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Transforming growth factor-β_1 regulates steady-state PTH/PTHrP receptor mRNA levels and PTHrP binding in ROS 17/2.8 osteosarcoma cells

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Abstract

The effect of transforming growth factor β_1 (TGF-β_1) on the expression of mRNA for the parathyroid hormone receptor and binding of iodinated parathyroid hormone-related protein in ROS 17/2.8 osteosarcoma cells was evaluated. TGF-β_1 stimulated a 2–7-fold increase in steady state mRNA levels for the parathyroid hormone receptor at a maximal dose of 5 ng/ml, with increased levels of expression at 6 h of TGF-β_1-incubation, and peak levels at 8–24 h. Receptor binding studies revealed a significant increase in PTHrP-specific binding with TGF-β_1 doses as low as 0.5 ng/ml and a 55% increase in numbers of receptors with no alteration in binding affinity with 5.0 ng/ml TGF-β_1. Time course studies indicated that receptor binding was increased at 24 h with peak levels reached at 48 h of treatment. PTH-stimulated cAMP levels were significantly increased in ROS 17/2.8 cells treated with TGF-β_1 (0.5 ng/ml) for 48 h. These data indicate that TGF-β_1 upregulates steady-state mRNA, ligand binding and PTH/PTHrP receptor signaling in rat osteosarcoma cells. The effects of TGF-β_1 on bone may be attributed in part to regulation of the PTH/PTHrP receptor at the molecular level.

Key words: Osteoblast; Parathyroid hormone; Receptor; Transforming growth factor-β

1. Introduction

Parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP) exert their effects on bone by binding to the same cell membrane receptor on osteoblasts (Jüppner et al., 1988; Nissenson et al., 1988). The cDNA for the PTH/PTHrP receptor has recently been cloned and sequenced and belongs to the family of G protein-coupled receptors with seven transmembrane domains (Jüppner et al., 1991). PTH and PTHrP bind to the PTH/PTHrP receptor with similar affinity resulting in intracellular accumulation of cAMP and inositol trisphosphate and increased levels of intracellular calcium (Abou-Samra et al., 1992; Jüppner et al., 1991). Characterization of the binding affinity and numbers of receptors per cell on osteoblasts has been extensively investigated and reviewed (Pun et al., 1990; Orloff et al., 1989). Information regarding local and systemic regulation of the PTH/PTHrP receptor is not well known. Transforming growth factor-β (TGF-β) is a polypeptide growth factor localized in high concentrations in bone (Pfeilschifter et al., 1990). It has been found to regulate several osteoblast phenotype characteristics including alkaline phosphatase activity, collagen synthesis, osteopontin and osteocalcin levels (Centrella et al., 1988). PTH opposes the effects of TGF-β on mitosis, collagen production, and alkaline phosphatase activity in rat calvarial cells (Centrella et al., 1988); however, little is known regarding the ability of TGF-β to modulate PTH or PTHrP effects. TGF-β_1

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has been reported to stimulate production of PTHrP from uterine cells, keratinocytes, and squamous cancer cells (Casey et al., 1992; Merryman et al., 1993; Allinson and Drucker, 1992; Kiriyama et al., 1993) and to accentuate the PTH-stimulated cAMP response in UMR 106 osteoblastic cells (Gutierrez et al., 1990). These findings suggest that TGF-β plays a key role in the functions of PTH/PTHrP in bone; however, the ability of TGF-β to alter expression of mRNA for the PTH/PTHrP cell surface receptor on osteoblasts is not known. The purpose of this study was to determine if TGF-β_1 alters PTH/PTHrP receptor mRNA levels and subsequently receptor binding and signaling in ROS 17/2.8 osteosarcoma cells.

2. Materials and methods

2.1. Cell culture

ROS 17/2.8 osteosarcoma cells (Majeska et al., 1980) were maintained in DMEM-F12 media containing 5% fetal bovine serum in a humidified, 5% CO_2, 37°C incubator. Cell culture media and supplements were obtained from Gibco (Grand Island, NY).

2.2. Northern blot analysis

ROS 17/2.8 cells were grown 24–48 h post-confluence prior to placement of TGF-β_1 (recombinant human, R & D Systems, Minneapolis, MN) or vehicle control in serum-free DMEM-F12 media for designated time periods. Total RNA was isolated from one 75 cm^2 flask per treatment by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987) and quantitated by A_{260}. Total RNA (20 μg) was denatured and electrophoresed in sample buffer (50% formamide, 10% MOPS buffer, 18% formaldehyde, 10% glycerol, 0.5% bromophenol blue, 1 μg ethidium bromide) on 1.2% agarose-formaldehyde gels (60 V, 4.5 h). The RNA was transferred to nylon membranes (Duralon U.V., Stratagene, La Jolla, CA) via passive transfer, and crosslinked by U.V. transillumination (Stratalinker, Stratagene Inc.). The nylon membranes were pre-hybridized for 1 h at 42°C (53% formamide, 5 × SSC, 100 mM Tris, 10 × Denhardt's, 0.2% SDS and 1 mg/ml denatured salmon sperm DNA) in a hybridization oven. The rat PTH/PTHrP receptor cDNA probe (R15B) (Abou-Samra et al., 1992) was prepared from a 1810 bp coding region cDNA fragment subcloned into pcDNA I (Invitrogen, San Diego, CA) and was labeled with [α^{32}P]dCTP (NEN Dupont, Boston, MA) using a random primer labeling kit (Stratagene) to 10^9 dpm/μg. The probe was added to the pre-hybridization tube with the membrane and hybridized overnight at 42°C. The membranes were washed 8 times for 15 min each wash at 50°C with decreasing concentrations of SSC (2 × –0.25 ×) in 0.1% SDS. The blots were exposed to Kodak X-OMAT film at −70°C with intensifying screens, and developed in an automatic film processor. Blots were also stripped and reprobed with a cDNA probe for 185 rRNA (de Papp and Stewart, 1993) as a control for even loading.

2.3. Binding assays

PTH/PTHrP receptor binding assays were performed as described (McCauley et al., 1992a). Briefly, ROS 17/2.8 cells, grown to confluence in 24-well plates, were pretreated with TGF-β_1 or control vehicle in DMEM-F12 media containing 1–2% fetal bovine serum for 48 h. For binding assays triplicate wells were incubated with 25 000–35 000 cpm of monoiodinated [^{125}I]PTHrP (1–36) (McCauley et al., 1992b) in addition to varying concentrations of nonradioactive PTHrP (1–36) (Bachem, Torrance, CA). The cells were incubated for 2 h at 4°C with gentle shaking. The unbound peptides were washed off the cell monolayer twice with Hank's buffered salt solution (HBSS), the cells were lysed with 0.5 M NaOH for 30 min, and the resultant suspension counted in a scintillation counter. Specific binding was calculated by subtracting the radioactivity bound in the presence of excess unlabeled PTHrP (1–36) (1.0 μM) from that observed in the presence of tracer only and expressed as a ratio of the total cpm added.

2.4. Adenylate cyclase stimulation assay

The adenylate cyclase stimulation assay and cAMP binding assay were performed as previously described (McCauley et al., 1992b). Briefly, ROS 17/2.8 cells were grown 24–48 h postconfluence in 24-well plates. TGF-β_1 or vehicle control was preincubated with confluent ROS cells in triplicate for 48 h with a media change at 24 h. The cells were rinsed and incubated with bPTH (1–34) (Bachem) (10 nM) or control vehicle for 10 min at 37°C in calcium- and magnesium-free HBSS containing 0.1% bovine serum albumin (BSA) and 1 mM isobutylmethylxanthine (IBMX). The media was aspirated and 250 μl ice-cold 5% perchloric acid was added to each well. The plates were incubated at −20°C overnight to extract the cAMP. After thawing, the extract was transferred to tubes, the pH was adjusted to 7.5 with 4 N KOH, and the extract was centrifuged to remove the precipitate. The neutralized extract was then assayed for cAMP content using a cAMP binding protein assay. Parallel wells were analyzed for cell number by trypan blue dye enumeration.

The cAMP binding protein assay was performed as follows. To each tube was added assay buffer (50 mM Tris, 5 mM EDTA, pH 7.4) 100 μl standards or un-

knowns, [^{3}H]cAMP (10000 cpm/tube), and cAMP binding protein sufficient to bind 40–60% of the radioactivity in a final volume of 250 μl. The tubes were incubated for 90 min on ice. Dextran-coated charcoal (0.5 mg/ml dextran and 5.0 mg/ml charcoal) was added to each tube (600 μl), incubated an additional 30 min on ice, then centrifuged to remove the unbound from the bound cAMP-binding protein–[^{3}H]cAMP complexes. The supernatant was decanted directly into scintillation vials and counted in a liquid scintillation spectrophotometer. Samples were run in duplicate and the concentration of cAMP was calculated by the log-logit method using Securia 1.0 (Packard Instruments, Indianapolis, IN). Data were expressed as pmol cAMP/10^3 cells.

2.5. Statistical analysis

Data for the % specific binding and adenylate cyclase assays were evaluated using Instat 1.1 (GraphPAD Software, San Diego, CA) and an MS/DOS computer. Analysis of variance followed by multiple comparisons using a Tukey's mean separation test were used to determine statistically significant ($p < 0.05$) differences between groups. Scatchard analysis of the binding curve for PTHrP was performed with LIGAND, a nonlinear curve fitting software program (Munson and Robard, 1980). Autoradiographs from the northern blot analysis were evaluated for relative signal density with NIH Image Software on a Macintosh computer.

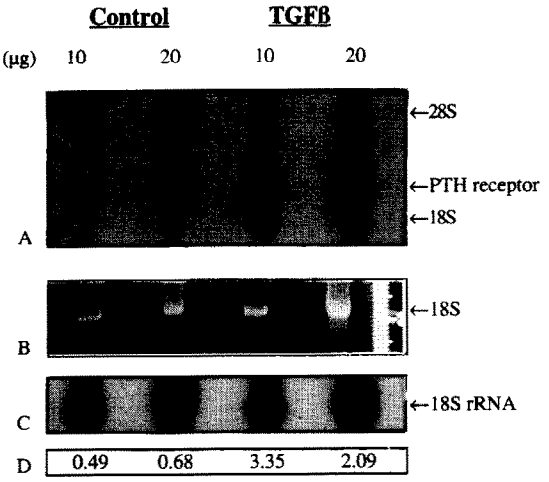

Fig. 1. Northern blot analysis of PTH/PTHrP receptor mRNA in ROS 17/2.8 cells. Total RNA was isolated from control and TGF-β_1-treated (5.0 ng/ml) ROS cells (12 h) and 10 or 20 μg of each was run on a 1.2% agarose-formaldehyde gel. The RNA was transferred onto a nylon filter and hybridized to a cDNA probe for the PTH/PTHrP receptor (R15B). (A) Autoradiograph of northern blot for PTH/PTHrP receptor, (B) ethidium bromide stained gel of autoradiograph in A, (C) autoradiograph of Northern blot for 18S rRNA, (D) densitometric ratio of signal intensity for PTH/PTHrP receptor vs. 18S rRNA. There was a 3–7-fold increase in PTH/PTHrP receptor mRNA with TGF-β_1 treatment.

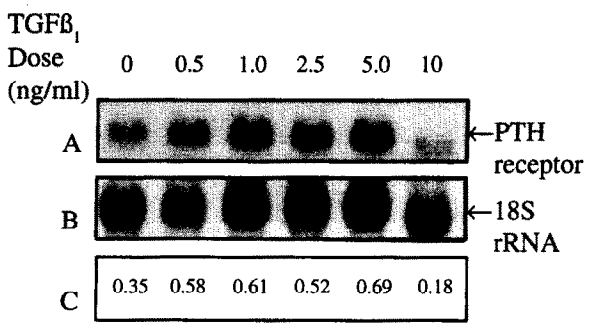

Fig. 2. Northern blot analysis of PTH/PTHrP mRNA expression in ROS 17/2.8 cells treated with TGF-β_1 (0–10 ng/ml) for 6 h. (A) Autoradiograph of Northern blot for PTH/PTHrP receptor, (B) autoradiograph of Northern blot for 18S rRNA, (C) densitometric ratio of signal intensity for PTH/PTHrP receptor vs. 18S rRNA. There was an increase in mRNA expression up to 5.0 ng/ml, and a reduction in expression at 10 ng/ml.

3. Results

3.1. Northern blot analysis

Northern blot analysis revealed a 3–7-fold increase in expression of mRNA for the PTH/PTHrP receptor when ROS cells were treated with TGF-β_1 (5.0 ng/ml) for 12 h (Fig. 1). TGF-β_1 stimulated a dose-dependent increase in mRNA expression up to 5.0 ng/ml, with a diminution of message expression at 10 ng/ml (Fig. 2). Time dependence studies revealed a two-fold increase in PTH/PTHrP receptor mRNA expression after 6 h of TGF-β_1 (5.0 ng/ml) treatment and up to three-fold increases in mRNA expression at 24 h of treatment (Fig. 3). When TGF-β_1-treated ROS 17/2.8 cells were incubated in the presence of cyclohexamide there was

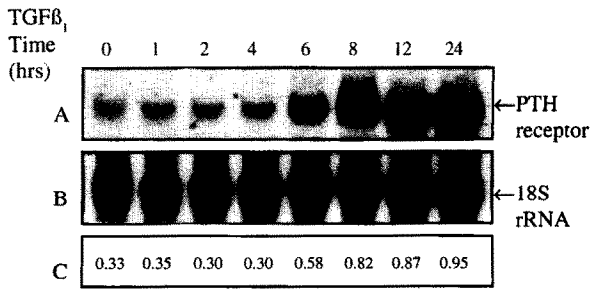

Fig. 3. Northern blot analysis of PTH/PTHrP mRNA expression in ROS 17/2.8 cells treated for 0–24 h with TGF-β_1 (5.0 ng/ml). (A) Autoradiograph of Northern blot for PTH/PTHrP receptor, (B) autoradiograph of Northern blot for 18S rRNA, (C) densitometric ratio of signal intensity for PTH/PTHrP receptor vs. 18S rRNA. Expression of PTH/PTHrP mRNA was increased 2-fold at 6 h and peaked at 8–24 h.

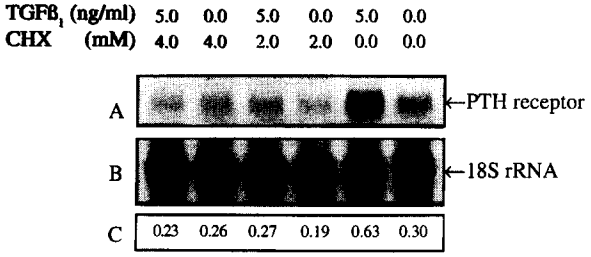

Fig. 4. Northern blot analysis of PTH/PTHrP receptor mRNA in ROS 17/2.8 cells treated for 6 h with TGF-β_1 (5.0 ng/ml) or control in the presence of cyclohexamide (CHX) (0, 2.0 or 4.0 mM). (A) Autoradiograph of Northern blot for PTH/PTHrP receptor, (B) autoradiograph of Northern blot for 18S rRNA, (C) densitometric ratio of signal intensity for PTH/PTHrP receptor vs. 18S rRNA. CHX was effective in blocking the TGF-β_1-mediated increase in PTH/PTHrP receptor mRNA.

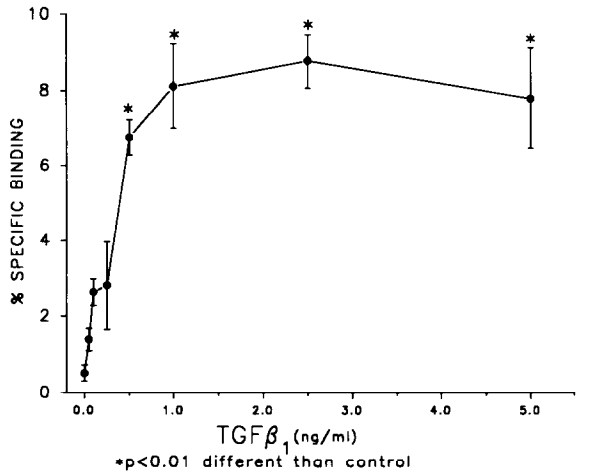

Fig. 5. Effects of TGF-β_1 (0–5.0 ng/ml; 48 h in media containing 1% FBS) on [^{125}I]PTHrP binding to ROS 17/2.8 cells. Specific binding is the total cpm bound with no added unlabeled PTHrP minus the cpm bound in the presence of excess unlabeled PTHrP (1 μM) and is expressed as a percent of the total cpm (25000) added per well. Data expressed as mean ± SEM of triplicate wells.

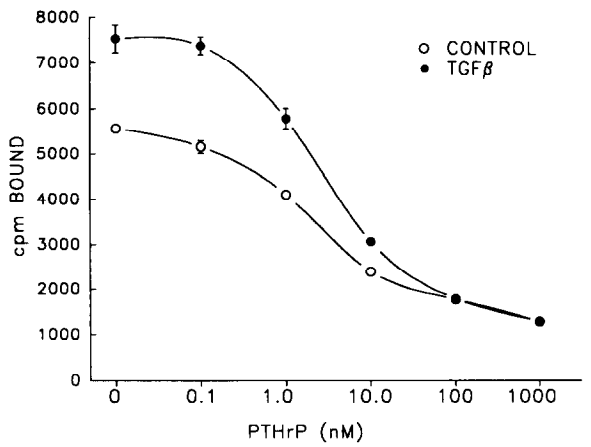

Fig. 6. Competitive inhibition of [^{125}I]PTHrP (35000 cpm/well) binding to ROS 17/2.8 cells pretreated (48 h in media containing 2% FBS) with TGF-β_1 (5.0 ng/ml) by unlabeled PTHrP (0–1000 nM). There was higher specific binding of PTHrP to the TGF-β_1-treated cells. Data expressed as mean ± SEM of triplicate wells.

Table 1
Scatchard analysis of PTHrP binding to ROS cells

Treatment	K_d (nM)	Number receptors/cell
Control	1.7	112,000
TGF-β_1 (5 ng/ml)	1.5	173,000

K_d values and number of receptors/cell were calculated from data in Fig. 6 by Scatchard analysis.

a loss of the TGF-β_1-upregulation of PTH/PTHrP receptor mRNA levels (Fig. 4). This indicates that TGF-β_1 is dependent on protein synthesis to mediate its effects on the PTH/PTHrP receptor.

3.2. Binding assays

TGF-β_1 stimulated an increase in specific binding of iodinated PTHrP to ROS 17/2.8 cells which was statistically significant with doses as low as 0.5 ng/ml TGF-β_1 (48 h pre-treatment) (Fig. 5). The treatments were routinely placed in cell culture media containing low levels of fetal bovine serum since higher serum levels stimulated PTH/PTHrP receptor levels and masked the TGF-β_1 effects (data not shown). Evaluation of PTHrP competitive binding (Fig. 6) by Scatchard analysis indicated that the binding affinity of PTHrP was similar for TGF-β_1-treated (5.0 ng/ml) and control cells (Table 1) and similar to that reported by other investigators for osteoblastic cells (Nissenson et al., 1988; Abou-Samra et al., 1989). Calculation of the number of receptors per cell revealed that there was an

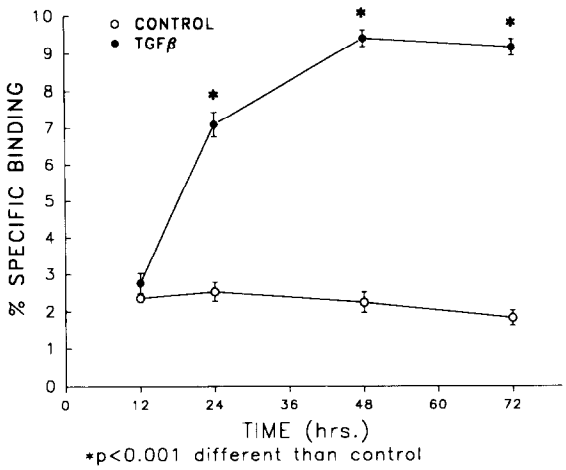

Fig. 7. Time course of [^{125}I]PTHrP binding to ROS 17/2.8 cells pretreated with TGF-β_1 (5.0 ng/ml in media containing 1% FBS). Specific binding is the total cpm bound with no added unlabeled PTHrP minus the cpm bound in the presence of an excess of unlabeled PTHrP (1 μM) and is expressed as a percent of the total cpm (25000) applied per well. There was a significant increase in % specific binding in TGF-β_1-treated cells at 24, 48 and 72 h. Data expressed as mean ± SEM of triplicate wells.

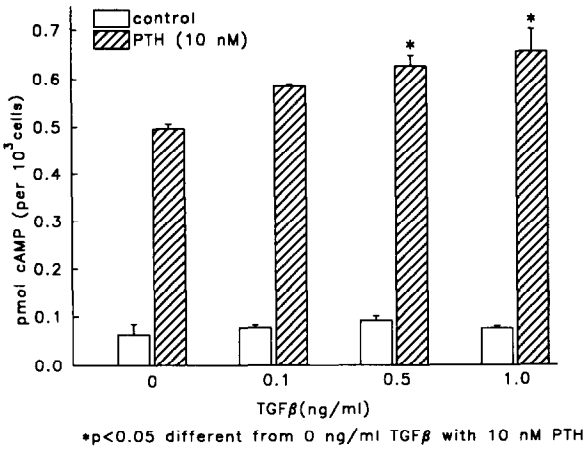

Fig. 8. Effects of 48 h pre-incubation with TGF-β_1 (0, 0.15, 0.25 ng/ml in media containing 1% FBS) on PTH-stimulated (10 min exposure) cAMP levels in ROS 17/2.8 cells. Levels of cAMP per 10^3 cells in triplicate samples are shown (mean + SEM). There was no significant difference in control levels of cAMP (no PTH-stimulation) with TGF-β_1 pre-incubation; however, with PTH-stimulation, cAMP levels were greater ($p < 0.05$) with 0.5 ng/ml and 1.0 ng/ml vs. 0 ng/ml TGF-β_1.

increase of approximately 55% in numbers of receptors when the ROS cells were pre-incubated with TGF-β_1 for 48 h. The cell number as determined by trypan blue dye exclusion in treatment and control groups was not different (data not shown). There was a significant increase in percent specific binding after 24 h of TGF-β-treatment (5.0 ng/ml) which increased up to 48 h; and remained increased at 72 h (Fig. 7).

3.3. *Adenylate cyclase stimulation assay*

The ability of TGF-β_1 to alter PTH-stimulated adenylate cyclase activity was evaluated in confluent ROS cells pre-incubated with TGF-β_1 (0, 0.1, 0.5, 1.0 ng/ml) followed by a 10 min PTH stimulation (10 nM). There was no difference in the basal levels of cAMP in cells pre-incubated with TGF-β_1; however, significantly greater levels of cAMP were found in response to PTH-stimulation in the 0.5 and 1.0 ng/ml TGF-β_1-pre-treated cells (Fig. 8).

4. Discussion

The present study provides the first evidence for upregulation of the PTH/PTHrP receptor by TGF-β_1 at the level of mRNA expression in osteoblastic cells. Previous reports have indicated that TGF-β enhanced PTH stimulation of adenylate cyclase in osteoblastic cells (ROS 17/2.8 and UMR-106) and TGF-β_1 inhibited adenylate cyclase and PTHrP binding in renal (OK) cells (Gutierrez et al., 1990; Schneider et al., 1992; Seitz et al., 1992; Law et al., 1993). Gutierrez et al. (1990) suggested that TGF-β did not alter PTH receptor affinity which is in agreement with our findings; however they also suggested that the modulatory effect of TGF-β was localized to nonreceptor components of the adenylate cyclase system. We found that PTH-stimulated cAMP levels, PTHrP receptor binding and PTH/PTHrP receptor mRNA upregulation were significantly elevated with similar levels of TGF-β_1 in ROS cells. This indicates that TGF-β_1 acts directly to increase PTH/PTHrP receptor numbers on osteoblastic cells by increasing the steady state PTH/PTHrP receptor mRNA levels. This is in support of findings by Schneider et al. (1992) which found an increase in PTH/PTHrP receptors and the G protein stimulatory subunit with TGF-β-treatment of UMR-106 osteoblastic cells.

TGF-β upregulates the binding of PTH on chondrocytes and osteoblastic cells, both of which require 24–48 h of incubation with TGF-β (Takigawa et al., 1991; Schneider et al., 1992). This is in agreement with our studies since specific binding increased over a 48 h period. Our study indicates that mRNA levels are increased in response to TGF-β_1 as early as 6 h, which is indicative of a rapid effect of TGF-β_1 on ROS cells; the resultant increase in the receptor determined by ligand binding reached a peak at 48 h.

The results of this study indicate that the upregulation of the PTH/PTHrP receptor mRNA is rapid; however, it is dependent on the synthesis of another protein(s) since cycloheximide inhibited the TGF-β_1-induced upregulation of PTH/PTHrP receptor mRNA. Alternately, the cycloheximide may have effected an increase in the transcription of a gene whose product inhibited PTH/PTHrP receptor gene transcription.

TGF-β has been reported to have a wide variety of effects on osteoblastic cells dependent on the state of differentiation of the cell. Clonal populations of rat osteoblastic cells exposed to TGF-β_1 respond differently to PTH stimulation based on the differentiation state. Mature osteoblastic cells have increased PTH-stimulated cAMP levels in response to TGF-β_1; whereas osteoblast precursors do not have altered PTH responses with TGF-β_1 treatment (Yamaguchi et al., 1991). Furthermore, preliminary data indicate that primary rat calvarial cells respond to TGF-β_2 with a decrease in PTH responsiveness (Jongen et al., 1993). Inconsistencies in TGF-β actions on osteoblasts in vitro suggest that the stage of differentiation, dose, and culture conditions may alter TGF-β effects. The results from our study are consistent with the ROS 17/2.8 cells representing a mature osteoblastic cell type that responds to TGF-β_1 with increased numbers of PTH/PTHrP receptors. Other phenotypic characteristics of a differentiated osteoblastic cell that have been reported to be stimulated by TGF-β_1 in ROS 17/2.8 cells include alkaline phosphatase activity, osteopontin,

and type I collagen (Centrella et al., 1988; Pfeilschifter et al., 1990; Noda and Rodan, 1987). Since TGF-β_1 is present in large amounts in the extracellular matrix of bone, and is released and activated during bone resorption, it has been thought to be an important factor in the process of coupling of bone resorption and bone formation (Pfeilschifter et al., 1990). The results from this study indicate that TGF-β_1 may exert its effects on the bone remodeling cycle at least in part by its ability to regulate PTH/PTHrP receptor mRNA, its translation product, and signaling pathway.

5. Acknowledgments

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