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Parathyroid Hormone and Transforming Growth Factor-β1 Coregulate Chondrocyte Differentiation In Vitro

E. Nasatzky,[1] E. Azran,[1] D. D. Dean,[2] Barbara D. Boyan,[2–4] and Z. Schwartz[1–3]

[1]Department of Periodontics, Hebrew University Hadassah Faculty of Dental Medicine, Jerusalem, Israel and Departments of [2]Orthopaedics, [3]Periodontics, and [4]Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, TX

Parathyroid hormone (1-34) (PTH(1-34) and transforming growth factor-β1 (TGF-β1) regulate chondrocyte proliferation, differentiation, and matrix synthesis. Both proteins mediate their effects in a dose- and time-dependent manner, and the effects are cell maturation specific. Moreover, similar signaling pathways are used, suggesting that there may be cross talk leading to coregulated cell response. To test this hypothesis, confluent cultures of rat costochondral resting zone and growth zone chondrocytes were treated with 0.22, 0.44, or 0.88 ng/mL of rhTGF-β1 for 24 h, followed by treatment with 10^{-11} to 10^{-8} *M* PTH(1-34) for 10 min or 24 h. [^{3}H]-Thymidine incorporation, specific activity of alkaline phosphatase (AP), and [^{35}S]-sulfate incorporation were measured. PTH(1-34) had no effect on [^{3}H]-thymidine incorporation by growth zone cells pretreated with 0.22 or 0.44 ng/mL of TGF-β1, but in cultures treated with 0.88 ng/mL, PTH(1-34) caused a dose-dependent decrease that was maximal at the lowest concentration tested. By contrast, PTH-(1-34) stimulated [^{3}H]-thymidine incorporation by resting zone cells, and this effect was additive with the stimulation caused by 0.22 ng/mL of TGF-β1. PTH(1-34) caused a synergistic increase in AP in growth zone cells treated with 0.44 or 0.88 ng/mL of TGF-β1, but not in cells treated with 0.22 ng/mL of TGF-β1. It had no effect on AP in resting zone cells pretreated with any concentration of TGF-β1. PTH(1-34) increased [^{35}S]-sulfate incorporation in growth zone and resting zone cell cultures treated with 0.22 ng/mL of TGF-β1 to levels seen in cultures treated with 0.88 ng/mL of TGF-β1 alone. These results support the hypothesis that PTH(1-34) and TGF-β1 coregulate growth plate chondrocytes and that the effects are cell maturation dependent.

Key Words: Parathyroid hormone; transforming growth factor β1; chondrocytes; differentiation; proliferation.

Received May 1, 2000; Revised June 12, 2000; Accepted June 22, 2000. Author to whom all correspondence and reprint requests should be addressed: Dr. Barbara D. Boyan, Department of Orthopaedics, MSC-7774, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900. E-mail: boyanb@uthscsa.edu

Introduction

Parathyroid hormone (PTH) produces a variety of important metabolic effects on skeletal tissue, including both bone and cartilage, which have been well documented and studied extensively *(1)*. Recent studies using PTH-related peptide (PTHrP) (–/–) knockout mice *(2,3)* have shown that PTHrP plays a fundamental role in regulating endochondral bone formation during embryonic development. Whether it plays a similar role in postfetal bone growth and endochondral ossification during bone wound healing is not yet known. However, in adolescent bone growth, the contribution of PTH to the process is also of considerable importance. In growing and adult mice, injection of PTH causes an anabolic effect, including initiation of chondrogenesis, hypertrophy and calcification, and premature closure of the growth plate *(4)*.

Cell culture studies demonstrate that PTH directly regulates chondrocyte proliferation, differentiation, and matrix synthesis. PTH stimulates chondrocyte proliferation *(5–7)*; however, the effects of PTH on chondrocyte metabolism and differentiation are not as clear. PTH has been shown to increase glycosaminoglycan production *(8–10)* and to stimulate the activities of alkaline phosphatase (AP)*(11)* and ornithine decarboxylase *(9,10)*. It induces the production of nuclear receptors for 1α,25-$(OH)_2D_3$ *(5,12)* and epidermal growth factor *(13)*. PTH has also been found to inhibit production of collagen *(14)*, including type X collagen *(15)*, and to decrease AP activity *(8,15–17)*, Ca incorporation into the matrix, and the number of nuclear receptors for 1α,25-$(OH)_2D_3$ *(15)*.

The effect of PTH on chondrocytes is mediated by receptors both in vivo and in vitro *(6,15,18–21)*. As chick

limb bud mesenchymal cells, which do not exhibit PTH receptors, become chondrocytes, PTH receptors are expressed on their cell membrane *(22)*. The mechanism by which PTH elicits its effects on chondrocytes appears to be complex. Several studies indicate that PTH stimulates cyclic adenosine monophosphate (cAMP) production *(5,8,11,23)*, eventually causing a change in protein kinase A. Other studies have found that some of the effects of PTH cannot be achieved only by changes in cAMP, such as increased [^{3}H]-thymidine incorporation *(24,25)* and proteoglycan production *(24,26,27)*, indicating that other mechanisms may be involved. These include inositol-triphosphate production and Ca ion flux *(17,24,27)*, phospholipase C activation through G proteins *(28)*, protein kinase C (PKC) *(11)*, and the PTH/PTHrP receptor *(29,30)*.

These disparate observations can be explained by differences in species, models, the timing and length of the addition of PTH, and the maturation state of the chondrocytes along the endochondral lineage. Recently, we have shown that the effect of PTH(1-34) on rat costochondral chondrocyte AP, phospholipase A_2, and of PKC specific activities are maturation dependent in terms of time course and signal transduction mechanism. Whereas both growth zone chondrocytes and resting zone chondrocytes exhibited a rapid response to PTH, only growth zone cells showed a long-term response. In growth zone cells, the effects of PTH were associated with changes in cAMP, whereas in resting zone chondrocytes, the effects of PTH appear to be associated with changes in PKC *(11)*.

Transforming growth factor-β1 (TGF-β1) is also a major regulator of chondrocyte maturation and differentiation. TGF-β1 is synthesized as a latent complex and is activated in the tissue *(31–35)*. It is potent in its ability to stimulate differentiation of mesenchymal cells *(36,37)*, causing them to differentiate into chondrocytes *(32)*, and then inhibiting their terminal differentiation *(36,38–42)*. TGF-β1 causes an increase in proliferation, but concentrations that stimulate [^{3}H]-thymidine incorporation (0.44 or 0.88 ng/mL) do not affect differentiation, and concentrations that modulate phenotypic expression (0.11 or 0.22 ng/mL) do not increase proliferation *(33)*. In addition to causing an increase in AP activity *(33)*, TGF-β1 increases collagen *(43)* and proteoglycan *(34,44,45)* production. Recently, it was found that the effects of TGF-β1 on chondrocytes are cell maturation dependent *(33,46)*. Moreover, TGF-β1 is produced by chondrocytes *(35,47,48)*; hence, part of its effect is probably via autocrine mechanisms. TGF-β1 stimulates PKC activity in chondrocyte cultures after a 6-h exposure to the growth factor, suggesting that some of its effects could be mediated through this signal transduction pathway *(34)*.

It is becoming increasingly clear that TGF-β1 has interactive effects with other growth factors and hormones known to regulate chondrocyte metabolism, such as fibroblast growth factor, insulin-like growth factor-1, and vitamin D metabolites *(46,49,50)*. Recently, we found that there is a synergistic enhancement of resting zone chondrocyte differentiation and PKC activity when cells are exposed to rhTGF-β1 and 24R,25-$(OH)_2D_3$ together *(46),* and that the activation of the latent TGF-β1 produced by the resting zone and growth zone chondrocytes is regulated by 1α,25-$(OH)_2D_3$ *(35)*. Moreover, we have shown that chondrocyte response to the systemic hormone 17β-estradiol can be modulated by rhTGF-β1 *(51)*.

Interaction between PTH and TGF-β1 has already been found in squamous cell carcinoma cells *(52,53)*, renal epithelial cells *(54)*, and osteoblasts *(55,56)*. Recently, in cultured rat articular chondrocytes, it was found that both human PTHrP and human PTH significantly attenuate TGF-β1-stimulated DNA synthesis *(57)*. In a three-dimensional differentiation model of growth cartilage cells, PTH reversibly suppressed both the induction of AP activity and calcium uptake stimulated by TGF-β1; and in rabbit costal chondrocytes, treatment with TGF-β1 resulted in increases in the number of PTH receptors without any change in their affinity *(58)*.

These latter studies examined heterogeneous populations of cells; thus, the effects of TGF-β1 and PTH on specific chondrocyte maturation states was not addressed, and the results reported reflected the aggregate response of cells with very different phenotypes. The aim of the present study was to examine whether the interaction between PTH and TGF-β1 influences proliferation and differentiation of growth zone or resting zone chondrocytes, and to determine whether the interaction is maturation dependent. We used a chondrocyte culture system in which chondrocytes at two different stages of maturation are isolated from two different zones of costochondral cartilage *(11,59,60)*.

Results

Overall Experimental Approach

For all studies, cells were first pretreated with vehicle, 0.22, 0.44, or 0.88 ng/mL of rhTGF-β1 for 24 h. In one-half of the experiments, this medium was then replaced with fresh media containing vehicle, 10^{-11}, 10^{-10}, 10^{-9}, or 10^{-8} *M* PTH(1-34) for an additional 24 h. For the other half of the experiments, PTH(1-34) was added to the TGF-β1-treated cultures 10 min prior to harvest. At harvest, [^{3}H]-thymidine incorporation, specific activity of AP, and [^{35}S]-sulfate incorporation were determined.

[^{3}H]-Thymidine Incorporation

When growth zone chondrocytes were treated with either rhTGF-β1 or PTH(1-34), [^{3}H]-thymidine incorporation was stimulated (Fig. 1A, B). Moreover, the effect of each agent was comparable, with the maximal dose of either TGF-β1 or PTH(1-34) causing approx 3.5–4.0 × 10^5 dpm/well of incorporation. The effect for rhTGF-β1 alone is shown in Fig. 1A by the cross-hatched bars, which indicate

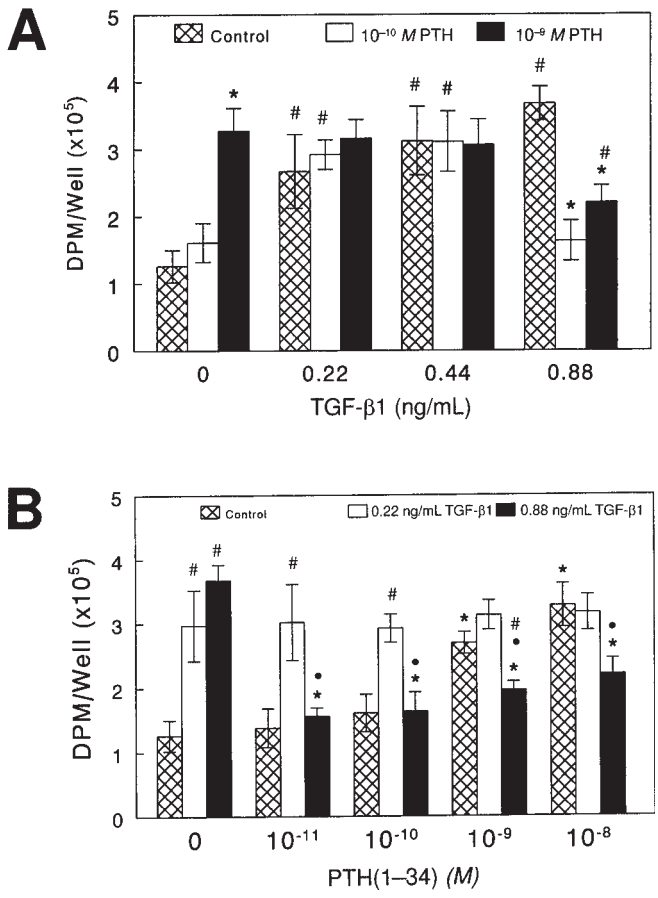

Fig. 1. Effect of pretreatment with rhTGF-β1, followed by treatment with PTH(1-34), on [^{3}H]-thymidine incorporation by growth zone chondrocytes. Fourth-passage cultures of growth zone chondrocytes were cultured to confluence and then pretreated with rhTGF-β1 ([A], 0.22, 0.44, and 0.88 ng/mL; [B] 0.22 and 0.88 ng/mL) for 24 h followed by treatment with bovine PTH(1-34) [A] 10^{-10} or 10^{-9} *M*; [B] 10^{-11}–10^{-8} *M*) for an additional 24 h. Four hours prior to harvest, the cultures were pulsed with [^{3}H]-thymidine. At harvest, radioisotope incorporation by the cells was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments, all yielding comparable results. $^*P < 0.05$, vs PTH(1-34) vehicle (control); $^{\#}P < 0.05$ vs pretreatment with rhTGF-β1 vehicle (control); • $P < 0.05$, vs pretreatment with 0.22 ng/mL of rhTGF-β1.

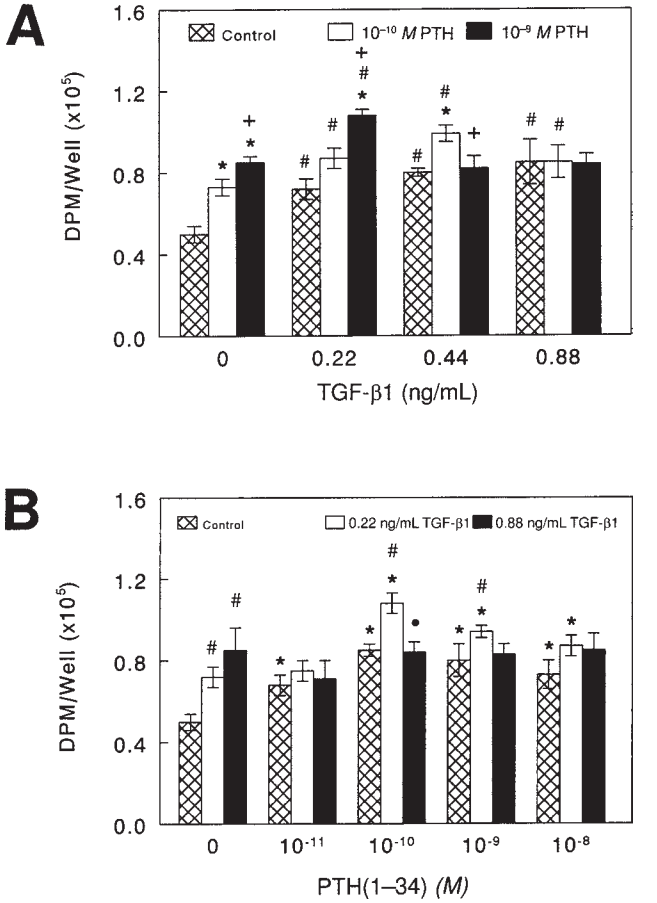

Fig. 2. Effect of pretreatment with rhTGF-β1, followed by treatment with PTH(1-34), on [^{3}H]-thymidine incorporation by resting zone chondrocytes. Fourth-passage cultures of resting zone chondrocytes were cultured to confluence and then pretreated with rhTGF-β1 ([A] 0.22, 0.44, and 0.88 ng/mL; [B] 0.22 and 0.88 ng/mL) for 24 h followed by treatment with bovine PTH(1-34) [A] 10^{-10} and 10^{-9} *M*; [B] 10^{-11}–10^{-8}*M*) for an additional 24 h. Four hours prior to harvest, the cultures were pulsed with [^{3}H]-thymidine. At harvest, radioisotope incorporation by the cells was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments, all yielding comparable results. $^*P < 0.05$ vs PTH(1-34) vehicle (control); $^{\#}P < 0.05$ vs pretreatment with rhTGF-β1 vehicle (control); • $P < 0.05$ vs pretreatment with 0.2 ng/mL of rhTGF-β1; $^+P < 0.05$ vs treatment with 10^{-10} *M* PTH(1-34).

that the cells were first pretreated with rhTGF-β1, followed by treatment with PTH(1-34) vehicle for 24 h. In Fig. 1B, the same TGF-β1 effect is shown on the *x*-axis at the left, where PTH(1-34) vehicle was added to the cultures. Similarly, the effect of PTH(1-34) alone is shown in Fig. 1B by the cross-hatched bars, and in Fig. 1A on the *x*-axis at the left.

In cultures pretreated with 0.22 or 0.44 ng/mL of TGF-β1 (Fig. 1A), PTH(1-34) had no additional effect on [^{3}H]-thymidine incorporation. However, in cultures pretreated with 0.88 ng/mL of rhTGF-β1, subsequent PTH(1-34) treatment inhibited the increase in proliferation owing to TGF-β1 (Fig. 1A). The inhibitory effect of PTH(1-34) on cells pretreated with 0.88 ng/mL of rhTGF-β1 was seen at all concentrations of hormone tested, but the maximal effect was observed at 10^{-11} *M* PTH(1-34) (Fig. 1B).

Resting zone cells responded similarly to treatment with either rhTGF-β1 or PTH(1-34) alone (Fig. 2). When resting zone cells were first pretreated with rhTGF-β1, followed by treatment with PTH(1-34) vehicle (Fig. 2A, cross-hatched bars; Fig. 2B, *x*-axis, on far left), [^{3}H]-thymidine incorporation was increased. As seen for growth zone cells, the effect of PTH(1-34) alone was also stimulatory (Fig. 2B, cross-hatched bars; Fig. 2A, *x*-axis on the left). Furthermore, each agent alone was comparable in its effect on [^{3}H]-thymidine incorporation.

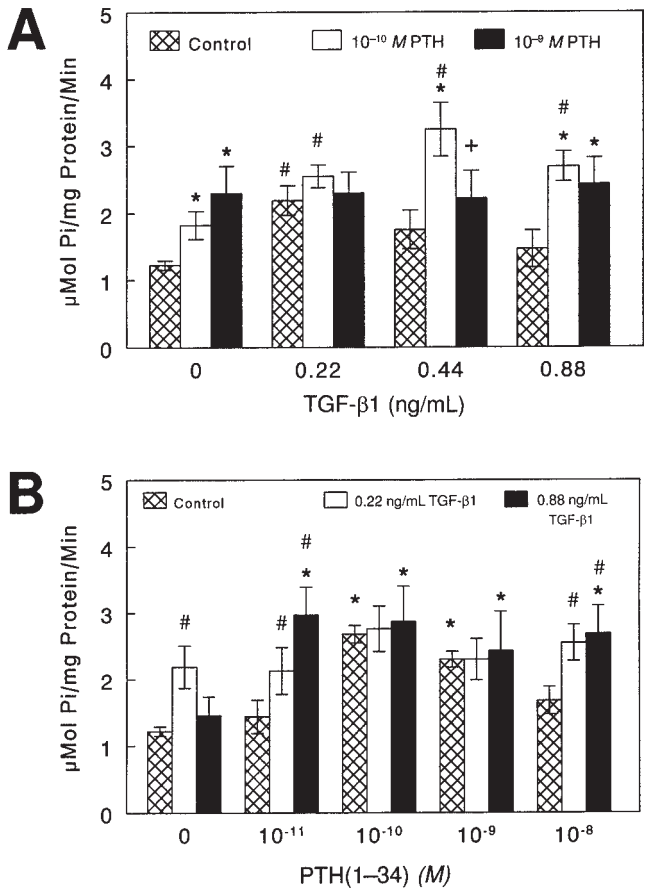

Fig. 3. Effect of pretreatment with rhTGF-β1, followed by treatment with PTH(1-34), on specific activity of AP in growth zone chondrocytes. Fourth-passage cultures of growth zone chondrocytes were cultured to confluence and then pretreated with rhTGF-β1 ([A] 0.22, 0.44, and 0.88 ng/mL; [B] 0.22 and 0.88 ng/mL) for 24 h followed by treatment with bovine PTH(1-34) ([A] 10^{-10}–10^{-9} *M*; [B] 10^{-11}–10^{-8} *M*) for an additional 24 h. At harvest, ALPase specific activity in the cell layers was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments, all yielding comparable results. $^{*}P < 0.05$ vs PTH(1-34) vehicle (control); $^{\#}P < 0.05$ vs pretreatment with rhTGF-β1 vehicle (control); $^{+}P < 0.05$ vs treatment with 10^{-10} *M* PTH(1-34).

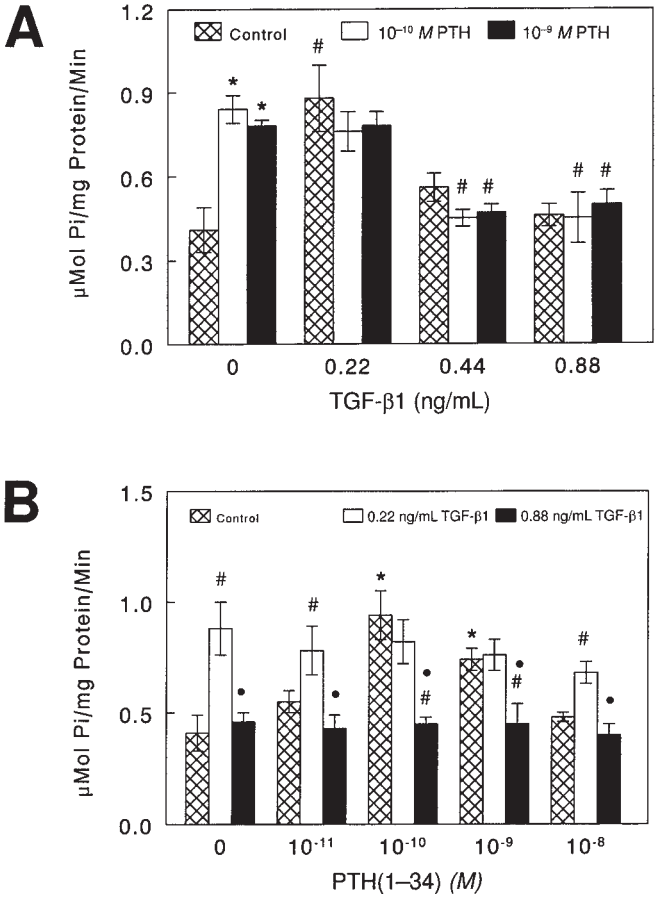

Fig. 4. Effect of pretreatment with rhTGF-β1, followed by treatment with PTH(1-34), on specific activity of AP in resting zone chondrocytes. Fourth-passage cultures of resting zone chondrocytes were cultured to confluence and then pretreated with rhTGF-β1 ([A] 0.22, 0.44, and 0.88 ng/mL; [B] 0.22 and 0.88 ng/mL) for 24 h followed by treatment with bovine PTH (1-34) ([A] 10^{-10} and 10^{-9} *M*; [B] 10^{-11}–10^{-8} *M*) for an additional 10 min. At harvest, ALPase specific activity in the cell layers was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments, all yielding comparable results. $^{*}P < 0.05$ vs PTH(1-34) vehicle (control); $^{\#}P < 0.05$ vs pretreatment with rhTGF-β1 vehicle (control); • $P < 0.05$ vs pretreatment with 0.22 ng/mL of rhTGF-β1.

[^{3}H]-Thymidine incorporation was increased by PTH(1-34) in a dose-dependent manner after pretreatment with rhTGF-β1 (Fig. 2A). At low concentrations of rhTGF-β1 (0.22 ng/mL), PTH(1-34) caused a further stimulation that was additive with the effect of rhTGF-β1. At 0.22 ng/mL of rhTGF-β1, the additive effect of PTH(1-34) was evident at 10^{-10} and 10^{-9} *M*, but not at the higher concentration of PTH(1-34) (Fig. 2B). In cultures treated with 0.88 ng/mL of rhTGF-β1, no effect of PTH(1-34) was evident.

Specific Activity of AP

By itself, rhTGF-β1 caused a dose-dependent increase in AP activity in growth zone cell cultures that was maximal at 0.22 ng/mL (cross-hatched bars in Fig. 3A). PTH(1-34) alone (cross-hatched bars in Fig. 3B) also stimulated enzyme activity, and the maximal effect was observed at 10^{-10} *M*.

In cultures pretreated with 0.22 ng/mL of rhTGF-β1 for 24 h, 24-h treatment with PTH(1-34) had no additional effect; however, in cultures pretreated with 0.44 ng/mL of rhTGF-β1, 10^{-10} *M* PTH(1-34) caused a synergistic increase in AP (Fig. 3A). This effect was still evident in cultures pretreated with 0.88 ng/mL of rhTGF-β1, but to a lesser extent. In cultures pretreated with rhTGF-β1 for 24 h, followed by PTH(1-34) for 10 min, the results were similar (data not shown).

In resting zone cells, rhTGF-β1 alone caused a biphasic increase in AP that was maximal in cultures treated with 0.22 ng/mL (cross-hatched bars in Fig. 4A). PTH(1-34) alone, when added to the cultures for 24 h, had no effect on

AP (data not shown); this effect on AP was also observed in cultures pretreated with rhTGF-β1 (data not shown). However, when PTH(1-34) was added to the cultures for 10 min, AP activity was increased, with maximal effects at 10^{-10} *M* (cross-hatched bars in Fig. 4B). This increase was comparable to the response of the cells to treatment with 0.22 ng/mL of TGF-β1 alone. In cells pretreated with any concentration of TGF-β1, PTH(1-34) treatment for 10 min had no effect (Fig. 4A).

[^{35}S]-Sulfate Incorporation

When growth zone chondrocytes were treated with TGF-β1 alone, there was a dose-dependent increase in [^{35}S]-sulfate incorporation into the cell layer that was maximal with 0.88 ng/mL (solid bars in Fig. 5A). Treatment with PTH(1-34) alone also stimulated proteoglycan production in the cell layer, with maximal effects at 10^{-8} *M* (solid bars in Fig. 5B). Addition of PTH(1-34) to TGF-β1-pretreated cultures increased [^{35}S]-sulfate incorporation in the cell layer to levels seen in cultures treated with either 0.88 ng/mL of rhTGF-β1 or PTH(1-34) alone. Resting zone chondrocytes behaved in a similar manner (data not shown).

Discussion

The results of the present study support the hypothesis that TGF-β1 and PTH(1-34) coregulate chondrocyte differentiation. This coregulation is dependent on the maturation state of the chondrocytes. PTH(1-34) treatment of TGF-β1-stimulated cells attenuated, and even abolished, the increase in [^{3}H]-thymidine incorporation that was seen in growth zone chondrocytes treated with either agent alone. However, in resting zone chondrocytes, PTH(1-34) caused a synergistic increase in [^{3}H]-thymidine incorporation by TGF-β1-stimulated cells. Furthermore, PTH(1-34) caused a synergistic increase in the specific activity of AP in growth zone chondrocytes pretreated with TGF-β1, whereas in resting zone chondrocytes, the effect of TGF-β1 dominated, and no subsequent effect of PTH(1-34) could be seen. In contrast to the cell-maturation-dependent effects already noted, PTH(1-34) did not alter the response of either cell type to TGF-β1 with respect to [^{35}S]-sulfate incorporation.

Our observations regarding [^{3}H]-thymidine incorporation in resting zone chondrocytes are similar to those described in the literature, in which PTH was shown to attenuate TGF-β1-stimulated DNA synthesis in rat articular chondrocytes *(61)*. However, there remains disagreement concerning the effects of PTH(1-34) on TGF-β1-dependent increases in AP activity. Whereas we found that PTH(1-34) caused a synergistic increase in enzyme activity of TGF-β1-pretreated growth zone cells, others found that PTH reversibly suppressed the induction of AP activity stimulated by TGF-β1 *(57)*. This latter study used a three-dimensional differentiation model of growth cartilage cells, which includes chondrocytes at various stages of differentiation and maturation. As already noted, the effect of

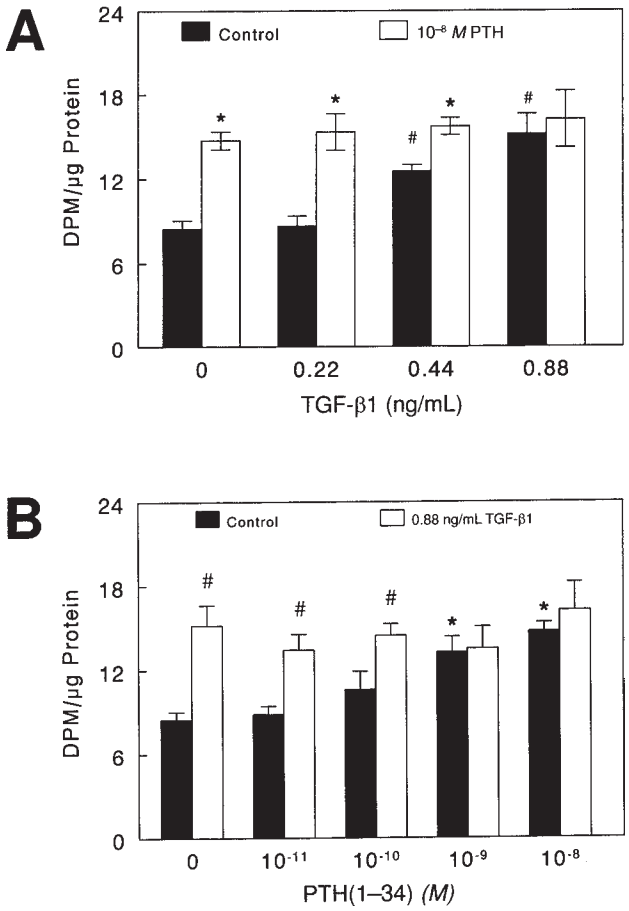

Fig. 5. Effect of pretreatment with rhTGF-β1, followed by treatment with PTH(1-34), on [^{35}S]-sulfate incorporation by growth zone chondrocytes. Fourth-passage cultures of growth zone chondrocytes were cultured to confluence and then pretreated with rhTGF-β1 ([A] 0.22, 0.44, and 0.88 ng/mL; [B] 0.88 ng/mL) for 24 h followed by treatment with bovine PTH(1-34) ([A] 10^{-8}; [B] 10^{-11}–10^{-8} *M*) for an additional 24 h. At harvest, [^{35}S]-sulfate incorporation by the cultures was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments, all yielding comparable results. *$P < 0.05$ vs PTH(1-34) vehicle (control); #$P < 0.05$, vs pretreatment with rhTGF-β1 vehicle (control).

PTH(1-34) on TGF-β1-stimulated AP was cell maturation dependent, with fewer mature resting zone cells responding to PTH(1-34) with a decrease in the TGF-β1-induced increase in enzyme activity. Thus, the discrepancy in reported results may reflect the fact that we examined subsets of the total population of growth plate chondrocytes, whereas the three-dimensional model examined the total growth plate response.

The results of the present study show, for the first time, that TGF-β1 pretreatment followed by PTH(1-34) synergistically enhances the early differentiation of these cells by inhibiting their replication and increasing their AP activity. We previously demonstrated that both TGF-β1 and PTH(1-34) promote differentiation of prehypertrophic and upper hypertrophic cells (growth zone cells) by

increasing AP *(11,33)*. In vivo, the activity of this enzyme increases prior to terminal differentiation *(62,63)* and is considered an early differentiation marker. The growth zone cells in our model do not express type X collagen mRNA (unpublished data), nor do they produce type X collagen under basal conditions or when stimulated by dexamethasone *(64)*; thus, we did not examine the effects of TGF-β1 or PTH(1-34) on this marker of the late hypertrophic chondrocyte in previous studies or in the present one. Both TGF-β1 *(33)* and PTH(1-34) *(11)* retard calcification in the growth plate by inhibiting matrix vesicle phospholipase A_2 activity. Thus, it is unlikely that TGF-β1 and PTH(1-34) promote late differentiation events when used in combination.

Proteoglycan production, based on [^{35}S]-sulfate incorporation in the cell layer, was unaffected by PTH(1-34) treatment of TGF-β1-stimulated cells, suggesting that the effect of PTH(1-34) was limited to events surrounding the transition of cells from a proliferative state to initiation of differentiation in the endochondral pathway. Both agents alone were anabolic in terms of matrix production. This anabolic effect was not altered during the transition in phenotypic expression along the endochondral differentiation pathway, at least within the time frame of the experiment.

The results support the hypothesis that TGF-β1 initiates a sequence of events that sensitizes the chondrocytes to PTH(1-34). These cellular responses may be mediated by either genomic or nongenomic mechanisms, or through both. It is likely that TGF-β1 upregulates PTH receptors or enzymes that modulate these receptors posttranslationally, potentially modulating the binding capacity or affinity of the receptors for PTH(1-34). Previous studies have shown that TGF-β increases the number of PTH receptors in rabbit costal chondrocytes, but does not change their affinity for the hormone *(58)*. Similar results were found in UMR 106-06 cells *(56)*.

Recently, we showed that both TGF-β1 *(34)* and PTH(1-34) *(11)* mediate some of their effects on chondrocytes through the regulation of PKC. Both agents stimulate PKC in resting zone cells. Moreover, PKC-dependent pathways mediate some of the physiologic effects observed in response to each of the factors. This may explain the synergistic effect of PTH(1-34) on [^{3}H]-thymidine incorporation in TGF-β1-pretreated resting zone cells. However, it does not explain the attenuation of AP activity in these cells. Furthermore, the regulation of PKC does not explain the synergistic effect on AP activity in growth zone chondrocytes, because in these cells, the effects of PTH(1-34) are associated with changes in cAMP or other unknown mechanisms *(11)*.

Both TGF-β1 and PTH(1-34) have been shown previously to increase AP activity specifically by increasing enzyme activity in extracellular matrix vesicles *(11,33)*. The synergistic increase in AP activity of growth zone chondrocytes observed in the present study was seen whether the TGF-β1-pretreated cells were treated with PTH(1-34) for 10 min or for 24 h. This suggests that TGF-β1 induced the formation of new, AP-enriched matrix vesicles, and at least part of the effect of PTH(1-34) was on preexisting enzyme. PTH(1-34) is known to stimulate 1α-hydroxylase activity in kidney cells, resulting in increased production of 1,25-$(OH)_2D_3$ *(65)*. Growth zone cells also possess 1α-hydroxylase activity *(66)* and secrete 1,25-$(OH)_2D_3$ *(67),* which can act directly on matrix vesicles to increase the specific of AP activity through rapid nongenomic mechanisms *(68)*.

The effects of PTH(1-34) on TGF-β1-pretreated chondrocytes described herein are specific to the maturation state of the cell in the endochondral pathway. Previous studies showed that the effects of TGF-β1 or PTH(1-34) alone on proliferation, specific activity of AP, and proteoglycan production are cell maturation dependent *(11,33)*. Each of these factors exerts its effects through different, but overlapping, signaling pathways *(11,69,70)*, which may account for the observed results in the present study. However, the exact mechanisms mediating this effect are not known. Several important questions are raised: Does TGF-β1 regulate PTH receptors in these chondrocytes? Does PTH(1-34) regulate TGF-β1 production or activation? Does PTH(1-34) modulate production of 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$, both of which are known to regulate the chondrocytes in a cell-maturation-specific manner *(71)*? And does the effect of PTH(1-34) on TGF-β1-pretreated cells involve genomic or nongenomic mechanisms or both? Resolution of these questions is presently under way.

The studies described herein used PTH(1-34) to challenge the cells. However, many recent studies have suggested that PTHrP may be of greater significance to the local regulation of chondrocyte differentiation and maturation *(3,29,30,72)*. Although this may be true for fetal growth plates, it is not necessarily the case for postfetal chondrocytes. Studies have shown that chondrocytes from adult rats respond preferentially to PTH and not to PTHrP *(73)*. In addition, a large body of more recent evidence is evolving that indicates changes in expression of the PTH/PTHrP receptor may play a significant role in regulating cell response to the hormone *(74,75)*. In any event, it is of great interest to know whether the mechanisms involved in embryonic growth and development are also involved in postfetal responses of cells in the endochondral pathway to PTH.

Material and Methods

Chondrocyte Cultures

The culture system used in the present study has been described previously *(59,76)*. Chondrocytes were derived from the costochondral resting zone and growth zone cartilage of 125-g male Sprague-Dawley rats. Cells were incubated at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 μg/mL of ascorbic acid, and 1% penicillin-streptomycin in an

atmosphere of 5% CO_2 and 100% humidity. Fourth-passage cells were used for these experiments because previous studies demonstrated that the chondrocytes retained their differential phenotype and responses to various hormones and growth factors through the number of passages in culture *(11,35,60,71)*.

Experimental Design

Fourth-passage resting zone or growth zone cells were grown to confluence. At confluence, the media were replaced, and the cells were incubated for 24 h with media containing 0.22, 0.44, or 0.88 ng/mL of rhTGF-β1 (R & D Systems, Minneapolis, MN) or vehicle alone. After 24 h, the media were replaced with media containing 10^{-11}–10^{-8} *M* bovine PTH(1-34) (Sigma, St. Louis, MO) or vehicle alone (control). The incubation was continued for another 24 h. Alternatively, the cultures were treated for 24 h with 0.22, 0.44, or 0.88 ng/mL of rhTGF-β1 or vehicle alone (control), and PTH(1-34) [10^{-11}–10^{-8} *M*] or vehicle alone (control) added to the cultures for the last 10 min. In each case, the cells were harvested and assayed as described subsequently.

[^{3}H]-Thymidine Incorporation

DNA synthesis by quiescent resting zone or growth zone cells was estimated by measuring [^{3}H]-thymidine incorporation into trichloroacetic acid (TCA)-insoluble precipitates *(77)*. Chondrocytes were grown to confluence in the wells of 96-well tissue culture plates, and quiescence was induced by culturing the cells for 48 h in DMEM containing 1% FBS. Then, the medium was replaced with medium containing TGF-β1, also containing 1% FBS, and the incubation continued for 24 h. This medium was then replaced with medium containing PTH(1-34) and 1% FBS for another 24 h. [^{3}H]-Thymidine (NEN/DuPont, Boston, MA) (50 μL) was added 4 h before harvest so that the final concentration in the medium was 2 μCi/mL. At harvest, the cell layers were washed twice with cold phosphate-buffered saline (PBS) and twice with 5% TCA and then treated with saturated TCA for 30 min. TCA-precipitable material was dissolved in 0.2 mL of 1% sodium dodecyl sulfate, and the radioactivity was measured by scintillation spectroscopy.

Specific Activity of AP

Preparation of Cell Layer

Cell layers were prepared according to the method of Hale et al. *(62)* as described previously *(76)*. Briefly, cells were cultured in 24-well culture dishes (Corning, Corning, NY). At harvest, the media were decanted, the cell layer was washed twice with PBS, and then removed using a cell scraper. After centrifugation, the cell layer pellet was washed twice with PBS and resuspended by vortexing in 500 μL of deionized water containing 25 μL of 1% Triton X-100. Enzyme assays were then performed using these cell layer lysates.

Enzyme Assay

AP (orthophosphoric monoester phosphohydrolase alkaline; EC 3.1.3.1) activity in the cell layer was measured as a function of release of paranitrophenol from paranitrophenylphosphate at pH 10.2 *(78)*. Protein in the cell layer was measured using the bicinchoninic acid method *(79)*.

[^{35}S]-Sulfate Incorporation

Proteoglycan synthesis was measured by [^{35}S]-sulfate incorporation according to the method of O'Keefe et al. *(80)*, as previously described *(81)*. Because more than 85% of the total radiolabeled proteoglycan is found in the cell layer *(81)*, we only examined the effect of TGF-β1 and PTH(1-34) in this compartment. Thus, our results do not reflect any changes that may have occurred in the conditioned media.

Fourth-passage growth zone and resting zone chondrocytes were grown to confluence in 24-well culture plates (Corning) and then treated with medium containing TGF-β1 containing 10% FBS for 24 h. This medium was replaced with medium containing PTH(1-34) for 24 h. Four hours prior to harvest, 50 μL of DMEM containing 18 μCi/mL of $^{35}SO_4$ (NEN/DuPont) and 0.814 m*M* carrier sulfate was added to each culture. At harvest, the conditioned media were removed and the cell layers (cells and matrix) collected in two 0.25-mL portions of 0.25 *M* NaOH. The protein content of the cell layer was determined using the BCA method *(79)*. The total volume was adjusted to 0.75 mL by the addition of 0.15 *M* NaCl, and the sample was dialyzed in a 12,000–14,000 molecular weight cutoff membrane against buffer containing 0.15 *M* NaCl, 20 m*M* Na_2SO_4, and 20 m*M* Na_2HPO_4 (pH 7.4) at 4°C. The dialysis solution was changed until the radioactivity in the dialysate reached background levels. The amount of $^{35}SO_4$ incorporated was determined by liquid scintillation spectrometry and was calculated as disintegrations per minute/milligram of protein in the cell layer.

Statistical Analyses

The data presented are from one of three replicate experiments. For any given experiment, each data point represents the mean ± SEM for six individual cultures. The data were analyzed by analysis of variance, and statistical significance was determined by comparing each data point to the control using Bonferroni's modification of student's *t*-test. Values of $P < 0.05$ were considered significant.

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