Inhibitory effects of 1,25-dihydroxyvitamin D₃ and 9-*cis*-retinoic acid on parathyroid hormone-related protein expression by oral cancer cells (HSC-3)

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Abstract

We investigated the effects of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and 9-*cis*-retinoic acid (9cRA) on parathyroid hormone-related protein (PTHrP) production and its mRNA expression by the human oral squamous carcinoma cell line (HSC-3). The major transcript of PTHrP was 1.5 kb in human HSC-3 cells. In the presence and absence of serum, 1,25(OH)₂D₃ produced dose-dependent inhibition of PTHrP gene expression and secretion. Significant inhibition by 1,25(OH)₂D₃ in the presence of serum was observed at 10⁻¹⁰ M for mRNA expression and 10⁻⁸ M for secretion, whereas under serum-free conditions, 1,25(OH)₂D₃ significantly suppressed PTHrP mRNA expression at 10⁻¹⁰ M and secretion at 10⁻⁹ M. Thus the remainder of the experiments were performed under serum-free conditions. After 24 h of incubation, 9cRA decreased dose-dependently PTHrP mRNA expression and PTHrP secretion. Addition of 10⁻⁷ M 1,25(OH)₂D₃ or 10⁻⁷ M 9cRA to HSC-3 cells signifi-

cantly suppressed PTHrP transcription within 1 h and the PTHrP secretion within 12 h. Maximal suppression of mRNA expression was maintained for 12–48 h. 9cRA caused a continuous decrease in PTHrP secretion for up to 48 h, whereas the inhibition of secretion by 1,25(OH)₂D₃ was transient and abolished by 48 h. Neither 1,25(OH)₂D₃ nor 9cRA altered the stability of PTHrP mRNA. The inhibitory effect of 1,25(OH)₂D₃ and 9cRA on PTHrP mRNA expression was additive, whereas no additive effect was observed with regard to PTHrP secretion. These results indicate that 1,25(OH)₂D₃ and 9cRA suppressed PTHrP production and mRNA expression in oral squamous cancer cells, and suggest that transcriptional suppression may act through binding of the heterodimer (vitamin D receptor–retinoid X receptor) to negatively responsive elements of the PTHrP gene.

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Introduction

Hypercalcemia is a frequent complication of human malignancies, affecting almost 15% of all cancer patients (Rodman & Sherwood 1985). Parathyroid hormone-related protein (PTHrP) is a newly recognized calcitropic hormone that has been implicated as a major causative factor in the pathogenesis of humoral hypercalcemia of malignancy (Stewart *et al.* 1980). PTHrP was isolated from human and animal tumors and the gene has been cloned (Moseley *et al.* 1987, Suva *et al.* 1987, Mangin *et al.* 1988, Thiede *et al.* 1988, Yasuda *et al.* 1989). PTHrP acts on the bone and kidney and elicits *in vivo* biologic effects such as bone resorption, phosphaturia and elevated excretion of cAMP in the urine as well as parathyroid hormone (Horiuchi *et al.* 1987, Kemp *et al.* 1987). Subsequently, PTHrP has been shown to be expressed in many normal tissues including normal human keratinocytes (Kremer *et al.* 1991), mammary tissues (Thiede & Rodan 1988,

Ferrari *et al.* 1992) and aortic smooth muscle cells (Hongo *et al.* 1991). Although PTHrP may regulate differentiation and proliferation in normal tissues, assessment of the modulation of PTHrP production and, particularly, of PTHrP regression in cancer cells may provide important clues for the management of humoral hypercalcemia of malignancy.

PTHrP expression is regulated by many factors including glucocorticoids (Ikeda *et al.* 1989, Lu *et al.* 1989), serum (Kremer *et al.* 1991, Liu *et al.* 1993), epidermal growth factor (EGF) (Heath *et al.* 1995), transforming growth factor-β (TGF-β) (Merryman *et al.* 1994), interleukin-6 and tumor necrosis factor-α (Rizzoli *et al.* 1994). The active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), plays not only a major role in regulating calcium homeostasis, but also has antiproliferative and differentiating effects in several cell types (Reichel *et al.* 1989). It has been shown that 1,25(OH)₂D₃ decreases PTHrP gene expression by a

human C-cell line (Ikeda *et al.* 1989) and a rat Leydig cell tumor H-500 (Liu *et al.* 1993). This inhibitory effect of 1,25(OH)₂D₃ appears to involve vitamin D-responsive elements (VDREs) of the 5' promoter region of the gene (Kremer *et al.* 1991, 1996a, Falzon 1996). Vitamin D receptor for 1,25(OH)₂D₃ (VDR) recognizes and binds VDREs as heterodimers with 9-*cis*-retinoic acid (9cRA) receptors (RXR) (Yu *et al.* 1991). These interactions between activated VDRs and RXRs are important in the activation of intracellular pathways by 1,25(OH)₂D₃. In fact, VDR-mediated increase in 25-hydroxyvitamin D₃ 24-hydroxylase gene expression is increased by a selective RXR ligand (Allegretto *et al.* 1995). It is likely that the VDR-RXR complex binds to negative regulatory elements of the PTHrP gene to elicit transcriptional suppression.

The human oral cancer cell line (HSC-3) was chosen for this study because it constitutively produces PTHrP in a monolayer culture without requiring a fibroblast feeder layer, as do some squamous carcinoma cell lines (Hoekman *et al.* 1990). Also, HSC-3 expresses EGF receptor (Kamata *et al.* 1986), an important protein in transformation. Since there has been little study of the regulation of PTHrP mRNA expression in human oral cancer cells in spite of extensive investigations in other squamous cell carcinomas, we examined the effect of 1,25(OH)₂D₃ and 9cRA on PTHrP mRNA expression and its production in HSC-3 cells.

Materials and Methods

Cell cultures

HSC-3 cells were established at the Tokyo Medical and Dental University (Kamata *et al.* 1986) and maintained in 25 cm² flasks containing Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% newborn calf serum (Gibco-BRL, Grand Island, NY, USA). Cultures were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO₂. The cells were passaged with Ca/Mg-free PBS and 0.1% trypsin, and split 1:10. Confluence was reached in 4–6 days. Cell viability was determined by the trypan blue exclusion assay. HSC-3 cells were cultured in DMEM supplemented with 10% newborn calf serum, and thereafter in serum-free DMEM containing 0.1% BSA for 72 h. Live and dead cells from each subculture were estimated by trypan blue exclusion at sequential time points during the incubation, and percentages of live cells were calculated.

Treatment of cell cultures with 1,25(OH)₂D₃ and 9cRA

At 70% confluence, the culture medium was replaced with serum-free DMEM for 24 h. Cells were exposed to the test substance in fresh serum-free DMEM with 0.1% BSA unless otherwise noted in the text. 1,25(OH)₂D₃

(Rossel Uclaf, Ramainville, France) and 9cRA (Biomol Research Lab, Plymouth Meeting, PA, USA) were dissolved in ethanol, and control cells received an equal volume of ethanol vehicle to give a final ethanol concentration of 0.1%. Cultures were incubated at 37 °C for the indicated period and medium was changed daily. In time-course studies, medium containing the test substance was changed every 12 h. Where indicated, 5,6-dichlorobenzimidazole riboside (DRB; from Sigma Chemical Co., St Louis, MO, USA) dissolved in dimethylsulphoxide was added 24 h after 10⁻⁷ M 1,25(OH)₂D₃, 10⁻⁷ M 9cRA or vehicle.

RIA for PTHrP

PTHrP concentrations in conditioned medium were measured using a PTHrP RIA kit (Incstar Corp., Stillwater, MN, USA). In brief, conditioned medium (200 µl) treated with 0.7% soybean trypsin inhibitor (aprotinin; Bayer, Leverkusen, Germany) was incubated with goat anti-human PTHrP-(1–40) for 4 h at 4 °C, then ¹²⁵I-labeled PTHrP-(1–34) was added and the incubation was continued for 20 h at 4 °C in duplicate. Since PTHrP is known to be an extremely labile protein, soybean trypsin inhibitor was added to all conditioned media to prevent PTHrP degradation during the assay. An anti-goat IgG donkey serum was added and the reaction was extended for 15 min at 25 °C. After centrifugation, the radioactivity of the bound fraction was counted. Recombinant human PTHrP-(1–84) was used as a standard in this assay.

Measurement of PTHrP mRNA

The DNA fragment used as a probe for RNA analysis was generated by reverse transcriptase PCR using a GeneAmp RNA PCR kit (Roche Molecular System Inc, Branchburg, NJ, USA). Antisense oligonucleotide-primed single-stranded cDNA was obtained by reverse transcription of total RNA extracted from HSC-3 cells. Specific primers necessary for generation of the double-stranded cDNA (sense, 5'-ATGCAGCGGAGACTGGTTCA-3', nucleotides 304–323; antisense, 5'-TTACCGTGAATC GAGCTCCA-3', nucleotides 812–831), encompassing the open reading frame of human PTHrP exon V and VI, were derived from the published sequence (Thiede *et al.* 1988) and synthesized. PCR was performed (Saiki *et al.* 1988) and the PCR products were separated on an agarose gel. The major product (528 bp) of the PCR amplification (528 bp) was cloned into pT7 Blue T-vector (Novagen, Madison, WI, USA). The nucleotide sequence was determined by the dideoxynucleotide chain termination method (Sanger *et al.* 1977). A clone with the confirmed sequence was used to generate the probe. Total RNA was isolated from the HSC-3 cells using the guanidinium thiocyanate-phenol-chloroform method (Puissant & Houdebine 1990).

Northern blot analysis of total RNA was employed to assess the integrity of the isolated RNA and to quantify the PTHrP mRNA. Total RNA (20 µg) was electrophoresed on a 1.2% agarose–2.2 M formaldehyde gel and transferred to a nylon membrane (Hybond N⁺; Amersham International, Amersham, Bucks, UK). Blots were hybridized with the cDNA probe which was ³²P-labeled by the random primer method using [α -³²P]dCTP (specific radioactivity 110 TBq/mmol) from ICN Biochemicals (Costa Mesa, CA, USA) and the Megaprime DNA labeling kit (Amersham). Hybridization was performed in 50% formamide, 5 × Denhardt's solution, 0.5% lauryl sulphate and 5 × SSPE (80 mM NaCl, 10 mM sodium phosphate and 1 mM EDTA) at 42 °C for 2 days. Membranes were washed to high stringency (0.2 × SSPE/0.1% SDS, 50 °C) and exposed to Hyperfilm (Amersham) at –80 °C with intensifying screens (Akeno *et al.* 1994). The relative intensities of mRNA signals detected on autoradiographs were quantified by densitometric analysis. The PTHrP mRNA abundance was normalized by reference to β -actin mRNA expression.

Statistical analysis

The data are presented as the mean $\pm$ S.E.M. Statistical analysis was performed using ANOVA followed by Fisher's protected least significant difference (Statview 4.02, Abacus Concepts Inc, Berkeley, CA, USA). $P < 0.05$ was considered to be statistically significant.

Results

Effect of 1,25(OH)₂D₃ on PTHrP mRNA expression and production

HSC-3 cells expressed PTHrP mRNA and secreted PTHrP into conditioned medium under basal conditions. We first examined the effect of 1,25(OH)₂D₃ on PTHrP mRNA expression by Northern blot analysis in HSC-3 cells cultured in the presence or absence of serum (10%)-containing medium at confluence (Fig. 1). A prominent transcript of PTHrP was 1.5 kb. PTHrP mRNA was expressed at low abundance in cells grown in the absence of serum and increased in cells in the presence of 10% serum. The inhibitory effect of 1,25(OH)₂D₃ on PTHrP mRNA abundance and production was dose-dependent in the presence and absence of serum. The suppression of PTHrP mRNA expression was significant at 10^{–10} M 1,25(OH)₂D₃ in the medium and reached a maximum at between 10^{–7} and 10^{–6} M. Accumulation of PTHrP in the medium also decreased with 1,25(OH)₂D₃ treatment in a dose-dependent manner (Fig. 2). The reduction in PTHrP was significant at 10^{–8} and 10^{–9} M concentrations of hormone in the presence and absence of serum respectively. The inhibitory effect of 1,25(OH)₂D₃ on PTHrP secretion was smaller under the serum-free condi-

Figure 1 Effect of 1,25(OH)₂D₃ on PTHrP mRNA abundance in the presence or absence of serum in HSC-3 cells. At 70% confluence the medium was changed to serum-free DMEM for 24 h and then to DMEM containing 0.1% BSA (– serum) or 10% newborn calf serum (+serum) with various concentrations of 1,25(OH)₂D₃. Cells were treated with the hormone for 24 h and mRNA was analyzed by Northern blots using human PTHrP cDNA (528 bp) and β -actin cDNA (a). (b) Densitometric determination of the Northern blots. The PTHrP mRNA concentration was normalized to that of the β -actin mRNA. The data are expressed as the mean $\pm$ S.E.M. of triplicate determinations. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with control (CTL).

tions than under the 10% serum-supplemented conditions. Because 1,25(OH)₂D₃ clearly inhibited PTHrP mRNA expression and production regardless of serum supplementation, the remaining experiments were performed under serum-free conditions.

Effect of 9cRA on PTHrP mRNA expression and production

The incubation of HSC-3 cells with 9cRA caused a decrease in PTHrP mRNA expression and immunoreactive PTHrP production. Figure 3 shows the

Figure 2 Effect of 1,25(OH)₂D₃ on PTHrP secretion by HSC-3 cells. At 70% confluence medium was changed to serum-free DMEM for 24 h and then to DMEM containing 0.1% BSA (– serum) or 10% newborn calf serum (+serum) with various concentrations of 1,25(OH)₂D₃. Cells were treated with the hormone for 24 h, and PTHrP secretion into the medium was determined using an N-terminal PTHrP RIA kit. Results are expressed as the mean ± S.E.M. of triplicate determinations, and significant differences from control values (CTL) are expressed as **P*<0.05 or ***P*<0.01.

concentration–response curves for PTHrP gene expression and the secretion measured after 9cRA treatment for 24 h. The mean percentage decrease with 10^{−7} and 10^{−6} M 9cRA was 70% with regard to mRNA abundance and 20% with regard to secretion. The reduction in PTHrP in response to 9cRA was significant at 10^{−9} M in terms of mRNA abundance and 10^{−8} M in terms of its secretion.

Time courses of PTHrP mRNA expression and production in response to 1,25(OH)₂D₃ or 9cRA

HSC-3 cells were treated with vehicle, or 10^{−7} M 1,25(OH)₂D₃ or 10^{−7} M 9cRA for up to 48 h. There was a significant decrease in PTHrP mRNA abundance as early as 1 h after initiation of treatment with 1,25(OH)₂D₃ or 9cRA (Fig. 4). The inhibition was already maximum at 12 h and was maintained for up to 48 h. To determine whether 1,25(OH)₂D₃ and 9cRA modified the stability of PTHrP mRNA, HSC-3 cells were exposed to serum-free medium, 10^{−7} M 1,25(OH)₂D₃ or 10^{−7} M 9cRA for 24 h and then treated with 75 μM DRB, an RNA polymerase II inhibitor, in the absence or presence of bioactive molecules. The decay of PTHrP mRNA was determined by Northern blot analysis (Fig. 5). Neither

Figure 3 Effect of 9cRA on PTHrP mRNA expression and its secretion by HSC-3 cells. (a) Northern blots of total RNA (20 μg) extracted from cells for PTHrP (upper panel) and β-actin (lower panel) mRNAs. The estimated sizes of PTHrP and β-actin mRNAs were 1.5 and 2.1 kb respectively. The membrane was reprobed for β-actin mRNA to determine RNA integrity and to assess sample loading. (b) Determination of PTHrP mRNA abundance (●) and its secretion (○) into the medium. The PTHrP mRNA concentrations were determined by densitometry of the Northern blots, and the PTHrP secretion was measured by the PTHrP RIA kit. Results are expressed as the mean ± S.E.M. of triplicate determinations, and significant differences from control values (CTL) are expressed as ***P*<0.01 or ****P*<0.001.

1,25(OH)₂D₃ nor 9cRA altered the stability of this transcript. Figure 6 shows the time courses for the effects of 1,25(OH)₂D₃ and 9cRA on PTHrP secretion in HSC-3 cells. Although PTHrP secretion gradually decreased in vehicle-treated (control) cells, significant inhibition of the secretion by 9cRA persisted for 12–48 h. The inhibitory effect of 1,25(OH)₂D₃ on PTHrP production in HSC-3 cells was marked for 12–24 h but was blunted by 48 h (Fig. 6). To determine whether the reduction in PTHrP accumulation by longer incubation of HSC-3 cells under serum-free conditions was due to decreases in the number of living cells, cell viability was

Figure 4 Time courses of the inhibition of PTHrP mRNA expression by 1,25(OH)₂D₃ or 9cRA in HSC-3 cells. After various incubation times (1–48 h) with vehicle (□), 10^{−7} M 1,25(OH)₂D₃ (●) or 10^{−7} M 9cRA (○) in serum-free DMEM containing 0.1% BSA, cells were harvested and total RNA was extracted. Northern blots were hybridized with ³²P-labeled PTHrP cDNA. The PTHrP mRNA concentration was normalized to that of β-actin mRNA. The data are expressed as the mean ± S.E.M. of triplicate determinations. ****P* < 0.001 compared with vehicle control.

Figure 5 Effects of 1,25(OH)₂D₃ and 9cRA on PTHrP mRNA decay in HSC-3 cells. Oral cancer cells were exposed to 1,25(OH)₂D₃ (10^{−7} M), 9cRA (10^{−7} M) or control medium for 24 h before the addition of 75 μM DRB. At selected time points after the addition of DRB, total RNA from vehicle- (□), 1,25(OH)₂D₃- (●) and 9cRA-treated (○) cells was subjected to Northern blot analysis with ³²P-labeled human PTHrP cDNA. The PTHrP mRNA concentration was normalized to that of β-actin mRNA.

examined. The percentage of live cells did not change significantly in cultures under serum-free conditions (+serum, 98.0 ± 0.3%; −serum (24 h) 97.8 ± 0.2%, (48 h) 97.7 ± 0.3%, (72 h) 97.2 ± 0.9%).

Combined effect of 1,25(OH)₂D₃ and 9cRA on PTHrP expression

We assessed the combined effect of 1,25(OH)₂D₃ with 9cRA on PTHrP gene expression and its production in HSC-3 cells. The decrease in PTHrP mRNA abundance induced by 1,25(OH)₂D₃ was enhanced by the addition of 9cRA, as indicated by the Northern blot analysis (Fig. 7a). PTHrP mRNA abundance and its secretion during 1,25(OH)₂D₃ and 9cRA treatment was quantified. The reduction caused by the effective concentration of each compound was clearly additive with regard to PTHrP mRNA expression (Fig. 7b), whereas the inhibitory effect of each substance on secretion was not additive (Fig. 7c).

Discussion

The results of this study demonstrate that 1,25(OH)₂D₃ and 9cRA down-regulated PTHrP mRNA expression and its production in oral cancer cells (HSC-3). The

additive effect of 1,25(OH)₂D₃ and 9cRA on PTHrP mRNA expression suggests that these agents may be acting through binding of the VDR–RXR heterodimer to VDRE.

There is high frequency of expression of both PTHrP mRNA and protein in squamous cell tumors irrespective of the calcium status of the patient (Ralston *et al.* 1991, Dunne *et al.* 1993). Immunohistochemical study shows that PTHrP is produced frequently in squamous cell tumors of the oral cavity (Dunne *et al.* 1995). Many investigations have been performed into the regulation of PTHrP expression using squamous carcinoma cell lines established from lung, kidney and testis. Previously, we screened 16 cell lines established from oral cancer and found that HSC-3 cells constitutively expressed PTHrP mRNA. It is noteworthy that HSC-3 cells responded to several factors including serum, 1,25(OH)₂D₃ and 9cRA with regard to PTHrP gene expression as reported previously for other cells (Ikeda *et al.* 1989, Liu *et al.* 1993, Merryman *et al.* 1993, Kremer *et al.* 1991, 1996b). Thus the regulation of PTHrP mRNA expression and production was investigated in the present study using HSC-3 cells.

Many factors regulate PTHrP gene expression and production in different tissues. In neuroendocrine tumors, 1,25(OH)₂D₃, glucocorticoids and chromogranin A

Figure 6 Time courses of the effects of 1,25(OH)₂D₃ and 9cRA on PTHrP secretion by HSC-3 cells. After various incubation times (12–48 h) with vehicle (□), 10⁻⁷ M 1,25(OH)₂D₃ (●) or 10⁻⁷ M 9cRA (○) in serum-free DMEM containing 0.1% BSA, the conditioned media after a 12 h period were collected and PTHrP concentrations determined by a PTHrP RIA kit. The data are expressed as the mean ± S.E.M. of triplicate determinations. **P*<0.05, ****P*<0.001 compared with vehicle control.

decrease PTHrP mRNA abundance, whereas calcitonin has been shown to increase gene expression (Ikeda *et al.* 1989). EGF, serum and phorbol esters up-regulate PTHrP expression in an osteoblastic cell line (Rodan *et al.* 1989) and Leydig cell tumors (Liu *et al.* 1993). In keratinocytes, PTHrP production decreases upon treatment with glucocorticoid and 1,25(OH)₂D₃ and is elevated by extracellular calcium, growth factors in serum and all-*trans*-retinoic acid (Kremer *et al.* 1991, 1996b). Cycloheximide has been reported to increase PTHrP gene expression and mRNA stability by inhibiting protein synthesis and removing the PTHrP gene from the control of a labile repressor protein (Ikeda *et al.* 1990). TGF-β has been reported to increase PTHrP production and mRNA expression in squamous carcinoma cells (Merryman *et al.* 1994). We found that 1,25(OH)₂D₃ and 9cRA suppressed PTHrP gene expression and production in oral squamous carcinoma cells and that the inhibitory effects were additive.

Neither 1,25(OH)₂D₃ nor 9cRA altered the stability of PTHrP mRNA but both elicited rapid decreases (as early as 1 h) in mRNA abundance in HSC-3 cells. These findings demonstrate that both compounds inhibited PTHrP gene expression by a transcriptional mechanism. These two compounds achieve their cellular effects through interaction with the appropriate receptors which are members of the steroid/thyroid hormone receptor

Figure 7 Effects of 1,25(OH)₂D₃ (1,25D) and 9cRA on PTHrP mRNA abundance and its secretion by HSC-3 cells. Cells were treated with 10⁻⁹ M 1,25(OH)₂D₃ and/or 10⁻⁸ M 9cRA in serum-free DMEM containing 0.1% BSA for 24 h. Cells were harvested and total RNA was extracted. (a) Northern blots of total RNA (20 μg) from cells for PTHrP (upper panel) and β-actin (lower panel) mRNAs. (b) Densitometric determination of the blots. The PTHrP mRNA concentration was normalized to that of β-actin mRNA. (c) Secretion of PTHrP into the medium. PTHrP amounts were measured by a PTHrP RIA kit. The data are expressed as the mean ± S.E.M. of triplicate determinations. ***P*<0.01 and ****P*<0.001 compared with vehicle control (CTL). †††*P*<0.001 compared with the group receiving combined treatment (9cRA+1,25(OH)₂D₃). NS, not significantly different.

superfamily (Petkovich *et al.* 1987, Evans 1988). This family includes not only VDR, the receptor for 1,25(OH)₂D₃, and the retinoic acid receptor (RAR), the receptor for all-*trans*-retinoic acid, but also RXR, the receptor for 9cRA, although 9cRA can also bind to RAR (Lala *et al.* 1996). Indeed, 1,25(OH)₂D₃ can exert its effect through two distinct pathways, the first being the formation of homodimers between two activated VDRs,

which will then bind to DNA and initiate a cascade of cellular events. Recently, a second and more generally used pathway was described, whereby the activated VDR forms a heterodimer with an RXR and then interacts with VDRE in the DNA, leading to gene transcription. A major part of the cellular effect of 1,25(OH)₂D₃ is mediated through interactions between the activated VDR and RXR (Carlberg *et al.* 1993, Green 1993). The DNA sequences in the PTHrP gene responsible for 1,25(OH)₂D₃-mediated transcriptional repression have been identified and characterized (Kremer *et al.* 1991, 1996a, Falzon 1996). In the present study, 1,25(OH)₂D₃ and 9cRA additively repressed PTHrP mRNA expression in HSC-3 cells, suggesting that RXRs heterodimerize with VDR, and the VDR–RXR heterodimers bind to the negative VDREs and down-regulate the transcription function of the PTHrP gene promoter. We also showed that transcriptional inhibition in response to 1,25(OH)₂D₃ was more pronounced in the presence of mitogenic stimuli such as serum. The mechanism by which transcriptional repression occurs may also involve interference with the binding of positive transcriptional factors such as serum, EGF and TGF- β .

It is interesting to note that prolonged exposure to 9cRA decreases PTHrP mRNA abundance and that this correlates with the marked reduction in protein accumulation in the conditioned medium. However, a longer period of 1,25(OH)₂D₃ treatment abolished the inhibition of PTHrP secretion even with the down-regulation of gene expression. These results suggest that the post-translational control mechanisms of PTHrP by 1,25(OH)₂D₃ and 9cRA are different. Proteins, such as proteinase inhibitors, would be induced in HSC-3 cells by sustained exposure to 1,25(OH)₂D₃, because 1,25(OH)₂D₃ requires a 48–96 h latency period for PTHrP accumulation (Merryman *et al.* 1993). The cause of the time-dependent reduction of PTHrP accumulation in the medium of vehicle-treated cells is not known. However, it was not due to a decrease in living cells because we observed no alterations in cell viability during culture. Moreover, as fractional accumulation (1 h) of PTHrP in the medium decreased time-dependently in 1–3-day cultures under serum-free conditions, this reduction cannot be due to altered lability of PTHrP. PTHrP production may decrease in post-confluent cells (Merryman *et al.* 1993, 1994).

Previous studies have shown mixed effects of 1,25(OH)₂D₃ on PTHrP expression. Inhibitory (Ikeda *et al.* 1989, Kremer *et al.* 1991, Liu *et al.* 1993), stimulatory (Merryman *et al.* 1993) and no effects (Werkmeister *et al.* 1993, Cramer *et al.* 1996) on PTHrP production have been reported. We found that 1,25(OH)₂D₃ inhibited PTHrP expression in the oral cancer cell line (HSC-3). Further, all-*trans*-retinoic acid significantly stimulates PTHrP mRNA expression and secretion in human cervical epithelial cells (Kremer *et al.* 1996b). Thus cell type-

specific differences in 1,25(OH)₂D₃ and retinoic acid regulation of PTHrP gene expression may be due to tissue-specific transcriptional factors and tissue-specific promoter utilization (Campos *et al.* 1992). It is well demonstrated that 1,25(OH)₂D₃ and retinoic acid elicit antiproliferative effects on many cell lines including squamous carcinomas. Since PTHrP acts as an autocrine/paracrine factor in normal and malignant cells, proliferation and differentiation of squamous cells may also be regulated by 1,25(OH)₂D₃, retinoic acid and PTHrP.

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