JCI The Journal of Clinical Investigation

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G J Strewler, … , R D Williams, R A Nissenson

J Clin Invest. 1983;71(3):769-774. https://doi.org/10.1172/JCI110825.

Research Article

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Human Renal Carcinoma Cells Produce Hypercalcemia in the Nude Mouse and a Novel Protein Recognized by Parathyroid Hormone Receptors

GORDON J. STREWLER, RICHARD D. WILLIAMS, and ROBERT A. NISSENSON,
Departments of Medicine and Urology, Veterans Administration Medical Center and University of California, San Francisco, California 94121

ABSTRACT When grown in nude mice, cultured renal carcinoma cells from a hypercalcemic patient produced marked hypercalcemia that was reversed by resection of tumor. Conditioned medium from this cell line contained a protein with activity in a renal adenylate cyclase bioassay for parathyroid hormone (PTH) which was blocked by the competitive PTH antagonist [^{8}norleucyl, ^{18}norleucyl, ^{34}tyrosinyl]bPTH (3–34)amide. However, the biologically active protein was eluted from gel filtration columns as a larger molecular size component than PTH and was not recognized by any of four region-specific PTH antisera. The properties of this factor resemble those of the postulated PTH-like substance(s) in humoral hypercalcemia of malignancy.

INTRODUCTION

The etiology of malignancy-associated hypercalcemia (MAH)[1] is uncertain. Immunoreactive PTH (iPTH) levels are either inappropriately high (1) or undetectable (2) depending on the radioimmunoassay (RIA) used. A recent study (2) in which 41/50 consecutive patients with MAH had elevated nephrogenous cyclic AMP (NcAMP) (a finding previously considered to be specific for hyperparathyroidism) has rekindled interest in the possibility that MAH in many patients may be due to secretion of a PTH-like substance.

In this report we describe a protein factor secreted by cultured renal carcinoma cells from a patient with MAH. This factor activates renal adenylate cyclase apparently via an interaction with the PTH receptor, but is not recognized by multiple PTH antisera. The same cells produce both subcutaneous tumors and marked hypercalcemia in nude mice. The origin and properties of this cell line suggest that the factor it secretes may be representative of the etiologic agents producing hypercalcemia in patients with MAH and high NcAMP.

METHODS

Cell lines. Line 786-O was derived from a primary renal adenocarcinoma in a 58-yr-old man with multiple lung metastases but without evidence of bone metastases. The patient had a total serum calcium of 10.6–12.5 mg/dl, albumin 3.0 g/dl, and phosphorus 3.5 mg/dl. The culture methods and a complete characterization of the cell line have previously been published (3).

Tumor production in nude mice. 3-wk-old nude mice of White Swiss origin obtained from the University of California, San Francisco animal care facility were injected subcutaneously with 2×10^{6} tumor cells in 0.1 ml of outgrowth medium (RPMI 1640 with 20% heat-inactivated fetal bovine serum, 200 IU/ml penicillin, and 200 μg/ml streptomycin). Control animals of the same age were injected with an equal volume of medium. Blood was obtained and

Address all correspondence to Dr. Strewler.
Received for publication 21 October 1982 and in revised form 6 December 1982.
[1] *Abbreviations used in this paper*: bPTH, bovine PTH; BSA, bovine serum albumin; iPTH, immunoreactive PTH; MAH, malignancy-associated hypercalcemia; NcAMP, nephrogenous adenosine-3',5'-monophosphate; PTH, parathyroid hormone.

tumor diameters were measured weekly. Tumor volumes were calculated as length × width^2/2. 20 d following tumor cell injection, some animals had complete resection of the tumor under ether anesthesia. A second group had sham operations consisting of a similar skin incision on the opposite flank.

Blood was obtained from the medial retroorbital sinus under ether anesthesia. Serum calcium was measured on triplicate 10 μl serum samples using a Calcette ultramicro calcium analyzer (Precision Systems, Inc., Sadbury, MA). Inter- and intraassay CV were <2.0%.

Medium preparation. Cells were grown to confluence in outgrowth medium, which was then replaced by serum-free RPMI 1640. After 2–3 additional days medium was removed, supplemented with 0.001% or 0.1% bovine serum albumin (BSA), lyophilized, and resuspended in 0.1 M acetic acid (5–10% of initial volume). Concentrated medium was desalted at 4°C on a 1.5 × 60-cm column of BioGel P-2 (Bio-Rad Laboratories, Richmond, CA). 50–100 mesh, with 0.1 M acetic acid/0.1% BSA as eluant. Fractions eluting in the void volume were pooled, lyophilized, and resuspended in 10 mM acetic acid for bioassay or in 10% human hypoparathyroid plasma for RIA.

Assays for PTH-like activity. PTH-like biological activity was evaluated by a guanyl nucleotide-amplified renal adenylate cyclase assay that can detect 10 pg of human PTH(1–84) (4). Unless otherwise indicated, bovine PTH(1–34) [bPTH(1–34)] (Beckman Instruments, Inc., Palo Alto, CA, 6,000 U/mg) was used as standard, and the results were expressed as nanogram-equivalents bPTH (1–34)/ml. Previous studies have shown bPTH(1–34) to be equipotent (on a molar basis) with both bPTH(1–84) and human PTH(1–84) in this system (4). Immunoreactive PTH was measured by the method of Arnaud et al. (5) using four region-specific PTH antisera (generously supplied by Dr. C. D. Arnaud, University of California, San Francisco). Antiserum GP-FM has specificity for the 53–84 amino-acid sequence (6); GP-1M recognizes the 43–68 region (7); CH-14 is specific for the amino-terminal 1–34 region (7); and CH-12 recognizes only intact PTH(1–84) (7).

RESULTS

All nude mice injected with 786-O cells developed subcutaneous tumors. Associated with tumor growth was the development of marked hypercalcemia (Fig. 1). Mean serum calcium levels increased from an initial value of 9.1±0.4 (SD) to 15.0±3.0 mg/dl after 20 d of tumor growth (n = 12, $P < 0.001$). Tumor resection, but not sham operation, restored serum calcium to normal within 2 d. The time between injection of cells and the development of hypercalcemia was variable, but serum calcium was significantly correlated with tumor volume (r = 0.58; $P < 0.05$). Control animals, injected with vehicle alone, remained normocalcemic (mean serum calcium 9.5±0.3 mg/dl pre- and 10.0±0.6 mg/dl 28 d postinjection, n = 7). Nude mice injected with 253-J cells derived from a human transitional cell cancer of the bladder (8) in a normocalcemic patient developed tumors but remained normocalcemic (data not shown).

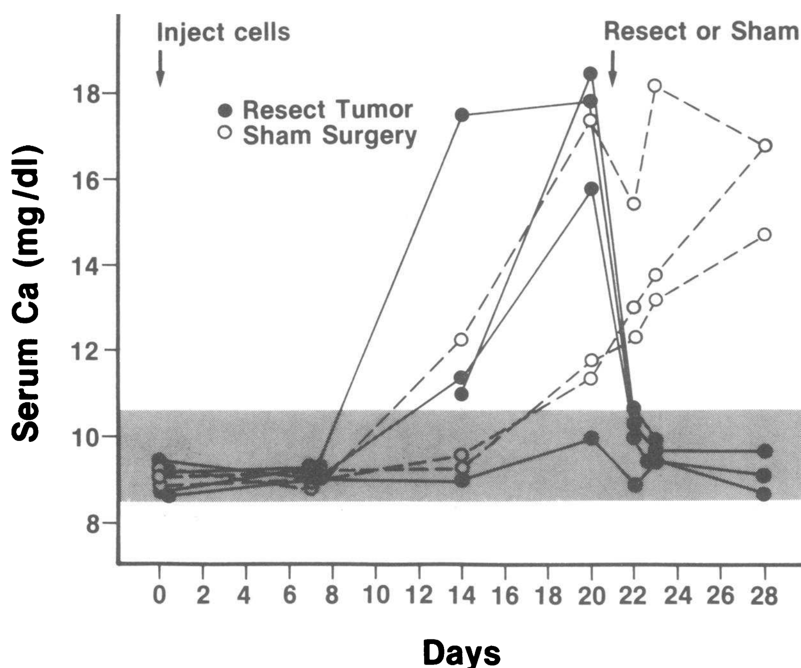

FIGURE 1 Serum calcium and response to resection (●) or sham surgery (○) in nude mice bearing tumors derived from 786-O cells. The normal range (mean±2 SD, n = 7) is shaded.

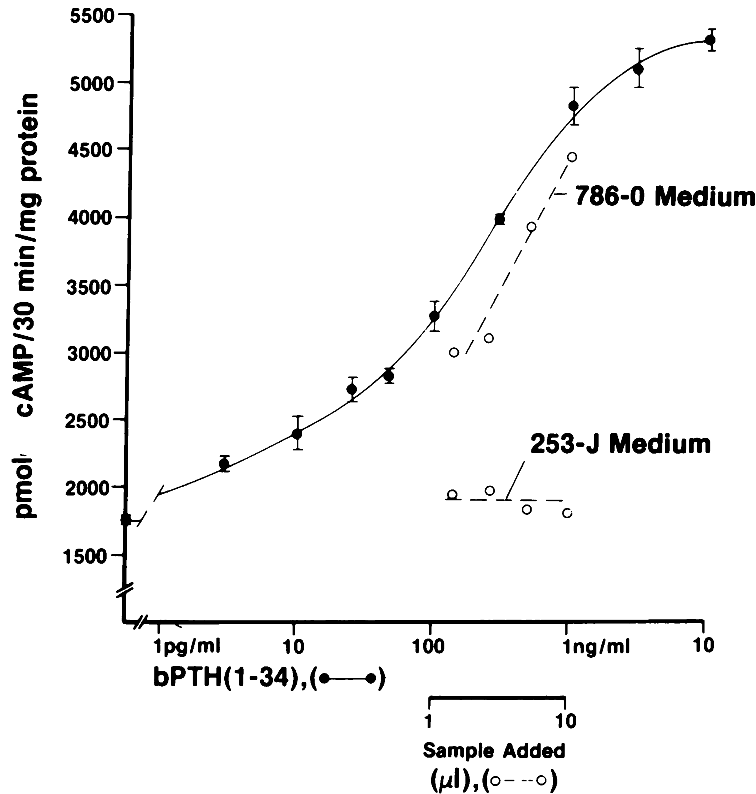

FIGURE 2 Adenylate cyclase activation by 786-O and 253-J medium. Values are the mean (±SE) of triplicate (●) or duplicate (○) determinations.

Medium from 786-O cells produced activation of renal adenylate cyclase, and the dose-response curve produced by multiple dilutions of medium was parallel to that obtained with bPTH(1–34) (Fig 2). In contrast, medium from 253-J cells produced no adenylate cyclase activation. Media from four additional human renal carcinoma cell lines and from a primary culture of normal human kidney cells were likewise inactive (not shown). [^{8}Norleucyl, ^{18}norleucyl, ^{34}tyrosinyl]-bPTH(3–34)amide (Peninsula Laboratories, San Carlos, CA), a competitive antagonist of PTH (9), completely abolished adenylate cyclase activation produced by the 786-O cell factor and by bPTH(1–84) (Fig. 3). Trypsin treatment of the factor also abolished its bioactivity (data not shown).

Gel filtration chromatography of 786-O medium disclosed two components of PTH-like bioactivity (Fig. 4). A major component was eluted between the ^{125}I-bPTH(1–84) marker and monomeric BSA, the later peak at absorbance of 280 nm. A minor component of bioactivity was eluted after ^{125}I-bPTH(1–84), but well before ^{125}I-bPTH(1–34).

Biologically active material was assayed in four PTH RIA with differing regional specificities. No iPTH was found despite addition of medium with enough bioactivity to exceed the detection limits of the RIA by 100- to 1,000-fold. Furthermore, iPTH was not found in gel filtration column effluent fractions corresponding to either the major or minor component of bioactivity (data not shown).

DISCUSSION

That hypercalcemia in the nude mouse bearing human tumor cells has a humoral basis is evidenced by the rapid normalization of serum calcium following resection of tumor. Although the humoral mechanism remains undefined, 786-O cells in culture were found to elaborate a factor that produced a PTH-like biological effect in vitro, whereas no such factor was released by 253-J cells that failed to produce hypercalcemia in vivo. Clearly the most parsimonious interpretation of the above observations, that the PTH-like protein causes hypercalcemia, must be explored.

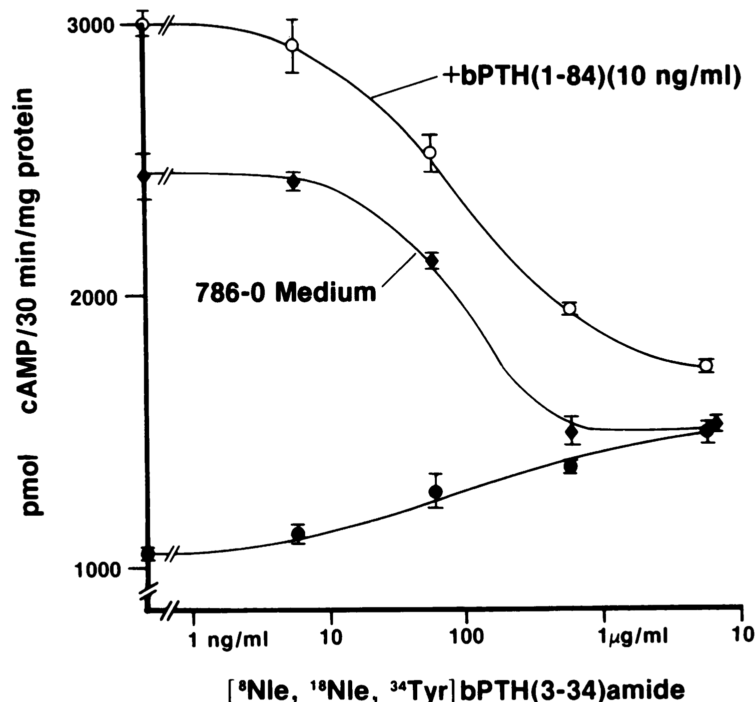

FIGURE 3 Inhibition by [[8]Nle, [18]Nle, [34]Tyr]bPTH(3–34)amide of adenylate cyclase activation produced by 786-O cell medium (6 ng-eq/ml; ♦) and bPTH (1–84) (10 ng/ml; ○) (MRC Lot 72/286, kindly provided by Dr. J. Zanelli). Also shown is the effect of [[8]Nle, [18]Nle, [34]Tyr]bPTH(3–34)amide alone (●). Values are the mean±SE of triplicate determinations.

The precise relationship of the 786-O factor to PTH is uncertain. Its activity in a PTH-specific assay and the inhibition produced by a PTH antagonist that acts competitively at PTH receptors (9) provides strong evidence that the 786-O factor interacts with renal PTH receptors. Yet the factor is distinguishable from native human PTH both by its failure to cross-react in four region-specific PTH RIA and by its larger apparent molecular size upon gel filtration chromatography. Nor is the protein identical to either of the known precursor forms of PTH, prepro- or pro-PTH, inasmuch as these have substantial immunoreactivity in COOH-region PTH RIA (11), and amino-terminal extensions, as in the precursors, markedly reduce renal adenylate cyclase-stimulating activity (12). It is conceivable that the 786-O factor is an altered PTH gene product lacking accessible sites for interaction with PTH antibodies. Alternatively, the factor could be a distinct gene product that interacts with PTH receptors due to sequence and/or conformational homology with PTH. Less likely is the possibility that the factor acts via a distinct receptor, even though the PTH antagonist inhibits adenylate cyclase activation by the factor.

Secretion of high-molecular weight PTH-like substances may be a relatively frequent occurrence in patients with MAH. MAH sera were found to contain large forms of iPTH, the quantity of which was correlated with serum calcium (13). With a cytochemical bioassay, sera of three patients with MAH and elevated NcAMP excretion contained a PTH-like substance larger than PTH(1–84) (14). It has been reported in preliminary form that squamous carcinomas from two patients with MAH contained a high-molecular weight (~70,000) substance that stimulated renal adenylate cyclase (15).

Stewart et al. (2) reported that 80% of unselected patients with MAH had elevated excretion of NcAMP, despite normal or low circulating levels of iPTH. Given the close relationship between PTH-responsive renal adenylate cyclase and NcAMP, it is possible that such patients are hypercalcemic as the result of a humoral tumor-derived substance similar or identical to that reported here. If so, the in vitro cell system we have described will permit the identification of the responsible factor. The nude mouse model of MAH will also facilitate study of the human syndrome and testing of new therapeutic modalities, such as specific PTH an-

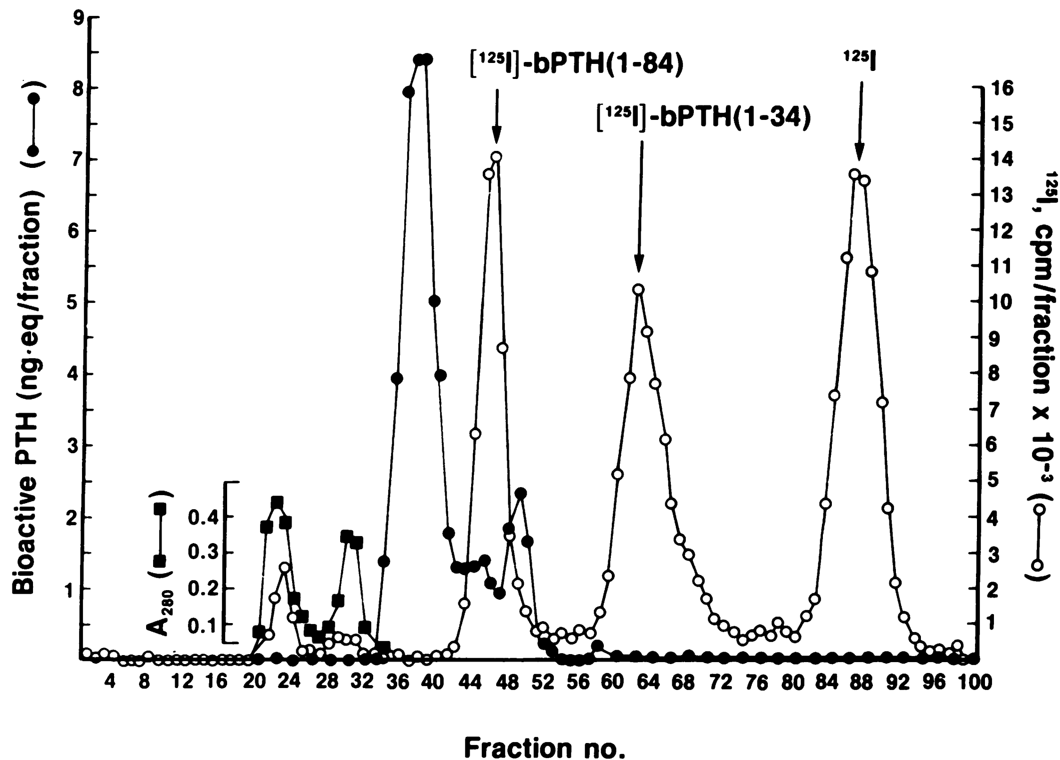

FIGURE 4 Chromatography of 786-O cell medium on Bio Gel P-150. Medium (160 ml) was concentrated and fractionated by ascending flow (6.5 ml/h) on 2.6 × 90-cm columns at 4°C in 0.2 M ammonium acetate (pH 4.6) (10). Biologically inactive, ^{125}I-labeled bPTH(1–84) and (1–34), as well as Na^{125}I were added to the sample as markers before chromatography. Fractions (5.5 ml) were lyophilized for 4 d and resuspended in 10 mM acetic acid/0.001% BSA for bioassay. Total recovery of bioactivity >50%.

tagonists, which may ultimately have widespread application to the treatment of patients with MAH.

ACKNOWLEDGMENTS

We are deeply grateful to Dr. Claude D. Arnaud for helpful discussions and critical review of the manuscript. We also appreciate the expert technical assistance of Skai Dombrovskis, Linda Zitzner, Elizabeth Wattenberg, and Elaina Harthcock, and the skillful secretarial help of Sally K. Danekas.

This work was supported by National Institutes of Health grants AM 27755, AM 27319, and AM 21614, and by funds from the Research Service of the Veterans Administration.

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