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Alteration of Secretion of Parathyroid Hormone-Related Peptide and Expression of Its mRNA in a Human Hepatoma Cell Line (HEP G2) Treated with Agents that Affect Cell Growth

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Previously, using human hepatoma cells (Hep G2), we found that immunoneutralization of secreted PTHrP increased cell growth. Here we asked whether PTHrP production was affected by agents that alter growth of Hep G2 cells. Immunoreactive PTHrP in medium and PTHrP mRNA expression were examined. Treatment of cells with 10 μM hydrocortisone or 1 ng/mL TGF-β1 for 72 h inhibited cell growth by 28 ± 6 and 36 ± 2% and increased PTHrP in medium by 128 ± 10 and 525 ± 27%, respectively. The increase in PTHrP produced by both agents was dose- and time-dependent, and the increased PTHrP was accompanied by dose- and time-dependent enhanced expression of PTHrP mRNA. In contrast, 10% fetal bovine serum (FBS) for 72 h increased cell growth by 38 ± 6% (vs serum-free medium) and decreased PTHrP production by 49 ± 4% whereas culture in high glucose (3–4g/L) increased cell growth by 43 ± 1% (vs 1 g/L glucose) and decreased PTHrP by 55 ± 0.4%. Inhibition of PTHrP by both FBS and glucose was dose-dependent; FBS also inhibited PTHrP mRNA. The results show that increased cell growth was associated with decreased PTHrP production, while decreased growth was accompanied by increased PTHrP production. The findings imply that PTHrP may help mediate growth effects of these agents on Hep G2 cells.

Key Words: Parathyroid hormone-related peptide (PTHrP); cell growth; hepatocyte; growth factors.

Introduction

Parathyroid hormone (PTH)-related peptide (PTHrP), originally isolated from human tumors, shares significant amino acid sequence homology at its N-terminus with PTH (Martin et al., 1991; Burtis, 1992; Orloff et al., 1994). Deduction of the amino acid sequence of PTHrP from the nucleotide sequence of the cloned gene has shown that there are three isoforms of the human peptide ranging in length from 139 to 173 residues (Burtis, 1992; Orloff et al., 1994). Although PTHrP can circulate in high amounts in some cancer patients and can interact with PTH/PTHrP receptors in bone and kidney to cause hypercalcemia and other features of hyperparathyroidism, the peptide does not circulate in appreciable amounts normally (Burtis, 1992; Orloff et al., 1994). Therefore, current ideas about normal functions of PTHrP include the notion that it acts as a local regulatory factor near its site of production rather than as a classical circulating hormone. The peptide and its mRNA are widely distributed, being present in almost every tissue examined (Kramer et al., 1991; Selvanayagam et al., 1991; deDapp and Stewart, 1993), so that most workers conclude that PTHrP plays some fundamental role in the development or function of many tissues. Although the exact nature of this role remains to be clarified, it is clear from PTHrP gene knockout studies and from PTHrP overexpression experiments that severe developmental defects occur in both instances (Karaplis et al., 1994; Wysolmerski et al., 1994, 1995; Vasavada et al., 1996). PTH-like biological effects mediated by the PTH/PTHrP receptor reside in the N-terminal region of the peptide (Martin et al., 1991; deDapp and Stewart, 1993; Orloff et al., 1994). However, since PTHrP has multiple potential cleavage sites and is processed enzymatically by tissues and cells, PTHrP apparently can serve as a precursor for smaller active peptides downstream of the N-terminus (Orloff et al., 1994). Mid-region and C-terminal analogs have unique biologic actions (Martin et al., 1991; Burtis, 1992; deDapp and Stewart, 1993), indicating that multiple biological actions can result from processing of the original translation product.

The human PTHrP gene consists of 9 exons and is located on chromosome 12 (Martin et al., 1991; Burtis, 1992; Orloff et al., 1994). The 5'-flanking region of DNA upstream of

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the transcription start site contains three separate promoters, two of which have TATA characteristics and one of which is GC-rich (Yasuda et al., 1989). The 3' region of the gene has AUUUA-rich motifs that are thought to explain the rapid turnover of PTHrP mRNA (Yasuda et al., 1989; Orloff et al., 1994). The 3' region of the mRNA can be spliced alternatively to yield the three different peptide isoforms (Yasuda et al., 1989; Martin et al., 1991; Burtis, 1992; Orloff et al., 1994). Transcriptional control of the PTHrP gene has not been extensively studied to date. However, previous reports have indicated that PTHrP gene expression is induced rapidly (Assinson and Drucker, 1992) and can be altered by a number of factors, including serum, TGF-β, glucocorticoids, EGF, and 1,25-dihydroxyvitamin D_3 (Lu et al., 1989; Liu et al., 1993; Glatz et al., 1994; Merryman et al., 1994). Overall, the findings have led some workers to characterize the gene immediate-early gene (Assinson and Drucker, 1992).

Our previous studies using the human hepatoma cell line Hep G2 showed that immunoneutralization of PTHrP secreted by the cells in culture resulted in enhanced growth (Li et al., 1996). We concluded from these studies that PTHrP acted in these liver cells as a growth suppressor, a finding in agreement with similar studies by others using cultured human keratinocytes (Kaiser et al., 1992). In the present study, we asked whether PTHrP could be a mediator of other agents that might affect the growth of Hep G2 cells. To test this hypothesis we selected four agents likely to affect cell growth and asked (1) whether they altered growth of Hep G2 cells, (2) whether they altered PTHrP production or secretion, and (3) if so, whether changes in cell growth and PTHrP appeared consistently related to one another. The findings reported here show that PTHrP and cell growth were inversely correlated and provide circumstantial evidence that the growth effects of the four agents studied may involve an alteration in PTHrP production.

Results

Cell Growth

The results of representative experiments are presented in Table 1. Not surprisingly, the findings show that increasing the concentration of FBS from 1 to 10% in the culture medium increased the growth of Hep G2 cells over the 3-d period tested. Likewise, elevation of the medium glucose concentration from 1 to 3.5 g/L increased cell growth. The combined results of three experiments showed that 10% FBS increased Hep G2 cell growth in 3 d by 38 ± 6%, while 3–4 g/L glucose enhanced growth by 43 ± 4%. In contrast to FBS and glucose, which enhanced cell growth, treatment of cells with $10^{-5}M$ hydrocortisone decreased cell growth by 28 ± 6% over a 3-d test period, and 1 ng/mL TGF-β decreased cell growth by 36 ± 2% ($n = 3$).

Table 1
Effects of Hydrocortisone (HC), TGF-β, Glucose, and Fetal Bovine Serum (FBS) on Growth of Hep G2 Cells in Culture

	Treatment	Cell number × 2000
Exp. 1	1% FBS	16.47 ± 0.68
	5% FBS	19.79 ± 0.80
	10% FBS	23.37 ± 1.08**
Exp. 2	Glucose, 1 g/L	13.09 ± 0.77
	Glucose, 3.5 g/L	17.27 ± 0.59*
Exp. 3	Vehicle control	17.75 ± 0.44
	HC, $10^{-7}M$	14.60 ± 0.80
	HC, $10^{-5}M$	12.73 ± 1.11**
Exp. 4	Vehicle control	14.93 ± 1.31
	TGF-β, 1 ng/mL	9.52 ± 0.17**

Values given as mean ± SEM ($n = 4$ wells/group). Cells were plated in 24-well plates at a density of 1.5×10^3 cells/well; they were cultured for 3 d in the presence of test agent before being counted (*see* Methods section for additional details). *$p < 0.05$, **$p < 0.01$ (vs 1% FBS, 1 g/L glucose, or vehicle control, respectively).

PTHrP Production

PTHrP production in response to each of the four test agents that affected growth of Hep G2 cells was assessed. The expression of PTHrP mRNA was examined to determine whether the growth effects might involve an alteration of PTHrP biosynthesis. The level of immunoreactive PTHrP in culture medium was measured to gain insight concerning potential effects of the test agents on synthesis and/or secretion of the peptide itself.

Figure 1A shows the effect of different concentrations of hydrocortisone on expression of PTHrP mRNA over a 2-d test period. Hydrocortisone produced a dose-related increase in PTHrP mRNA over the dose range 10^{-9}–$10^{-5}M$ compared to vehicle alone. Figure 1B shows the results of a time-course study in which the effect of $10^{-5}M$ hydrocortisone on PTHrP mRNA was examined over a time interval ranging from 4 to 96 h. The results show that PTHrP mRNA was increased at all time intervals studied. Figure 2A shows results of studies in which the effect of hydrocortisone on immunoreactive PTHrP in the growth medium of Hep G2 cells was evaluated. Over the 2-d test period, hydrocortisone produced a dose-related increase in PTHrP, which was evident over the concentration range of 10^{-9}–$10^{-5}M$. Furthermore, as shown in Fig. 2B, a dose of $10^{-5}M$ hydrocortisone produced an increased PTHrP level in the culture medium at 24 h, and the level rose progressively over the 96-h study interval.

Results in Fig. 3A show that TGF-β caused a dose-related increase in PTHrP mRNA over concentration range of 0.01–3 ng/mL, and, as shown in Fig. 3B, a dose of 1 ng/mL TGF-β produced an elevated expression of PTHrP mRNA at all time periods examined ranging from 2 to 72 h. Figure 4A

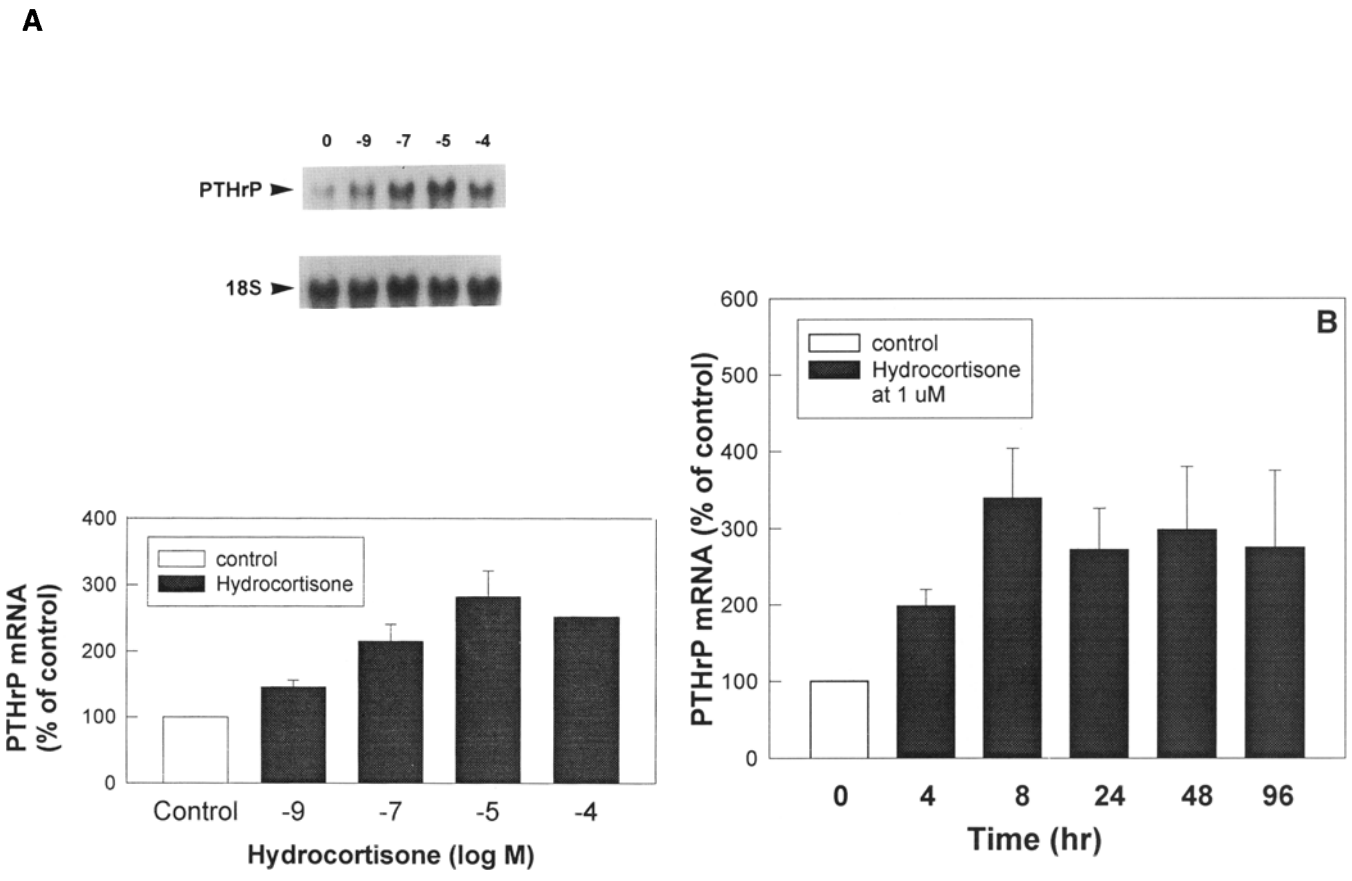

Fig. 1. PTHrP mRNA in Hep G2 cells. **(A)** Upper panel shows representative results with RNA isolated from cells treated for 2 d with various doses of hydrocortisone (HC) or with vehicle (0 dose). Northern blot of PTHrP mRNA is compared to the same blot reprobed for 18S RNA to assess loading. Doses of HC (log M) are shown above the blots. Lower panel shows laser densitometric analysis of blots from 3 studies ($n = 3$ except for $10^{-4}M$ where $n = 1$) where the PTHrP signal was normalized to the 18S signal, and the ratios were expressed as a percent of control (vehicle treatment). Here and in subsequent figures, values are given as mean ± SEM. **(B)** Densitometric analysis of Northern blots for PTHrP and 18S RNA from 3 studies where cells were treated with $2 \times 10^{-5}M$ HC for time-periods ranging from 4 to 96 h compared to vehicle control (0 time). Statistical analysis showed significant effects of treatment and time ($p < 0.05$).

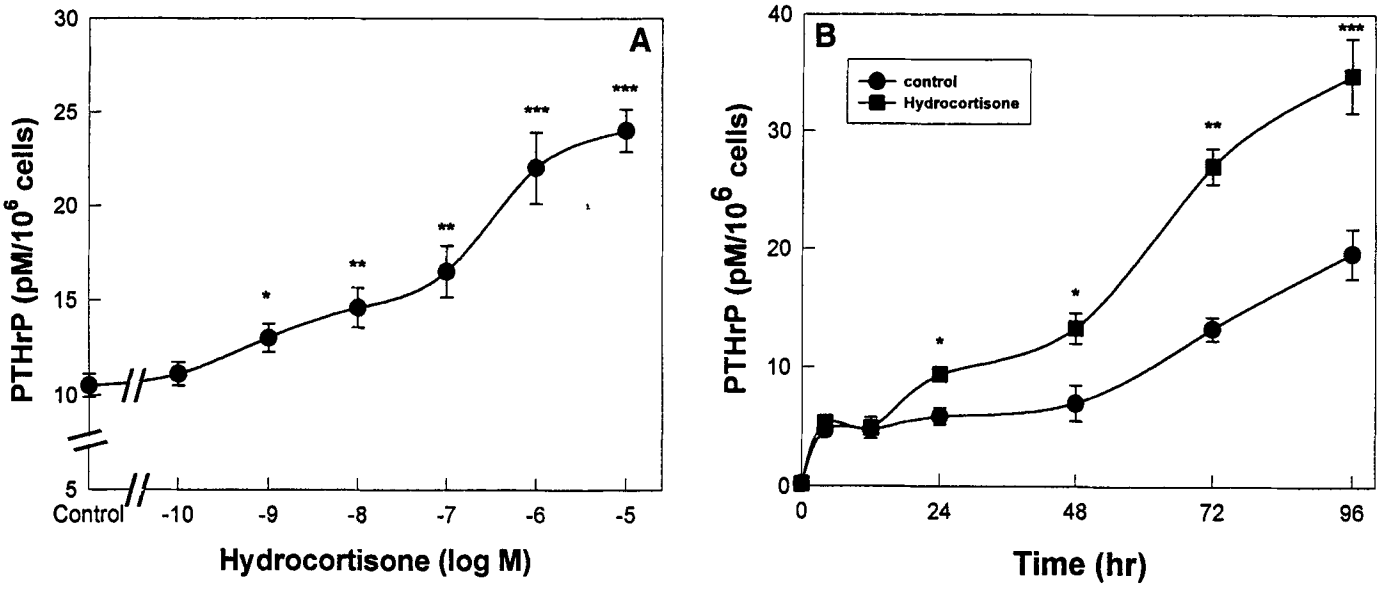

Fig. 2. Immunoreactive PTHrP in Hep G2 cell growth medium. **(A)** Medium from cells treated various doses of HC or vehicle (control) for 3 d. Here and in subsequent similar figures, values are given as mean ± SEM ($n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control or 0 time. **(B)** Medium from cells treated with $10^{-5}M$ HC ($n = 4$) for time-periods ranging from 6 to 96 h compared to vehicle control (0 time). In this figure and in subsequent figures, if no error bar is visible, it was smaller than the symbol representing the mean.

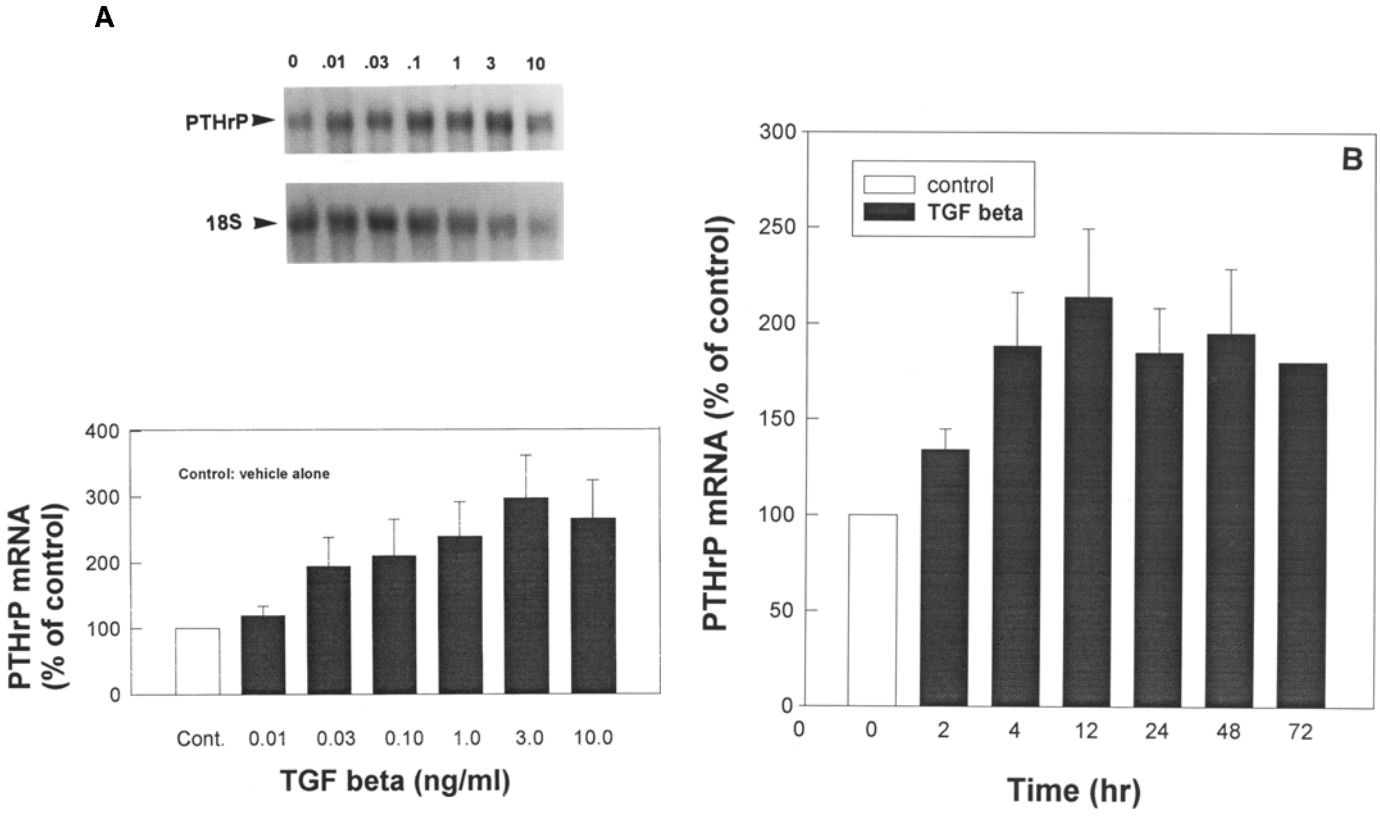

Fig. 3. PTHrP mRNA in Hep G2 cells. **(A)** Upper panel shows representative results with RNA isolated from cells treated for 2 d with various doses of TGF-β1 (TGF-β) or with vehicle (0 dose). Northern blot of PTHrP mRNA is compared to the same blot reprobed for 18S RNA to assess loading. Doses of TGF-β (ng/mL) are shown above the blots. Lower panel shows densitometric analysis of blots from 3–4 studies where the PTHrP signal was normalized to the 18S signal, and the ratios were expressed as a percent of control (vehicle treatment). **(B)** Densitometric analysis of Northern blots for PTHrP and 18S RNA from 3–4 studies (except for 72 h where $n = 1$) in which cells were treated with 1 ng/mL TGF-β for time-periods ranging from 2 to 72 h compared to vehicle control (0 time). Statistical analysis showed significant effects of treatment and time ($p < 0.05$).

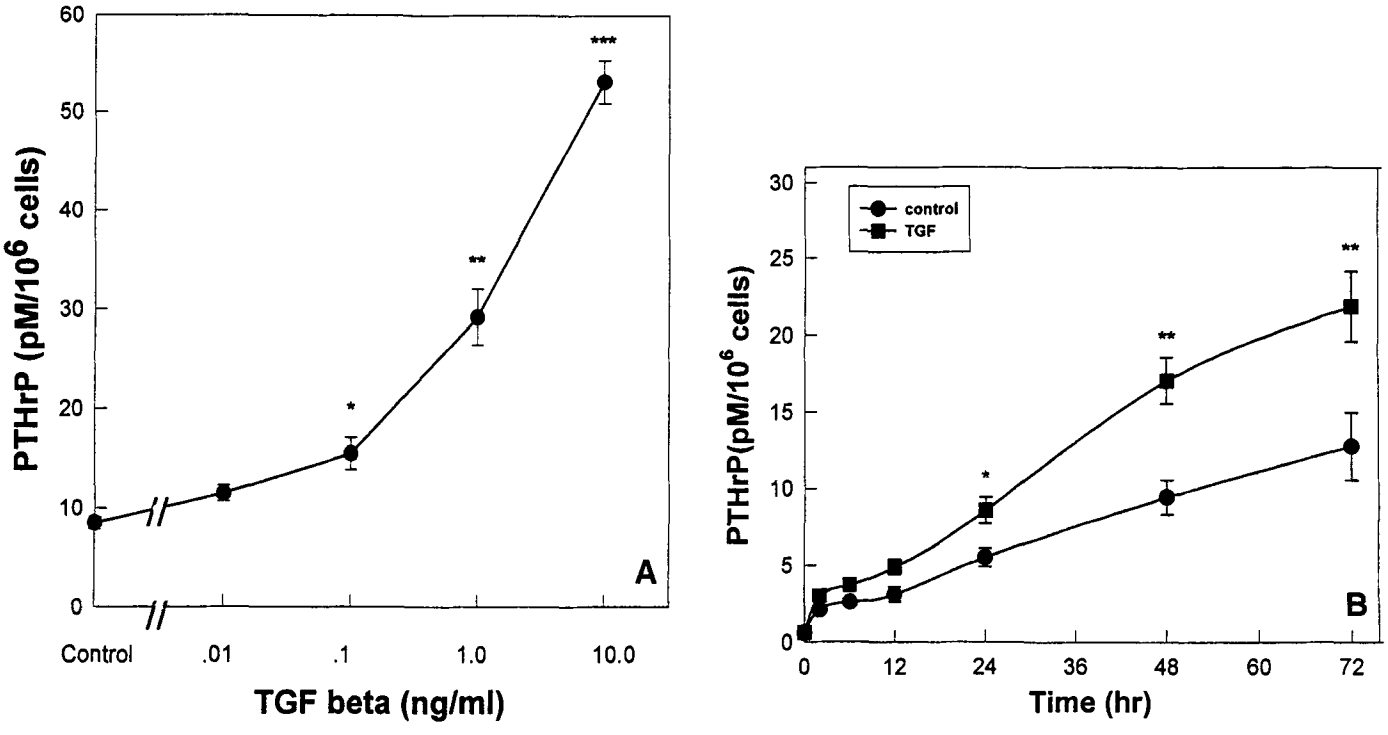

Fig. 4. Immunoreactive PTHrP in Hep G2 cell growth medium. **(A)** Medium from cells treated with various doses of TGF-β or vehicle (control) for 3 d ($n = 4$). **(B)** Medium from cells treated with 1 ng/mL TGF-β for time-periods ranging from 2 to 72 compared to vehicle control (0 time).

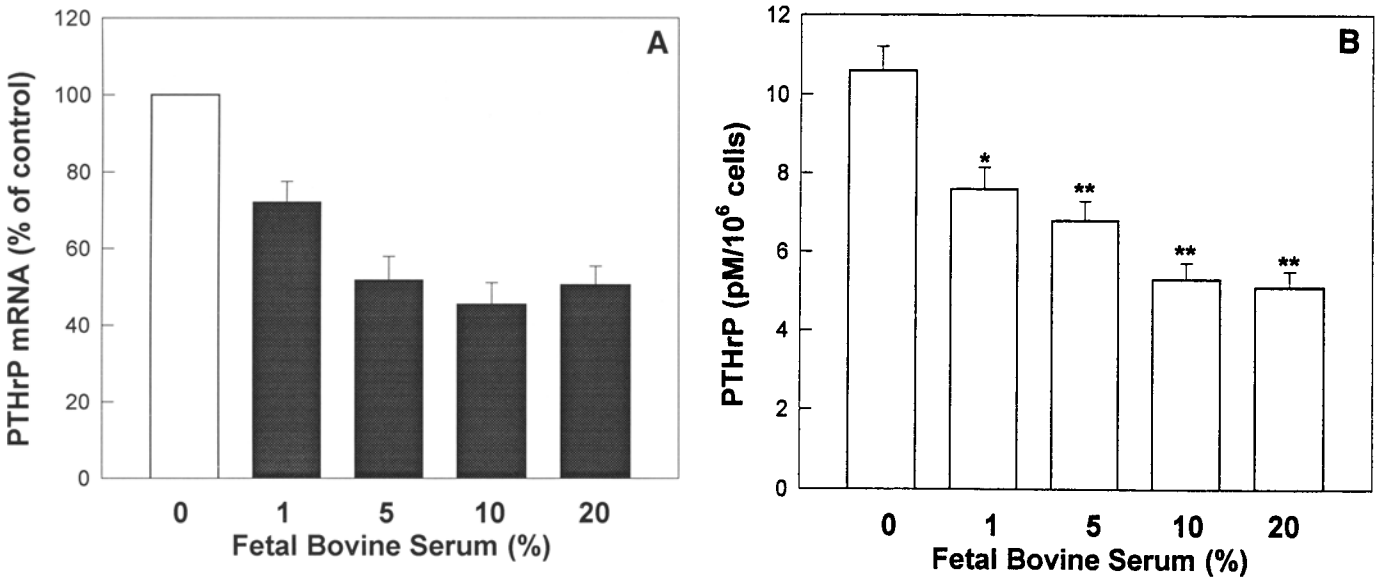

Fig. 5. PTHrP mRNA in cells and immunoreactive PTHrP in growth medium after exposing Hep G2 cells to serum-free medium for 24 h and then exposing them to medium containing fetal bovine serum (FBS) at the concentrations (v/v) shown. **(A)** Densitometric analysis of blots from 4–5 studies that normalized to the same blot reprobed for 18S RNA. Ratios were expressed as a percent of control (0% FBS). RNA was isolated after 1 d of treatment. Statistical analysis showed a significant effect of treatment ($p < 0.01$). **(B)** PTHrP in growth medium of cells exposed to different concentrations of FBS for 2 d ($n = 4$). $*P < 0.05$, $**P < 0.01$ vs 0% FBS.

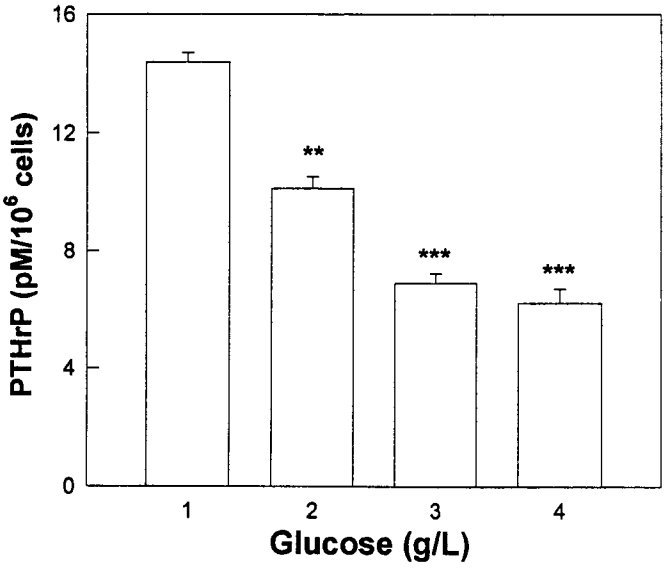

Fig. 6. Immunoreactive PTHrP in Hep G2 cell growth medium. Cells were grown for 3 d in medium containing different concentrations of glucose ($n = 4$). $**P < 0.01$, $***P < 0.001$ vs 1 g/L.

illustrates dose-related effects of TGF-β on immunoreactive PTHrP in medium; a significant increase occurred in response to 0.1 ng/mL and the greatest increase was observed with 10 ng/mL, the highest dose tested. Figure 4B shows that the effect of 1 ng/mL TGF-β to increase medium PTHrP was observed over a 24–72-h period, with the amount of PTHrP increasing progressively over the time interval studied.

Figure 5 shows that FBS, over a concentration range of 1–10% in the culture medium, caused a dose-related decrease in both cellular PTHrP mRNA (Fig. 5A) and in peptide immunoreactivity in the growth medium (Fig. 5B). A concentration of 20% FBS produced no additional effect compared to 10%. Like FBS, an elevation of medium glucose from 1 to 3 g/L caused a progressive decrease in medium PTHrP (Fig. 6); however, in studies not shown we were unable to document any decrease in PTHrP mRNA in response to an elevation in medium glucose.

Discussion

Our results with the four agents tested in cultured Hep G2 cells clearly show an inverse relationship between effects on growth and the production of PTHrP. Both hydrocortisone and TGF-β enhanced the expression of PTHrP mRNA, elevated immunoreactive PTHrP secreted into culture medium, and significantly decreased cell growth. The effects of these two growth inhibitors to elevate PTHrP mRNA and peptide were both dose- and time-dependent. In contrast, increasing the level of FBS in the growth medium led to a decrease in both cellular PTHrP mRNA and medium peptide immunoreactivity and significantly stimulated cell growth. Likewise, raising the level of glucose in the culture medium enhanced cell growth and decreased PTHrP secretion, although a corresponding decrease in PTHrP mRNA was not detected. Effects of increased FBS and glucose were dose-dependent also. Previous studies in our laboratory showed that immunoneutralization of PTHrP secreted by Hep G2 cells resulted in enhanced growth of the cells (Li et al., 1996). These earlier results suggested that PTHrP could act as an autocrine/paracrine growth suppressor in this cell line. The

current studies support this view and further imply that PTHrP might act to mediate at least some of the effects of other growth regulators as well. However, additional studies with more growth regulators will be required to test this idea more fully.

The present findings establish that synthesis and secretion of PTHrP by Hep G2 cells can be regulated and therefore is not simply constitutive. We showed previously that the peptide secreted into the medium has N-terminal biological activity, since it could elevate cAMP in rat osteosarcoma cells in culture (Li et al., 1996). The earlier findings and those presented here have established that a peptide extending at least to residue 72 must be secreted, since the immunoassay employed is a two-antibody "sandwich" assay. However, we do not know whether longer forms of the peptide are secreted or whether the cells process PTHrP posttranslationally and secrete smaller, active fragments of the originally produced peptide. Also, we do not know from these studies which regions of the peptide are responsible for the growth effects. Our findings of an inhibitory effect of PTHrP on Hep G2 cell growth are in close agreement with those of Kaiser et al. (1992), who showed an enhanced growth of cultured human keratinocytes after abolition of PTHrP production using antisense technology. In their subsequent studies, these workers (Kaiser et al., 1994) were able to reverse antisense-enhanced growth by adding full-length PTHrP (1–141) back to the culture medium, but growth remained unaffected by readdition of PTHrP (1–34), PTHrP (38–64), or PTHrP (67–86). From these findings, the authors concluded that domains of the peptide molecule other than those residing in the fragments tested must be involved, and they offered the novel speculation that a bipartite nuclear targeting sequence present in the carboxyl-terminal region of the peptide might be involved in the growth effects (Kaiser et al., 1994). In a subsequent report, Henderson et al. (1995) provided evidence that this targeting amino acid sequence in PTHrP (residues 87–107), which has sequence homology to nuclear targeting sequences in human retroviruses, was functional and prolonged survival of cultured chondrocytes by delaying the apoptosis normally induced by serum deprivation. Nevertheless, the exact region of the PTHrP molecule responsible for the growth inhibitory effect of PTHrP on Hep G2 cells remains to be identified. Whether full length peptide is required or whether smaller analogs will suffice awaits additional study. It is possible that smaller fragments could modulate growth, since such effects have been reported in other cell systems using either PTHrP (1–34) or PTHrP (107–111) (Garcia-Ocuna et al., 1995; Whitfield et al., 1996).

Exact mechanisms by which PTHrP alters cell growth remain to be identified and are likely to be complex, particularly since PTHrP has been reported to both decrease and increase cell growth, depending on the cell system being studied (Kaiser et al., 1992; Rabbani et al., 1995; Li et al., 1996). Additionally, Whitfield et al. (1996) reported that PTHrP (107–111) stimulated membrane-associated protein kinase Cs and either inhibited or stimulated growth of murine keratinocytes, depending on whether the cells were cycling or quiescent at the time of treatment. In Hep G2 cells, we have not been able to implicate either cAMP or intracellular Ca^{2+} as second messengers in growth suppression of Hep G2 cells by the peptide (Li et al., 1996). Likewise, our current study provides no insight into the exact mechanisms by which the four agents tested increase or decrease Hep G2 cell growth. Nevertheless, the agents all altered PTHrP secretion in a predictable fashion, and three of the agents affected PTHrP mRNA. Whether effects on steady-state mRNA resulted from an effect on transcriptional activity of the PTHrP promoter was not studied. However, FBS is a well-known stimulator of growth; FBS contains numerous growth factors, and the rat PTHrP gene has been shown to contain a serum response element (Kremer et al., 1991). Also, both dexamethasone and TGF-β have been reported to affect PTHrP gene transcription (Lu et al., 1989; Liu et al., 1993; Glatz et al., 1994; Merryman et al., 1994). Reports that TGF-β upregulates transcriptional activity (Merryman et al., 1994) could help explain our observed increase in Hep G2 cell PTHrP mRNA with TGF-β treatment, but reports of downregulation of transcription with dexamethasone (Lu et al., 1989; Liu et al., 1993; Glatz et al., 1994) would not account for our increase in PTHrP mRNA with hydrocortisone treatment.

While additional studies are needed to elucidate mechanisms involved in PTHrP alteration of Hep G2 cell growth, our earlier studies showed that PTHrP can restrict growth of Hep G2 cells (Li et al., 1996), and the present findings are consistent with the idea that altered production or secretion of PTHrP may help account for the effects of some other agents that regulate growth of these cells. Since Hep G2 cells are well recognized as a useful model of the normal hepatocyte (Javitt, 1990), our current studies may relevance to growth and development of normal liver cells as well.

Materials and Methods

Reagents

Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Tissue culture plasticware was obtained from Corning (Corning, NY). Dulbecco's Modified Eagle's Medium (DMEM) and hydrocortisone were ordered from Sigma (St. Louis, MO). Porcine platelet transforming growth factor-β1 (TGF-β) was purchased from R&D Systems (Minneapolis, MN). All chemicals were analytical grade and were obtained from standard suppliers.

Cell Culture

The human hepatoma cell line, Hep G2, was obtained from the American Type Culture Collection (Rockville, MD). For routine culture, the cells were grown in a humidi-

fied atmosphere containing 5% CO_2 at 37°C supplemented with 10% FBS; medium was changed twice a week. For mRNA studies, cells were grown in T-75 flasks; for cell growth experiments or studies of secreted PTHrP, cells were cultured in 24-well multiplates.

Cell Growth

From preliminary studies not shown, four potential cell growth regulators were selected for further study; two (TGF-β and HC) appeared to suppress growth, and two (serum and glucose) were apparent growth enhancers. Exact protocols varied according to the agent being studied; in general, cells were plated in 24-well plates at a density of 1.5×10^3 cells/well and allowed to attach for 12 h before fresh medium was added. To examine effects of serum, cells were deprived of serum for 24 h after they had reached 60–70% confluence; then cells were treated with medium containing different concentrations of FBS for 24–48 h. To study effects of the other three agents, cells were grown to about 70% confluence in medium containing either 5% FBS (for TGF-β experiments) or 10% FBS (for HC and glucose studies); then fresh medium containing test agent or vehicle was added, and the cells were cultured for the desired time interval. At the end of the culture period, cells were harvested by trypsinization and counted in a Coulter Counter (Coulter Electronics, Hialeah, FL).

PTHrP mRNA

Total cellular RNA from Hep G2 cells was isolated with RNAzol B (Biotecx Labs, Houston, TX) according to the manufacturer's directions. Isolated RNA was dissolved in diethylpyrocarbonate-treated water and stored at –70°C for future use. For analysis by Northern blot, 25 μg total RNA were electrophoresed in a 1.2% agarose gel containing 3% formaldehyde, transferred to a nylon filter, and cross-linked with UV irradiation. Filters were prehybridized in a solution of 6X SSC (1X = 150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0), 5X Denhardt's solution (10% polyvinylpyrrolidone, 0.1% BSA, 0.1% Ficoll [Sigma]), 0.2% SDS, and 100 μg/mL denatured salmon sperm DNA for 4 h at 68°C. Hybridization to the ^{32}P-labeled probe was performed in the same solution for 16–18 h at 68°C. Filters were washed twice for 30 min each, first in 2X SSC, 0.1% SDS at room temperature and then in 0.2X SSC, 0.1% SDS at 68°C, and then they were autoradiographed for 12–24 h at –70°C using Kodak X-Omat film (Eastman Kodak, Rochester, NY).

The mRNA probe was a 231-bp cDNA generated by RT/PCR as described previously in detail (Li et al., 1995, 1996); the synthetic primers were designed to amplify a homologous region of the rat and human PTHrP gene, and the amplimer was authenticated by dideoxy sequencing (Li et al., 1996). For use, the amplimer was labeled with ^{32}P by asymmetric PCR (Scully et al., 1990). In order to provide a reference signal for mRNA quantification, either ethidium bromide stained 18S and 28S RNA were photographed immediately after electrophoresis or blots were stripped and rehybridized with a ^{32}P-labeled human 18S ribosomal RNA probe. For each blot, the PTHrP mRNA signal was normalized to the reference signal by densitometry.

PTHrP Immunoreactivity

Growth medium from cultured Hep G2 cells (conditioned medium) was assayed for PTHrP as described previously (Li et al., 1996). Briefly, the medium was analyzed using a kit purchased from Nichols Institute (San Juan Capistrano, CA). The method detects human PTHrP by using an immunoradiometric sandwich assay employing 2 antisera, one directed toward the 1–40 amino acid region of PTHrP and another that recognizes residues 60–72. The standard provided was human PTHrP 1–86. Immunoreactivity in conditioned growth medium was compared to fresh unconditioned medium containing FBS as the control.

Statistical Analysis

For cell growth and PTHrP measurement, representative experiments are shown. However, to establish reproducibility, each finding of interest was verified in 2–3 separate experiments. Data from PTHrP measurement and cell growth studies are presented as mean ± SEM and were analyzed by ANOVA followed by a Bonferroni multicomparison post test (Morrison, 1983). For PTHrP mRNA analysis, data from separate experiments (blots) were combined and analyzed nonparametrically using a Kruskal-Wallis ANOVA (Mendenhall et al., 1990). All statistical analyses were performed using computer software from GraphPad (San Diego, CA). Figures were plotted using SigmaPlot (San Raphael, CA). $P < 0.05$ was considered significant for establishing differences.

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