naoki Kubota (Chugai Pharmaceutical Co., Tokyo). $(Bu)_2$cAMP, all-trans RA, actinomycin D, and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO).

Chondrocyte cultures. Chondrocytes were isolated from growth plates of ribs of 4-week-old male Japan white rabbits as described previously [26]. The experimental procedures about animal care and treatment were performed under the permission, rules, and guidelines of Hiroshima University. Cells were seeded at a density of 7 x 10^4 cells/22.7-mm plastic tissue culture dish and grown in α-MEM (Sanko Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Mitsubishi Kagaku Co., Tokyo, Japan), 32 U/ml penicillin, 60 μg/ml kanamycin (Meiji Seika Co., Tokyo, Japan), and 250 ng/ml amphotericin B (Dainippon Pharmaceutical Co., Osaka, Japan) at 37°C under 5% CO_2 in air. Chondrocytes on days 6-26 were preincubated in serum-free α-MEM for 24 h. Thereafter, the cells were incubated in the fresh serum-free α-MEM containing growth factors for 0-24 h before the end of incubation. RA was dissolved in ethanol and diluted with PBS. Other growth factors were dissolved in saline containing 0.1% BSA.

Preparation of cDNA probes. Rabbit cDNA clone used as hybridization probe for GAPDH was prepared as described previously [27]. Rabbit cDNAs for Ihh (411 bp), PTH/PTHrP receptor (442 bp), osteopontin (557 bp), and type X collagen (599 bp) were generated by the reverse

transcription-polymerase chain reaction (RT-PCR) from mRNA of rabbit growth plate chondrocytes using a suitable pair of primers: 5'-CAAGCAGTTCAGCCCCAACG-3' and 5'-ACGTGGGCCTTGGACTCGTA-3' for Ihh; 5'-AGCCAACATAATGGAGTCAG-3' and 5'-CTCTTCCTCTGTGAGGCGCT-3' for PTH/PTHrP receptor; 5'-GAAAGCCACGACCACATGGA-3' and 5'-TTGGAACTTTCCTGACTATC-3' for osteopontin; 5'- CCCAACACCAAGACACAGTT-3' and 5'-ATCACCTTTGATGCCTGGCT-3' for type X collagen. These oligonucleotides were synthesized by Kurabo Co. (Osaka, Japan), based on the sequence of mouse Ihh (EMBL/GenBank/DDBJ database Accession No. U85610), rat PTH/PTHrP receptor (No. M21967), rabbit osteopontin (No. D11411), and human type X collagen (No. X65120). The PCR products were subcloned into the pGEM T-easy vector (Promega, Madison, WI) and subjected to sequencing for their identification.

Northern blot analysis. Northern blot analysis was performed using total RNA preparations extracted from cultured rabbit chondrocytes with the guanidine thiocyanate method [28] and the following cDNA probes; Ihh, PTH/PTHrP receptor, osteopontin, type X collagen, and GAPDH. The RNA samples were denatured by 2.2 M formaldehyde and 50% formamide, electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, and transferred to Nytran nylon membranes (Schleicher & Schuell, Inc., Dassel, Germany). After transfer, the membranes were stained with methylene blue to confirm the equal loading of total RNA on each lane [29]. The membranes were hybridized with the ^{32}P-labeled probes in hybridization solution containing 6 x saline-sodium citrate, 5 x Denhardt's, 10 mM EDTA, 1% SDS, and 0.5 mg/ml sonicated salmon sperm DNA at 68°C. The membranes were washed with 0.1 ¥ saline-sodium citrate containing 0.5% SDS at 50°C and were exposed to Kodak BMX films at -80°C. The radioactivities of the hybridized bands were measured using the Bio-imaging Analyzer System BAS2000 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Real time quantitative RT-PCR analysis. Real time quantitative RT-PCR analyses for Ihh and PTHrP were performed using an ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Inc., Foster City, CA). The principles and protocols for RT-PCR analyses using this system have been described previously [30, 31]. First strand cDNA was synthesized using SuperScript II RNase H-reverse transcriptase (Life Technologies, Inc., Rockville, MD) with total RNA (1 μg). GAPDH was chosen as an internal standard to control for variability in amplification due to differences in the starting mRNA concentrations. Sequences for all primers and probes used in these analyses were as follows; 5'-GGAGGAGTCCTTGCATTATGAGG-3' and 5'-TAATACACCCAGTCGAAGCCG-3' for Ihh cDNA amplification; 5'-GAACACCAACTCCTCCATGACAA-3' and 5'-AGACCTCGGAGGTAGCTCTGATC-3' for PTHrP cDNA amplification; 5'-AACTCACTGGCATGGCCTT-3' and 5'-GCTTCACCACCTTCTTGATG-3' for GAPDH cDNA amplification. The sequence of TaqMan™ fluorogenic probes used were 5'-6FAM-ACGTCCGACCGTGACCGCAATAAGTA-TAMRA-3', 5'-6FAM-TGCACCACCTGATAGCCGAAATCCACAC-TAMRA-3', and 5'-6VIC-TGCCGCCTGGAGAAAGCTGCTAAGTA-TAMRA-3' for Ihh, PTHrP, and GAPDH, respectively.

RT-PCR Southern blot analysis. The RT-PCR for Ptc1 was performed using total RNA from rabbit chondrocyte cultures. The oligonucleotide primers for PCR amplification of Ptc1 were as follows; 5'-AGCTGGGAGGAAATGCTGAA-3' and 5'-ACGTGGGCCTTGGACTCGTA-3'. In pilot studies, we determined the optimal cycles in the log phase of amplification of the Ptc1 gene: 25 cycles for Ptc1. The PCR condition was 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 3 minutes.

RESULTS

Expression of Ihh, PTH/PTHrP receptor, Osteopontin, Type X collagen mRNA During Chondrocyte Differentiation

To dissect the complicated molecular and cellular events during chondrocyte differentiation in the growth plate, we incubated chondrocytes isolated from growth plates of rabbit ribs in high-density cultures. This culture system contains mixtures of chondrocytes from each zone of the growth plate. However, the chondrocytes are capable of recapitulating all stages of the differentiation program in vitro [26, 32]. In this system, the cultures became confluent on day 6. The PTH/PTHrP receptor mRNA level started to increase on day 14 and reached a maximum on

day 18. Thereafter, the receptor mRNA level decreased on day 22 (Fig. 1). The mRNA levels of osteopontin and type X collagen started to increase on day 14 and reached a maximum or plateau on day 22. On day 18, chondrocytes were spherical and surrounded by a reflactile matrix. On day 22, the cells became hypertrophic. The calcification took place on day 26 (data not shown). From these observations, we regarded cells on day 18 as the prehypertrophic stage of chondrocytes. In this culture system, the Ihh mRNA level started to increase on day 14 and the maximal level was observed on day 18 as well as the PTH/PTHrP receptor mRNA level (Fig. 1).

Effects of Various Factors on Ihh mRNA Expression in Chondrocytes

To explore the endogenous modulators for the expression of Ihh in the culture system, we examined the effects of PTH (1-84), RA, BMP-2, bFGF, TGF-β, insulin, or ConA on the expression of Ihh mRNA in the chondrocytes on day 18 by Northern blot analysis, since these compounds markedly modulate chondrocyte differentiation *in vitro* and/or *in vivo* [33-39]. Of these factors, PTH showed the most prominent inhibition of Ihh expression (Fig. 2A, lane 2), whereas RA and BMP-2 enhanced Ihh expression (Fig. 2A, lanes 3 and 4). Although bFGF and TGF-β slightly decreased Ihh expression (Fig. 2A, lanes 5 and 6), their effects were less than that of PTH at least in this culture system. Insulin or ConA did not show any significant effects on the expression of Ihh (Fig. 2A, lanes 7 and 8) in repeated experiments. The GAPDH mRNA level was not affected by the factors used, except RA, which significantly decreased the GAPDH mRNA level. The suppression of GAPDH expression by RA has been shown in chondrocyte cultures [27] and adipocyte cultures [40]. Since BMPs have been reported to increase Ihh expression in chondrocyte cultures [24, 25], we focused on the effects of PTH/PTHrP and RA.

Effects of PTH / PTHrP Fragments and $(Bu)_2$cAMP

Next we examined the effects of various fragments of PTH/PTHrP on the Ihh expression. PTH/PTHrP fragments containing the N-terminal region such as PTH (1-34) and PTHrP (1-34), and $(Bu)_2$cAMP also abolished Ihh expression (Fig. 2B, lanes 2-5). However, the C-terminal fragment of PTH, PTH (35-84), did not show any significant effect on the Ihh expression (Fig. 2B, lane 6). Other C-terminal fragments of PTH such as PTH (39-68), PTH (60-84), and PTH (71-84) also had little effect on Ihh mRNA expression even at a high concentration (data not shown). These findings suggest that the N-terminal region is principally responsible for the action of PTH/PTHrP on Ihh expression, as well as on chondrocyte differentiation by increasing the intracellular cAMP.

Time Course and Dose Dependency of Inhibition of Ihh mRNA Expression by PTH (1-84)

Northern blot analysis (Fig. 3A) shows that the inhibition of Ihh mRNA expression by 10^{-7} M PTH (1-84) was significant at 2 h and marked at 4 h, and then the Ihh mRNA expression was almost abolished at 24 h. Real time quantitative RT-PCR (Fig. 3B) showed almost the same pattern of change in the Ihh mRNA level as that of Northern blot analysis. Based on the quantitative analysis, the Ihh mRNA level in cultures exposed to PTH for 24 h was approximately 100-fold lower than that in the control cultures (Fig. 3B). PTH (1-84) also decreased the level of Ptc1 mRNA within 4 h (Fig. 3A). However, PTH (1-84) did not exhibit the suppression of type X collagen expression until 12-24 h. Although PTH (1-84) suppressed the expression of osteopontin mRNA at 4-6 h, this effect was slower and less than that of Ihh or Ptc1 expression. In other words, in the PTH (1-84)-exposed chondrocytes, the inhibition of Ihh and Ptc1 expression consistently preceded that of the expression of the hypertrophy-related genes such as osteopontin and type X collagen. The abundance of GAPDH mRNA was not inhibited by PTH (1-84), indicating the specificity of the PTH effect on Ihh mRNA expression. PTH (1-84) did not show any significant effect on the level of Ihh mRNA at a concentration of 10^{-9} M, but it exhibited the maximal inhibition at a concentration of 10^{-8} M (Fig. 3C), indicating the critical concentration of PTH (1-84) to exhibit an inhibitory effect on the Ihh mRNA level was between 10^{-9} and 10^{-8} M.

Inhibition of Ihh mRNA Expression by PTH (1-84) at Various Stages of Chondrocyte Differentiation

In order to evaluate whether the inhibitory effect of PTH (1-84) on the Ihh expression was stage-specific, growth plate chondrocytes on days 6 to 26 were exposed to PTH (1-84) for 24 h, and

RNAs were prepared from these cells for real-time quantitative RT-PCR. Incubation with PTH (1-84) for 24 h strongly suppressed Ihh expression in all stages of chondrocytes examined (Fig. 4A), indicating that chondrocytes in all stages retain the ability to respond to PTH/PTHrP and endogenous PTHrP level may be important to regulate Ihh expression.

Expression of PTHrP mRNA During Chondrocyte Differentiation

We next asked whether a relationship exists between Ihh and PTHrP levels during chondrocyte differentiation in this culture system. The expression level of PTHrP mRNA was also examined by the real-time quantitative RT-PCR method (Fig. 4B). The mRNA level of PTHrP, an inhibitor of Ihh expression, was high on day 6, and gradually decreased on days 10 and 14. The minimum level of PTHrP mRNA was observed on day 18, whereas the Ihh mRNA level reached a maximum in the corresponding samples (Fig. 4A). Thereafter, the PTHrP mRNA level slightly increased on days 22 and 26 (Fig. 4B). These findings demonstrate a stage-dependent reciprocal relationship between Ihh and PTHrP.

Time Course and Dose Dependency of Enhancement of Ihh mRNA Expression by RA

In contrast to the inhibitory action of PTH/PTHrP, RA markedly increased the Ihh mRNA level. The induction reached a maximum at 3 h in both Northern blot analysis (Fig. 5A) and real time quantitative RT-PCR (Fig. 5B) and then slightly decreased. RA also enhanced Ptc1 mRNA expression concomitantly with Ihh mRNA. The mRNA levels of osteopontin and type X collagen were also increased by RA, but an obvious increment was not observed until 6 h (Fig. 5A). In other words, the enhancement of Ihh and Ptc1 mRNA by RA preceded that of the expression of the hypertrophy-related genes such as osteopontin and type X collagen. Fig. 5C shows that the critical concentration of RA to increase the Ihh mRNA level was 10^{-8}-10^{-7} M. Although RA lowered the GAPDH mRNA level (Fig. 5A bottom) as described above, the equal loading of RNA preparations in each lane was confirmed by staining the blotted membranes with methylene blue (data not shown).

We also examined the effect of RA plus PTH on Ihh mRNA expression at various concentrations of these factors. Even in the presence of 10^{-8} and 10^{-7} M RA, PTH (1-84) at a low concentration (10^{-8} M) was sufficient to abolish Ihh expression below the control level. PTH/PTHrP dominated over the stimulatory effect of RA at least under these culture conditions (Fig. 6), suggesting the importance of the inhibitory actions of PTH/PTHrP on Ihh expression.

Effects of PTH (1-84) and RA on Ihh mRNA Expression in Presence of Actinomycin D or Cycloheximide

To clarify whether PTH (1-84) caused a change in Ihh mRNA stability, we next examined the effect of PTH (1-84) on the Ihh mRNA level in the presence of actinomycin D. The decay of Ihh mRNA was determined by real time quantitative RT-PCR (Fig. 7). The Ihh mRNA decay occurred at similar rates regardless of the presence of PTH (1-84) in the presence of actinomycin D. These findings indicate that PTH did not alter the stability of this transcript.

To elucidate whether the PTH inhibition and the RA stimulation of Ihh expression required newly synthesized protein, the reduction of Ihh mRNA by PTH and the induction of Ihh mRNA by RA were assessed in the presence of a protein synthesis inhibitor, cycloheximide (10 μg/ml). Cycloheximide did not affect the PTH-induced inhibition of Ihh mRNA expression, demonstrating that *de novo* protein synthesis was not required for such inhibition (Fig. 8A). In contrast, the enhancement of Ihh mRNA by RA was prohibited by cycloheximide, demonstrating that *de novo* protein synthesis was required for such induction (Fig. 8B).

DISCUSSION

The rabbit growth plate chondrocyte culture system exhibited the Ihh expression depending on the culture days showing a maximum in the prehypertrophic stage (Figs. 1 and 4A). This *in vitro* observation agrees with the up-regulation of Ihh expression during the prehypertrophic stage *in vivo* [10]. Because our chondrocyte culture system does not include non-cartilaginous tissues such

as bone or the perichondrium, endogenous factors must be responsible for the expression of Ihh in the prehypertrophic stage. Interestingly, the endogenous level of PTHrP mRNA was high in the proliferating stage (day 6) and markedly decreased in the prehypertrophic stage (days 18-22) (Fig. 4B). Moreover, the down-regulation of Ihh mRNA by the addition of PTH was observed in all stages of chondrocytes. Therefore, the inverse profile of PTHrP expression to Ihh expression could explain the up-regulation of Ihh mRNA during the prehypertrophic stage. The enhancement of Ihh expression by RA and BMP may also be responsible for the expression pattern of Ihh mRNA in the culture system, although their contribution in this system remains to be clarified.

The PTH/PTHrP receptor level was high in the prehypertrophic stage *in vivo* and *in vitro*. Nonetheless Ihh expression was not down-regulated during this stage *in vivo* [10] and *in vitro* (this study). These findings suggest that the ligand (PTHrP) level in this stage is too low to abolish Ihh expression, which is markedly enhanced by RA and BMP, and that the PTHrP level may be more critical to regulate Ihh expression than the PTHrP receptor level.

Another interesting finding in this study is the down-regulation of Ihh and Ptc by PTH/PTHrP consistently occurred earlier than that of osteopontin or type X collagen (Fig. 3A). This observation together with the finding that *de novo* protein synthesis was not required for the suppression by PTH indicates that the effect of PTHrP on Ihh expression is not secondary to the inhibition of chondrocyte hypertrophy. Previous studies have shown that incubation of embryonic limb explants with PTHrP for several days results in suppression of Ihh and type X collagen expression [10]. Our findings indicate that the decreased Ihh expression is not ascribed to a general inhibition of hypertrophy by PTHrP.

Since PTH/PTHrP suppressed Ihh expression via the cAMP pathway (Fig. 2B), it is noteworthy that the consensus sequence of CRE (cAMP response element) is found at 1,148 bp upstream from the translation initiation sites in the 5'-flanking region of the human Ihh gene (GenBank/EMBL/DDBJ DNA database; AB021874). The consensus sequences of RARE (retinoic acid response sequence) are also found at 1,114 bp and 1,183 bp upstream. Taken together with a rapid response of Ihh mRNA expression to both PTH/PTHrP and RA, it is probable that the Ihh expression is directly regulated at the transcriptional level by the signals from these factors.

RA and PTH/PTHrP have opposite effects on chondrocyte matrix synthesis and hypertrophy [13-17]. We showed here that these also have the opposite effect on Ihh expression. In contrast to PTH, RA increased Ihh expression in growth plate chondrocytes within 3 h. This stimulation of Ihh expression by RA was abolished by adding PTH simultaneously, suggesting that a local concentration of PTHrP may be more critical to control Ihh expression than that of RA. However, incubation with RA for 24-120 h has been shown to decrease the number of PTH receptors in chondrocyte cultures [41]. Thus chondrocytes previously exposed to RA for 24-120 h may not show the PTH inhibition of Ihh expression. In any case, the findings in this study suggest that Ihh is one of the important targets for both RA and PTH/PTHrP.

Previous studies have shown that Ihh secreted from prehypertrophic chondrocytes induces PTHrP in the perichondrium and, thereby, it negatively regulates hypertrophic conversion of proliferating chondrocytes [10]. On the other hand, the findings in the present study suggest that PTHrP secreted by periarticular perichondrium and proliferating chondrocytes directly suppresses Ihh expression in chondrocytes to prevent excess synthesis of PTHrP in response to Ihh.

Ihh has both PTHrP-dependent and -independent actions in the growth plate [23, 42] (Fig. 9). Ihh suppresses hypertrophy via the production of PTHrP in the periarticular perichondrium [10]. In addition, Ihh enhances chondrocyte proliferation and bone collar formation, and these actions do not appear to be dependent upon PTHrP synthesis [42]. Accordingly, PTHrP must also suppress the PTHrP-independent actions of Ihh such as bone collar formation in developing cartilage. Ihh-deficient mice showed the absence of bone collar in the perichondrium of the growth plate [23]. Interestingly, chimeric mice containing both wild-type and PTH/PTHrP receptor (-/-) cells, showed ectopic bone collar formation in the proliferating layer near the PTH/PTHrP receptor (-/-) cells that ectopically expressed Ihh at high levels [43]. PTHrP-deficient mice also showed ectopic bone collar in the perichondrium of rib resting cartilage and excess bone collar formation in the growth plate [6]. These observations, together with the present findings, suggest that PTHrP inhibits bone collar formation by suppressing Ihh synthesis by chondrocytes. The PTHrP-null mice did not show increased chondrocyte proliferation, perhaps because many growth factors besides of PTHrP and Ihh participate in the control of chondrocyte proliferation.

The significance of the stimulation of Ihh expression by RA is unknown. However, Ihh-deficient mice showed reduced chondrocyte proliferation and differentiation, and the absence of bone collar

formation [23]. RA may modulate chondrocyte proliferation and differentiation as well as bone collar formation via the enhancement of Ihh expression. However, this modulation requires the *de novo* synthesis of unidentified factor(s).

In conclusion, the direct action of PTH/PTHrP on Ihh expression has dual roles: it serves as a negative feedback mechanism for PTHrP synthesis in cartilage, and modulates Ihh-induced phenomena such as bone collar formation (Fig. 9). However, RA may promote the PTHrP-independent actions of Ihh on bone formation [44, 45] in some situations. The information obtained in this study will be useful for understanding the role of the PTHrP-Ihh system in endochondral bone formation.

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Footnotes

Abbreviations used are: Ihh, indian hedgehog; PTHrP, parathyroid hormone related peptide; RA, retinoic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Ptc1, patched 1; BMP, bone morphogenetic protein; bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor; ConA, concanavalin A

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Figure legends

FIG. 1. Changes in Ihh, PTH/PTHrP receptor (PPR), osteopontin (OPN), and type X collagen (TypeX) mRNA levels during chondrocyte proliferation and differentiation *in vitro*. (A) Total RNAs were isolated from growth plate chondrocyte cultures on days 6, 10, 14, 18, 22, and 26. The samples were separated by agarose gel electrophoresis, transferred to Nytran membranes, and hybridized to respective ^{32}P-labeled cDNA probes. The membranes were exposed for different lengths of time to ensure linearity of exposure. Equal loading of total RNA on each lane was confirmed by staining the membranes with methylene blue (data not shown). (B) The relative expression of Ihh (●), PTH/PTHrP receptor (■), osteopontin (○), and type X collagen (□) compared to GAPDH, as quantified by BAS2000. Three independent experiments were performed and gave similar results.

FIG. 2. (A) Effects of various growth factors on Ihh mRNA expression in growth plate chondrocyte cultures. Cells (day 18) were treated with vehicle (lane 1), PTH (1-84) (100 nM, lane 2), RA (100 nM, lane 3), BMP-2 (50 ng/ml, lane 4), bFGF (1 ng/ml, lane 5), TGF-β (10 ng/ml, lane 6), insulin (1 μg/ml, lane 7), and ConA (10 μg/ml, lane 8) for 24 h. Total RNA (20 μg) was subjected to Northern blot analysis using Ihh cDNA and GAPDH cDNA as probes. (B) Effects of PTH/PTHrP fragments and $(Bu)_2$cAMP on Ihh mRNA expression in growth plate chondrocyte cultures. Cells (day 18) were exposed to vehicle (lane 1), PTH (1-84) (lane 2), PTH (1-34) (lane 4), PTHrP (1-34) (lane 5), or PTH (35-84) (lane 6) at 100 nM, or 10 mM $(Bu)_2$cAMP (lane 3) for 24 h. Total RNA (20 μg) was subjected to Northern blot analysis. Three independent experiments were performed and gave similar results.

FIG. 3. (A) Time course of the inhibition of Ihh, Ptc1, osteopontin, and type X collagen mRNA expression by PTH (1-84) in growth plate chondrocyte cultures. Cells (day 18) were exposed to PTH (1-84) at 100 nM for the indicated periods in serum-free α-MEM. Total RNA (20 μg) from these cells was subjected to Northern blot analysis for Ihh, osteopontin, type X collagen, and GAPDH, or RT-PCR Southern blot analysis for Ptc1. (B) The findings of real time quantitative RT-PCR analysis of the Ihh mRNA abundance were normalized to those of the GAPDH mRNA. (C) Effects of increasing concentrations of PTH (1-84) on the Ihh mRNA expression in growth plate chondrocyte cultures. Cells were exposed to the indicated concentrations of PTH (1-84) in serum-free α-MEM for 24 h. Total RNA (20 μg) from these cells was subjected to Northern blot analysis for Ihh and GAPDH. Three independent experiments were performed and gave similar results.

FIG. 4. (A) The effect of PTH (1-84) on Ihh mRNA level in growth plate chondrocyte cultures at various stages of differentiation. Chondrocytes were exposed to PTH (1-84) at 100 nM for 24 h in serum-free α-MEM on days 6, 10, 14, 18, 22, and 26, and the Ihh mRNA level was examined by real time quantitative RT-PCR analysis. Closed bars, control culture (vehicle); open bars, PTH-treated. (B) The mRNA level of PTHrP in the control culture was examined by real time quantitative RT-PCR analysis. Three independent experiments were performed and gave similar results.

FIG. 5. (A) Time course of the enhancement of Ihh, Ptc1, osteopontin, and type X collagen mRNA expression by RA in growth plate chondrocyte cultures. Cells (day 12) were exposed to RA at 100 nM for the indicated periods in serum-free α-MEM. Total RNA (20 μg) from the cells was subjected to Northern blot analysis for Ihh, osteopontin, type X collagen, and GAPDH, or RT-PCR Southern blot analysis for Ptc1. (B) The findings of real time quantitative RT-PCR analysis of the Ihh mRNA. In this experiment, the levels of Ihh mRNA were not normalized by the GAPDH mRNA level, because RA strongly suppressed the GAPDH expression as shown in Northern blot analysis, and reported previously [27]. (C) Effects of increasing concentrations of RA on the Ihh mRNA level in growth plate chondrocyte cultures. Cells were exposed to the indicated concentrations of RA in serum-free α-MEM for 6 h. Total RNA (20 μg) from the cells was subjected to Northern blot analysis for Ihh and GAPDH. Three independent experiments were performed and gave similar results.

FIG. 6. Effects of the combination of PTH (1-84) and RA on Ihh mRNA expression. Cells (day 18) were exposed to the respective factors at indicated concentrations for 6 h in serum-free α-MEM.

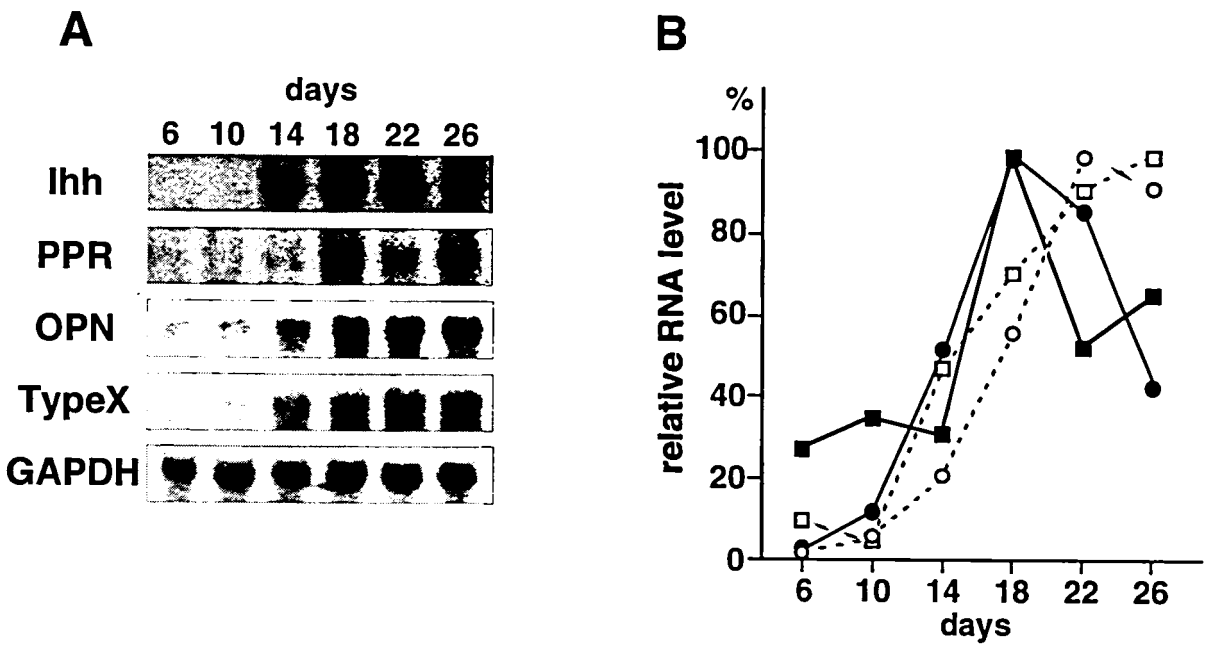
Total RNA (20 μg) from these cells was subjected to Northern blot analysis for Ihh and GAPDH. Three independent experiments were performed and gave similar results.

FIG. 7. Effects of actinomycin D on the decay of Ihh mRNA in the presence or absence of PTH (1-84) in growth plate chondrocyte cultures. Cells (day 18) were exposed to actinomycin-D (5 μg/ml) in the presence (○) or absence (■) of PTH (1-84) (100 nM) for the indicated periods. Total RNA was prepared and subjected to real time quantitative RT-PCR analysis. Three independent experiments were performed and gave similar results.

FIG. 8. Effects of cycloheximide (CHX) on Ihh mRNA level in growth plate chondrocyte cultures exposed to PTH or RA. Cells (day 18) were exposed to each factor for 6 h. CHX (10 μg/ml) was added 30 min before the addition of PTH (1-84) (A, 100 nM) or RA (B, 100 nM). Total RNA (20 μg) from these cells was subjected to Northern blot analysis for Ihh and GAPDH. Three independent experiments were performed and gave similar results.

FIG. 9. The inhibition of Ihh expression by PTHrP has dual roles. It serves as a negative feed back mechanism for PTHrP synthesis, since Ihh enhances PTHrP synthesis in the periarticular perichondrium [10]. In addition, PTH may modulate Ihh-induced phenomena such as bone collar formation by suppressing Ihh expression. RA may participate in the Ihh actions by activating Ihh expression.

Fig 1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days
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Ihh
PPR
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TypeX
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B
%
relative RNA level
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80
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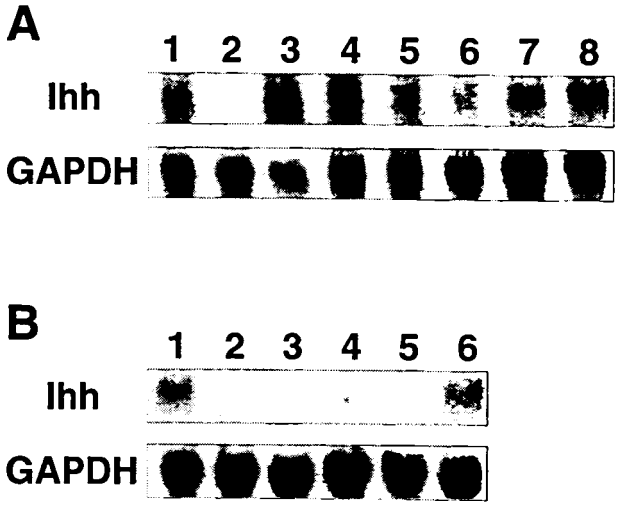
Fig 2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Ihh
GAPDH
B
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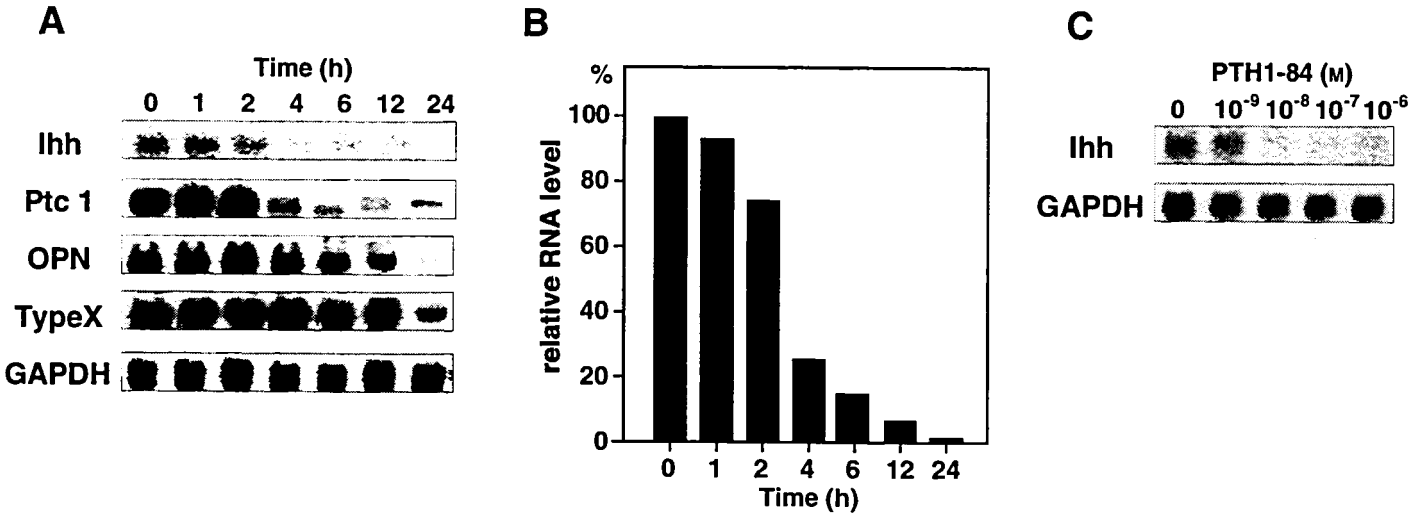
Ihh
GAPDH

Fig 3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Time (h)
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Ihh
Ptc 1
OPN
TypeX
GAPDH
B
%
100
80
60
40
20
0
relative RNA level
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Time (h)
C
PTH1-84 (M)
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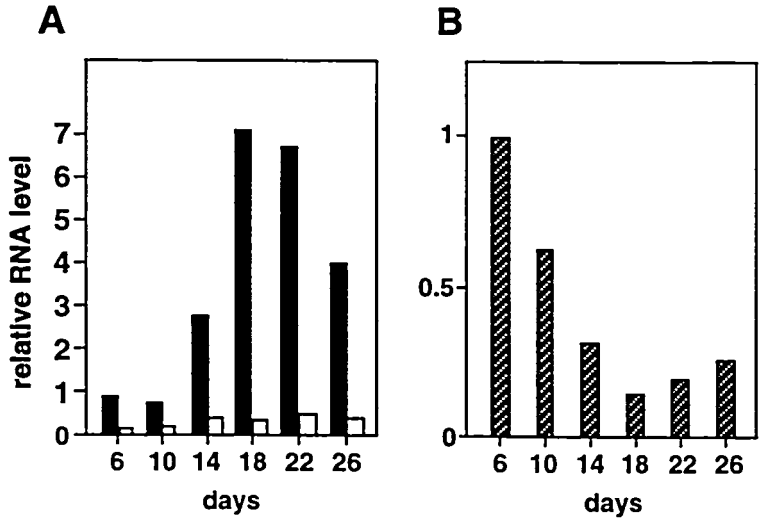
Ihh
GAPDH

Fig 4


A
B
relative RNA level
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6
5
4
3
2
1
0
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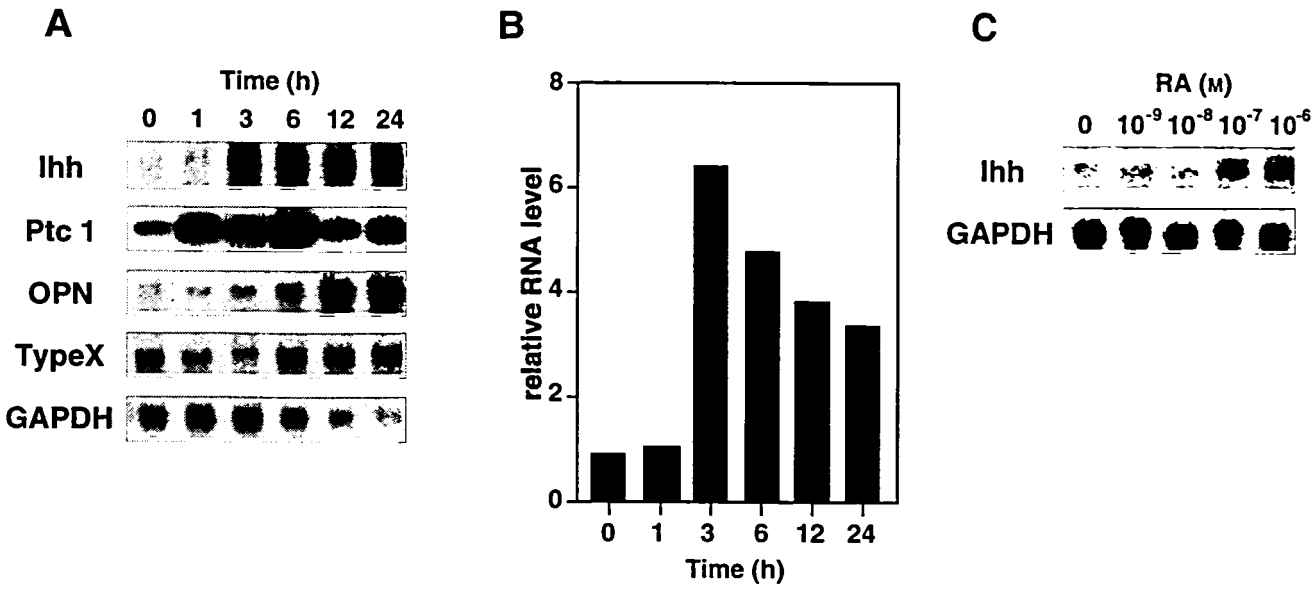
days
1
0.5
0
6 10 14 18 22 26
days

Fig 5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Ihh
Ptc 1
OPN
TypeX
GAPDH
B
relative RNA level
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Time (h)
C
RA (M)
0 10-9 10-8 10-7 10-6
Ihh
GAPDH

Fig 6

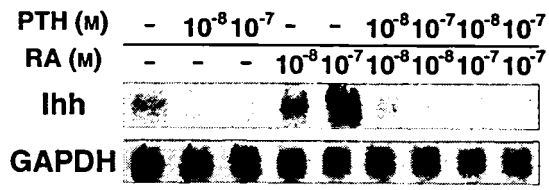
PTH (M) - 10-8 10-7 - - 10-8 10-7 10-8 10-7
RA (M) - - - 10-8 10-7 10-8 10-8 10-7 10-7
Ihh
GAPDH

Fig 7

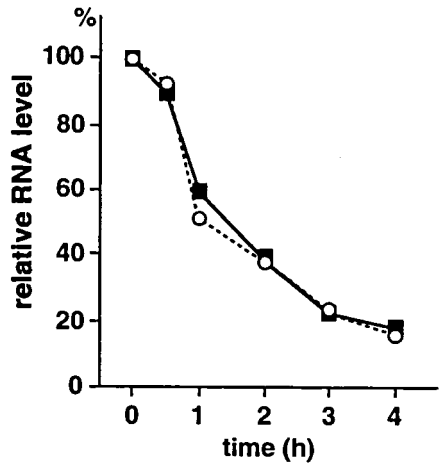

Fig 8

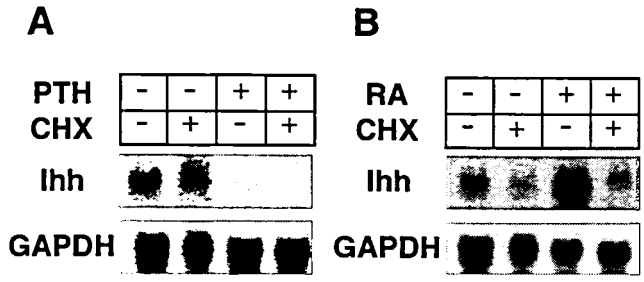

Fig 9