Jpn. J. Cancer Res. 82, 1008–1014, September 1991

Effects of Glucocorticoids and Calcitonin on Parathyroid Hormone-related Protein (PTHrP) Gene Expression and PTHrP Release in Human Cancer Cells Causing Humoral Hypercalcemia[1]

Keizo Kasono,[2] Osamu Isozaki,[2] Kanji Sato,[2,5] Yasuko Sato,[3] Kazuo Shizume,[3] Kazuaki Ohsumi[4] and Hiroshi Demura[2]

[2]*Department of Medicine, Institute of Clinical Endocrinology, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162,* [3]*Research Institute of the Foundation for Growth Science in Japan, 9-4 Wakamatsu-cho, Shinjuku-ku, Tokyo 162 and* [4]*Mitsubishi Yuka BCL, 3-30-1 Shimura, Itabashi-ku, Tokyo 174*

Glucocorticoids are widely used for the treatment of malignancy-associated hypercalcemia to delay the occurrence of an escape phenomenon inherent in calcitonin therapy. Using parathyroid hormone-related protein (PTHrP)-producing squamous carcinoma cells (T3M-1 and EC-GI) established in our laboratory, we investigated the *in vitro* effects of glucocorticoids and calcitonin on PTHrP mRNA expression in the cells and release of PTHrP into the culture medium. The PTHrP gene was constitutively expressed in the logarithmic growth phase in both squamous carcinoma cell lines. When these cells became superconfluent, PTHrP mRNA expression was greatly diminished in T3M-1 cells but was not distinctly diminished in EC-GI cells. Hydrocortisone inhibited the PTHrP mRNA expression in T3M-1 cells and EC-GI cells in a dose-dependent manner. In accordance with the decreased expression of PTHrP mRNA, the release of immunoreactive as well as bioactive PTHrP also decreased in the conditioned medium of glucocorticoid-treated cells. The minimal effective concentration of prednisolone was about 10^{-7} *M*, which is readily attainable in the serum of patients treated with the agent. Calcitonin and indomethacin did not affect the PTHrP mRNA expression or PTHrP release into the medium. Calcitonin did not modulate the hydrocortisone-induced inhibition of PTHrP production. These *in vitro* findings suggest that the combined use of glucocorticoids and calcitonin plays a beneficial role in the treatment of malignancy-associated hypercalcemia, since the steroid hormone can suppress PTHrP mRNA expression and release of bioactive PTHrP in certain PTHrP-producing tumors.

Key words: Parathyroid hormone-related protein (PTHrP) — Malignancy-associated hypercalcemia — Glucocorticoid — Calcitonin

Hypercalcemia is occasionally a life-threatening complication of malignant diseases. When radical resection of a tumor is impossible, current therapy for malignancy-associated hypercalcemia (MAH) includes saline hydration, forced diuresis with loop diuretics, and administration of inhibitors of bone resorption such as calcitonin together with glucocorticoids.[1] Indomethacin is also effective in some patients with MAH.[1]

Calcitonin is a potent inhibitor of osteoclastic bone resorption and is most widely used in patients with MAH. However, its inhibitory effect of bone resorption is brief because of an escape phenomenon.[2,3] To strengthen the effect of calcitonin on osteoclastic bone resorption, the combined use of calcitonin and glucocorticoid is recommended.[4,5] This is based on the *in vitro* observation of Raisz *et al.*[6] that glucocorticoids prolong the inhibition of ^{45}Ca release induced by calcitonin. Recently, Deftos *et al.*[7] reported that calcitonin stimulates the release of immunoreactive parathyroid hormone-related protein (PTHrP) induced by phorbol-12-myristate-13-acetate from PTHrP-producing cells, suggesting that the treatment with calcitonin of patients with MAH may aggravate humoral hypercalcemia in certain of the patients.

Using PTHrP-producing carcinoma cells (T3M-1, EC-GI) established from patients with hypercalcemia,[8,9] we have investigated the effects of glucocorticoids and calcitonin on PTHrP mRNA expression and release of immunoreactive and bioactive PTHrP into the culture medium.

MATERIALS AND METHODS

Source of tumor cells EC-GI cells were derived from a squamous cell carcinoma of the esophagus of a 65-yr-old man who had marked hypercalcemia.[8] T3M-1 cells were

[1] Part of this work was presented at the 12th Annual Meeting of the American Society for Bone and Mineral Research, Atlanta, August 1990 (abstract 486).

[5] To whom requests for reprints should be addressed.

established from a patient with submandibular carcinoma who also had hypercalcemia.[9] These cells produced PTHrP and developed hypercalcemia in the tumor-bearing mice.[10, 11]

Cell culture EC-GI or T3M-1 cells were cultured in F-12 medium (Gibco, Grand Island, NY) supplemented with 5% fetal calf serum (FCS) (Filtron, Australia). The media were changed twice a week. When the cells became subconfluent, media were changed to F-12 medium containing 0.2% bovine serum albumin, glucocorticoids (Sigma Chemcal Co., St. Louis, MO), salmon calcitonin (Sigma) and/or indomethacin (Sigma). Cells were incubated for an additional 24 or 48 h.

Northern blot hybridization Total cellular RNA was isolated essentially as described by Chirgwin *et al.*[12] Poly A^+ RNA was selected by oligo(dT)-cellulose chromatography,[13] and samples of 10 μg of RNA were electrophoresed in an agarose gel containing 0.66 *M* formaldehyde and transferred to nylon filters (Nytran, 0.45 μm pore size, Schleicher & Schuell, Keene, NH) as suggested by the manufacturer. For hybridization with the PTHrP probe, the membranes were prehybridized for at least 3 h at 60°C in 50% formamide/5×SSPE (1×SSPE is 0.15 *M* NaCl, 0.01 *M* NaH_2PO_4, 1 m*M* EDTA, pH 7.4)/5× Denhardt's solution (1×Denhardt's solution is composed of 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 200 μg/ml salmon sperm DNA, and 1% SDS. The radiolabeled cRNA for PTHrP described later was added (5×10^5 cpm/ml) and allowed to hybridize at 60°C for 18–24 h in 50% formamide/5×SSPE/2× Denhardt's solution/50 μg/ml salmon sperm DNA, 50 μg/ml transfer RNA, 10 μg/ml poly(A), 1% SDS. The filter washing was performed under a stringent condition at 65°C in 0.2×SSPE. Then the filters were subjected to autoradiography. For the evaluation of the amounts of RNA on filters, the filters were rehybridized with β-actin probe after stripping off the PTHrP probe as suggested by the manufacturer. The hybridization and washing for actin mRNA were described previously.[14, 15]

A cDNA clone, kindly provided by Dr. William I. Wood (Genentech Inc., San Francisco, CA), and containing a 537-base-pair fragment encoding PTHrP[16] inserted into the pSP65 plasmid, was used as a template for the pSP6 RNA polymerase-directed synthesis of a ^{32}P-cRNA, as described by Melton *et al.*[17] The probe had a specific activity of about 1×10^9 cpm/μg RNA. A rat β-actin cDNA fragment obtained from Dr. Bruce Patterson (National Institutes of Health, Bethesda, MD) was labeled with ^{32}P-dCTP by random primer labeling with a specific activity of 1×10^9 cpm/μg DNA.

Assay for PTHrP released into the medium EC-GI cells were cultured with F-12 medium containing 0.2% BSA, hydrocortisone, calcitonin or indomethacin as described in each experiment. After 24–48 h culture, the conditioned medium was collected and stored at −20°C until assayed for immunoreactive as well as bioactive PTHrP.

Bioactive PTHrP released into the medium was determined by measuring adenylate cyclase-stimulating activity using ROS 17/2.8-5 subclonal cells as described previously.[18] Cells were grown in 24-well plastic dishes (2 cm^2/well). Cyclic AMP produced in ROS 17/2.8-5 cells during a 10 min incubation was determined by using a radioimmunoassay kit (Eiken, Japan) and data were expressed as picomoles of cyclic AMP per well.

Immunoreactive PTHrP released into the medium was also determined by radioimmunoassay specific for the N-terminal region of PTHrP.[19, 20] Antiserum for the N-terminal region of PTHrP was prepared by immunizing rabbits with human PTHrP(1-34) conjugated to bovine thyroglobulin. The antiserum was highly specific for the N-terminal region of PTHrP(1-34): the antiserum did not recognize [Tyr^{126}]PTHrP(126-141) (synthesized in Toray Research Center, Kamakura), PTH(1-34), or PTH(65-84) (obtained from Peptide Institute, Osaka). [Tyr^0]PTHrP(1-34) was radioiodinated by the chloramine T method. The assay buffer was 0.1 *M* Tris-HCl buffer (pH 8.0) containing 0.1% BSA, 10 m*M* EDTA, 0.1% Tween 20 and 0.02% sodium azide. The RIA incubation mixture consisted of 100 μl of samples or standard PTHrP(1-34) (Peninsula Laboratory, Belmont, CA), and 100 μl of anti-PTHrP(1-34) antiserum (final dilution 1:90,000). Incubation was carried out at 4°C for 24 h, followed by addition of 100 μl of [^{125}I][Tyr^0]-PTHrP(1-34). Bound and free [^{125}I][Tyr^0]PTHrP(1-34) were separated by a polyethyleneglycol double-antibody precipitation. The sensitivity of the assay was 2 pg/tube. Data were expressed as picomoles equivalent to PTHrP(1-34)/ml.

Effect of calcium concentration on cell growth Ca-free F-12 medium was prepared in our laboratory. EC-GI or T3M-1 cells were cultured in 24-well dishes (2×10^4 cells/2 cm^2 well). On the following day, the culture medium was replaced with 1 ml of Ca-free F-12 medium containing 10% FCS supplemented with various concentrations of calcium (0, 0.25, 0.5, 1, 2, 3, and 4 m*M*). The calcium concentration of the FCS was 11 mg/dl (2.75 m*M*). Medium was replaced every three days. The numbers of cells grown in the medium with various calcium concentrations (0.275–3.875 m*M*) were counted using a Coulter counter at 2, 4, 6 and 10 days.

RESULTS

PTHrP gene expression in T3M-1 and EC-GI cells at various cell densities As shown in Fig. 1, PTHrP gene was mainly expressed in the logarithmic phase of cell growth in T3M-1 and EC-GI cells. Both cell lines expressed four mRNA bands, the sizes of which cor-

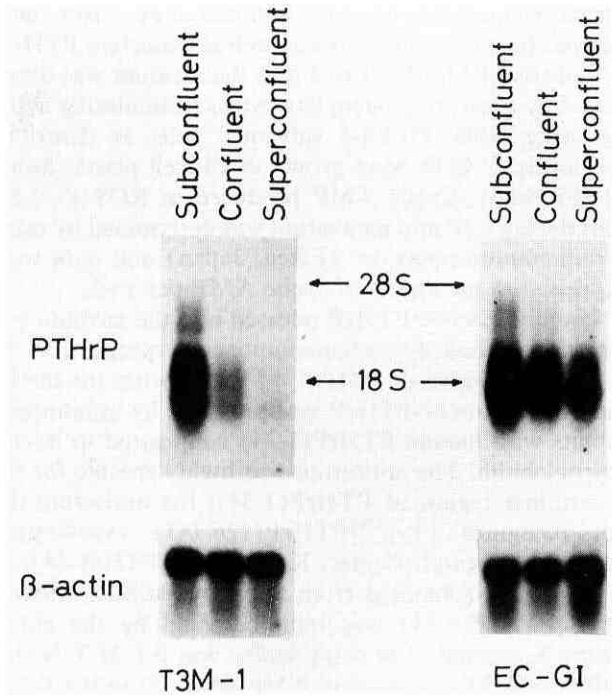

Fig. 1. Northern blot analysis of T3M-1 or EC-GI cell-derived mRNAs from cells at different cell densities. T3M-1 cells and EC-GI cells were cultured with F-12 medium supplemented with 10% FCS. When these cells became subconfluent, confluent or superconfluent, the medium was replaced with F-12 medium supplemented with 0.2% BSA, and the cells were cultured for an additional 48 h. Then, poly(A)$^+$RNA was prepared as described in "Materials and Methods." The upper panel shows the Northern blot analysis of poly(A)$^+$RNA (10 μg) from T3M-1 or EC-GI cells at different cell densities, using a PTHrP antisense RNA probe. The lower panel shows the same membrane rehybridized to a β-actin probe.

responded to those reported previously.[21] In T3M-1 cells, PTHrP gene expression was greatly diminished after the cells had reached confluency, whereas EC-GI cells expressed a considerable amount of PTHrP mRNA even when the cells became superconfluent. Therefore, we performed all the following experiments in the logarithmic growth phase.

Release of immunoreactive and bioactive PTHrP into the medium PTHrP levels in the conditioned medium of EC-GI cells, determined by RIA specific for the N-terminal region of PTHrP, increased gradually after 4 h incubation (Fig. 2). T3M-1 cells also secreted immunoreactive PTHrP into the medium, but to a smaller extent compared with EC-GI cells. In accordance with immunoreactive PTHrP levels in the medium, adenylate cyclase-stimulating activity in the conditioned medium also increased in a time-dependent manner, suggesting that EC-GI cells as well as T3M-1 cells secrete bioactive PTHrP into the medium.

Effects of hydrocortisone on PTHrP gene expression and production When T3M-1 cells or EC-GI cells were cultured with medium containing various concentrations of hydrocortisone, the steroid dose-dependently inhibited PTHrP gene expression in both cell lines (Fig. 3, upper panel). Hydrocortisone had no effect on the level of β-actin mRNA used as an internal control (Fig. 3, lower panel).

In accordance with inhibition of PTHrP mRNA expression, hydrocortisone also inhibited the release of immunoreactive as well as bioactive PTHrP into the medium (Fig. 4). The minimal effective concentration was 10^{-7} *M*. Hydrocortisone (10^{-9}–10^{-6} *M*) did not affect cell growth of EC-GI or T3M-1 cells (data not shown).

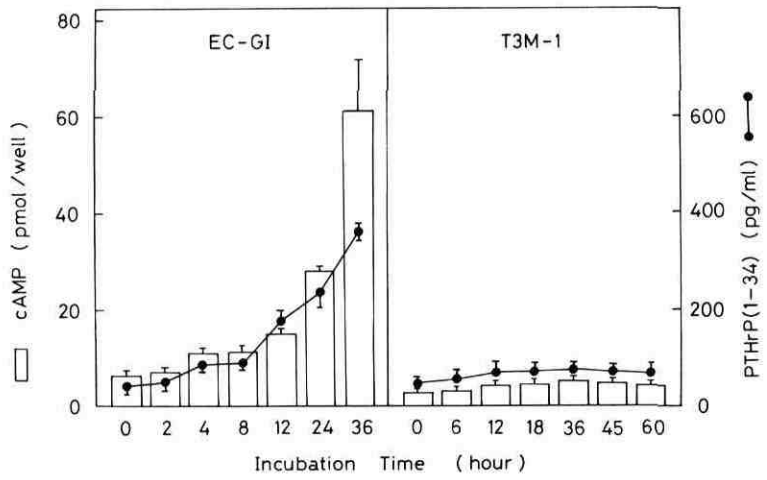

Fig. 2. Measurement of PTHrP released into culture medium. After EC-GI and T3M-1 cells became subconfluent, the media were changed to F-12 medium containing 0.2% bovine serum albumin and the cells were cultured for 36–60 h. Conditioned media at the indicated incubation period were stored at −20°C until assayed for bioactive and immunoreactive PTHrP. Open columns show the cyclic AMP produced in ROS 17/2.8-5 cells during 10-min incubation. Solid lines show the concentration of PTHrP in the media conditioned by EC-GI cells (left) and T3M-1 cells (right). PTHrP was determined by RIA specific for the N-terminal region of PTHrP. Data are means ± SE of quadruplicate cultures.

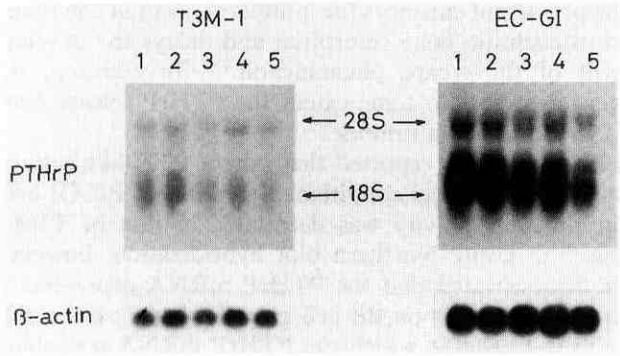

Fig. 3. Effect of hydrocortisone on PTHrP mRNA expression. After EC-GI and T3M-1 cells became subconfluent, the cells were cultured with F-12 medium supplemented with 0.2% BSA and various concentrations of hydrocortisone for 2 days. Then, mRNAs were prepared as described in "Materials and Methods." The upper panel shows the Northern blot analysis of total RNA (10 μg) from T3M-1 (left) or EC-GI (right) cells using a PTHrP antisense RNA probe. The lower panel shows the same membrane rehybridized to a β-actin probe. Hydrocortisone concentrations were 0 (lane 1), 10^{-9} *M* (lane 2), 10^{-8} *M* (lane 3), 10^{-7} *M* (lane 4) and 10^{-6} *M* (lane 5).

Table I. Effects of Steroid Hormones on PTHrP Release from EC-GI Cells

	Concentration (*M*)	iPTHrP (pg/ml)
Unconditioned medium		<10
Conditioned medium		
Control	(−)	113±7.5
Hydrocortisone	10^{-8}	112±10
	10^{-7}	63±3.2*
	10^{-6}	61±8.1*
Prednisolone	10^{-8}	111±5.2
	10^{-7}	63±3.2*
	10^{-6}	58±3.3*
17β-Estradiol	10^{-6}	93±3.8
Testosterone	10^{-6}	92±2.0

When EC-GI cells became subconfluent, the cells were cultured with F-12 medium supplemented with 0.2% BSA and steroid hormones. After 48-h culture, immunoreactive PTHrP (iPTHrP) concentration was determined by a radioimmunoassay specific for the N-terminal region of PTHrP. iPTHrP was expressed as pg/ml equivalent to PTHrP(1–34). Data are means±SE of triplicate cultures. * $P<0.01$.

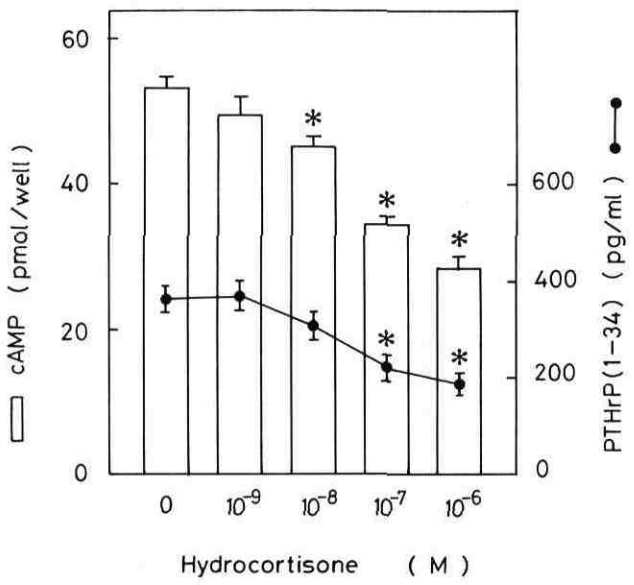

Fig. 4. Effect of hydrocortisone on PTHrP release. After EC-GI cells became subconfluent, the cells were cultured with F-12 medium supplemented with 0.2% BSA and various concentrations of hydrocortisone. After 48-h culture, conditioned medium was stored and the adenylate cyclase-stimulating activity was determined in PTH-responsive ROS 17/2.8-5 cells (open column). Immunoreactive PTHrP concentration in the conditioned medium was determined by radioimmunoassay specific for the N-terminal region of PTHrP (●—●). Data are means±SE of quadruplicate cultures. * $P<0.05$.

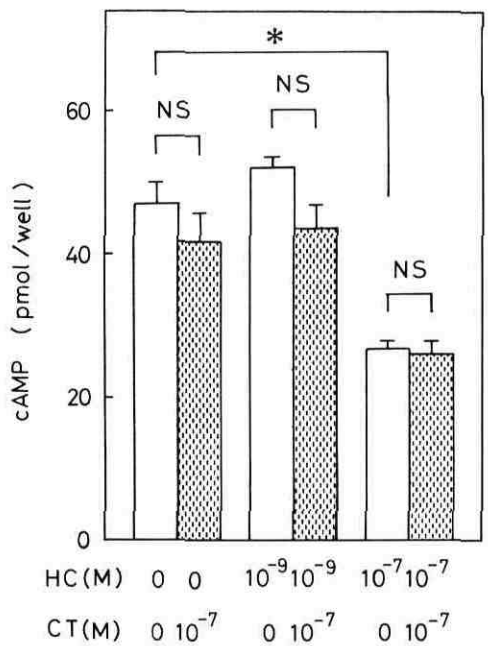

Fig. 5. Combined effects of hydrocortisone and calcitonin on PTHrP release. When EC-GI cells became subconfluent, the cells were cultured with F-12 medium supplemented with 0.2% BSA, hydrocortisone (10^{-9} or 10^{-7} *M*), and/or salmon calcitonin (10^{-7} *M*) for 2 days. After 48-h culture, the conditioned media were assayed for adenylate cyclase-stimulating activity using ROS 17/2.8 cells. Data are means±SE of quadruplicate cultures. NS: not significant, * $P<0.05$.

Prednisolone (10^{-7} *M*) also elicited a similar inhibitory effect on PTHrP release from EC-GI cells in a dose-dependent manner, whereas estrogen and androgen had no effect on PTHrP release even at 10^{-6} *M* (Table I).

Combined effects of hydrocortisone and calcitonin on PTHrP production We next examined the combined effects of hydrocortisone and calcitonin on release of PTH-like activity in EC-GI cells. Hydrocortisone significantly inhibited PTHrP release at 10^{-7} *M* (Fig. 5). Calcitonin (10^{-7} *M*) had no effect on PTHrP release. Consistent with these findings, calcitonin did not affect PTHrP gene expression in EC-GI cells (data not shown). Furthermore, calcitonin did not modulate the decreased PTHrP release induced by hydrocortisone (Fig. 5).

Effect of indomethacin on PTHrP production in EC-GI cells Indomethacin at 10^{-6} *M* showed no effect on PTHrP production in EC-GI cells (data not shown).

Effect of calcium concentration of the medium on tumor cell growth Since cell growth of certain PTHrP-producing tumor cells is stimulated in a hypercalcemic medium,[22] cell growth of T3M-1 and EC-GI cells was investigated in medium containing 0.25–3.875 m*M* calcium concentration. Cell growth was not stimulated by high calcium concentration.

DISCUSSION

Using two PTHrP-producing cell lines derived from patients with MAH, we demonstrated that glucocorticoids directly decreased PTHrP mRNA expression. Our data are in accordance with a report that glucocorticoid decreased PTHrP mRNA levels in a human carcinoid cell line (NCI-H 727).[23] Furthermore, we have demonstrated that a decrease in PTHrP mRNA expression was accompanied by a decreased release of immunoreactive as well as biologically active PTHrP in the culture medium. The minimum prednisolone concentration in the medium necessary to inhibit PTHrP mRNA expression (10^{-7} *M*) is readily attainable in patients treated with a therapeutic dose of glucocorticoids.[24]

Recently, Deftos *et al.*[7] reported that calcitonin stimulated immunoreactive PTHrP release from BEN cells, a lung cancer cell line from which PTHrP cDNA was originally cloned.[16] In contrast to BEN cells, calcitonin did not affect PTHrP mRNA expression or PTHrP release in EC-GI cells as well as T3M-1 cells. Furthermore, calcitonin did not modulate the decreased PTHrP release induced by glucocorticoid. These *in vitro* findings suggest that glucocorticoids may have dual effects in the treatment of MAH. As is well known, glucocorticoid enhances the inhibitory effect of calcitonin on osteoclastic bone resorption and delays the development of the escape phenomenon.[2,4] In addition, the agent decreases to some extent the PTHrP release from PTHrP-producing tumors.

Previously, we reported that potent PTH-like activity was detected in the conditioned medium of EC-GI cells but no such activity was detectable in that of T3M-1 cells.[10,25] Using Northern blot hybridization, however, we demonstrated that the PTHrP mRNA expression is largely dependent on the cell growth phase, particularly in T3M-1 cells, in which the PTHrP mRNA was abundant in the logarithmic phase but hardly detectable in the superconfluent phase. This is why we could not demonstrate PTH-like bioactivity in the conditioned medium of T3M-1 cells in the preliminary report,[25] since the conditioned medium was always collected in the superconfluent phase.

Recently, Shirakawa *et al.*[26] claimed that adult T cell leukemia cells (MT-2), which produce PTHrP and IL-1α,[27,28] proliferate more rapidly in culture medium containing a high calcium concentration (2–5 m*M*). Both squamous carcinoma cells used in this series of experiments also produce PTHrP and IL-1α,[10,11] both of which synergistically stimulate bone resorption *in vitro* and elicit marked hypercalcemia *in vivo*.[29] However, unlike MT-2 cells, neither EC-GI cells nor T3M-1 cells proliferated more rapidly in the high calcium medium.

In summary, although the chief beneficial effect of glucocorticoids in the treatment of patients with MAH is to prolong the inhibition of osteoclastic bone resorption induced by calcitonin, we have demonstrated that glucocorticoids also directly decrease PTHrP mRNA expression and release of bioactive PTHrP in certain PTHrP-producing tumor cells, suggesting additional beneficial effects in attenuating humoral hypercalcemia. These results provide a rational basis for the treatment of MAH patients with calcitonin together with glucocorticoids.

ACKNOWLEDGMENTS

The authors thank Dr. I. Wood (Genentech, San Francisco, CA) for supplying cDNA for PTHrP. This work was supported by a Grant-in-Aid for Scientific Research (02671109 and 02771814) from the Ministry of Education, Science and Culture of Japan, and a Grant-in-Aid for Cancer Research (I-33) from the Ministry of Health and Welfare of Japan.

(Received February 19, 1991/Accepted June 19, 1991)

REFERENCES

1) Neer, R. M. and Potts, J. T. Medical management of hyperparathyroidism and hypercalcemia. *In* "Endocrinology," ed. L. J. DeGroot, Second edition, pp. 1002–1012 (1989). W. B. Saunders Co., Philadelphia.
2) Wener, J. A., Gorton, S. J. and Raisz, L. G. Escape from inhibition of resorption in cultures of fetal bone treated with calcitonin and parathyroid hormone. *Endocrinology,* **90,** 752–759 (1972).
3) Fujii, Y., Sato, K., Kasono, K., Satoh, T., Fujii, T. and Shizume, K. Prolonged and ubiquitous inhibition by interferon-γ of bone resorption induced by parathyroid hormone-related protein, 1,25-dihydroxyvitamin D_3, and interleukin-1 in fetal mouse forearm bones. *Calcif. Tissue Int.,* **47,** 178–182 (1990).
4) Binstock, M. L. and Mundy, G. R. Effect of calcitonin and glucocorticoids in combination on the hypercalcemia of malignancy. *Ann. Intern. Med.,* **93,** 269–272 (1980).
5) Mundy, G. R. Treatment of hypercalcemia due to malignancy. *In* "Calcium Homeostasis: Hypercalcemia and Hypocalcemia," Second edition, pp. 116–136 (1990). Martin Dunitz, London.
6) Raisz, L. G., Trummel, C. L., Wener, J. A. and Simmons, H. A. Effect of glucocorticoids on bone resorption in tissue culture. *Endocrinology,* **90,** 961–967 (1972).
7) Deftos, L. J., Angeletti, R. H., Chalberg, C. and Tu, S. PTHrP secretion is stimulated by CT and inhibited by CgA peptides. *Endocrinology,* **125,** 563–565 (1989).
8) Sato, K., Kasono, K., Ohba, Y., Yashiro, T., Fujii, Y., Yoshida, M. A., Tsushima, T. and Shizume, K. Establishment of a parathyroid hormone-like factor-producing esophageal carcinoma cell line (EC-GI). *Jpn. J. Cancer Res.,* **78,** 1044–1048 (1987).
9) Sato, K., Mimura, H., Han, D., Kakiuchi, T., Ueyama, Y., Ohkawa, H., Okabe, T., Kondo, Y., Ohsawa, N., Tsushima, T. and Shizume, K. Production of bone-resorbing activity and colony-stimulating activity *in vivo* and *in vitro* by a human squamous cell carcinoma associated with hypercalcemia and leukocytosis. *J. Clin. Invest.,* **78,** 145–154 (1986).
10) Sato, K., Fujii, Y., Kasono, K., Tsushima, T. and Shizume, K. Production of interleukin-1α and a parathyroid hormone-like factor by a squamous cell carcinoma of the esophagus (EC-GI) derived from a patient with hypercalcemia. *J. Clin. Endocrinol. Metab.,* **67,** 592–601 (1988).
11) Sato, K., Fujii, Y., Kakiuchi, T., Kasono, K., Imamura, H., Kondo, Y., Mano, H., Okabe, T., Asano, S., Takaku, F., Tsushima, T. and Shizume, K. Paraneoplastic syndrome of hypercalcemia and leukocytosis caused by squamous carcinoma cells (T3M-1) producing parathyroid hormone-related protein, interleukin-1α, and granulocyte colony-stimulating factor. *Cancer Res.,* **49,** 4740–4746 (1989).
12) Chirgwin, M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry,* **18,** 5294–5300 (1979).
13) Aviv, H. and Leder, P. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc. Natl. Acad. Sci. USA,* **69,** 1408–1412 (1972).
14) Church, G. M. and Gilbert, W. Genomic sequencing. *Proc. Natl. Acad. Sci. USA,* **81,** 1991–1995 (1984).
15) Isozaki, O., Kohn, L. D., Kosak, C. A. and Kimura, S. Thyroid peroxidase: rat cDNA sequence, chromosomal localization in mouse, and regulation of gene expression by comparison to thyroglobulin in rat FRTL-5 cells. *Mol. Endocrinol.,* **3,** 1681–1692 (1989).
16) Suva, L. J., Winslow, G. A., Wettenhall, R. E. M., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J. and Wood, W. I. A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science,* **237,** 893–896 (1987).
17) Melton, D. A. Translation of messenger RNA in injected frog oocytes. *Methods Enzymol.,* **152,** 288–296 (1987).
18) Sato, K., Han, D., Ozawa, M., Fujii, Y., Tsushima, T. and Shizume, K. A highly sensitive bioassay for PTH using ROS 17/2.8 subclonal cells. *Acta Endocrinol.,* **116,** 113–120 (1987).
19) Imamura, H., Sato, K., Shizume, K., Satoh, T., Kasono, K., Ozawa, M., Ohmura, E., Tsushima, T. and Demura, H. Urinary excretion of parathyroid hormone-related protein fragments in patients with humoral hypercalcemia of malignancy and hypercalcemic tumor-bearing nude mice. *J. Bone Miner. Res.,* **6,** 77–82 (1991).
20) Horiuchi, J., Ohsumi, K., Imamura, H., Sato, K., Sakurai, H. and Demura, H. Radioimmunoassay for parathyroid hormone-related protein (PTHrP). *Folia Endocrinol. Jpn.,* **66,** 350 (1990) (in Japanese).
21) Mangin, M., Webb, A. C., Dreyer, B., Posillico, T. J., Ikeda, K., Weir, E. C., Stewart, A. F., Bander, N. H., Milstone, L., Barton, D. E., Francke, U. and Broadus, A. E. Identification of a cDNA encoding a parathyroid hormone-like poptide from a human tumor associated with humoral hypercalcemia of malignancy. *Proc. Natl. Acad. Sci. USA,* **85,** 597–601 (1988).
22) Shirakawa, F., Yamashita, U., Oda, S., Chiba, S., Eto, S. and Suzuki, H. Calcium dependency in the growth of adult T-cell leukemia cells *in vitro*. *Cancer Res.,* **46,** 658–661 (1986).
23) Lu, C., Ikeda, K., Deftos, L. J., Gazdar, A. F., Mangin, M. and Broadus, A. E. Glucocorticoid regulation of parathyroid hormone-related peptide gene transcription in a human neuroendocrine cell line. *Mol. Endocrinol.,* **12,** 2034–2040 (1989).
24) Miyachi, Y., Yotsumoto, H., Kano, T., Mizuchi, A.,

Moto, T. and Yanagibashi, K. Blood levels of synthetic glucocorticoids after administration by various routes. *J. Endocrinol.*, **82**, 149–157 (1979).

25) Sato, K., Fujii, Y., Kakiuchi, T., Kasono, K. and Shizume, K. Production of interleukin 1α (IL-1α)-like activity and colony-stimulating activity by clonal squamous cell carcinomas derived from patients with hypercalcemia and leukocytosis. *In* "Calcium Regulation and Bone Metabolism: Basic and Clinical Aspects," Vol. 9, ed. D. V. Cohn, T. J. Martin and P. J. Meunier, pp. 149–154 (1987). Elsevier Scientific Publishers, Amsterdam.

26) Shirakawa, F., Yamashita, U., Oda, S., Chiba, S., Eto, S. and Suzuki, H. Calcium dependency in the growth of adult T-cell leukemia cells *in vitro*. *Cancer Res.*, **46**, 658–661 (1986).

27) Shirakawa, F., Yamashita, U., Tanaka, Y., Watanabe, K., Sato, K., Haratake, J., Fujihira, T., Oda, S. and Eto, S. Production of bone-resorbing activity corresponding to interleukin-1α by adult T-cell leukemia cells in humans. *Cancer Res.*, **48**, 4284–4287 (1988).

28) Motokura, T., Fukumoto, S., Takahashi, S., Watanabe, T., Matsumoto, T., Igarashi, T. and Ogata, E. Expression of parathyroid hormone-related protein in a human T cell lymphotrophic virus type 1-infected T cell line. *Biochem. Biophys. Res. Commun.*, **154**, 1182–1188 (1988).

29) Sato, K., Fujii, Y., Kasono, K., Ozawa, M., Imamura, H., Kanaji, Y., Kurosawa, H., Tsushima, T. and Shizume, K. Parathyroid hormone-related protein and interleukin-1α synergistically stimulate bone resorption *in vitro* and increase the serum calcium concentration in mice *in vivo*. *Endocrinology*, **124**, 2172–2178 (1989).