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Research Article

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Humoral Hypercalcemia of Malignancy

Release of a Prostaglandin-stimulating Bone-resorbing Factor In Vitro by Human Transitional-Cell Carcinoma Cells

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Abstract

Secretion by tumor cells of circulating bone-resorbing factors may frequently underlie the hypercalcemia that occurs in patients with malignancy. Efforts to identify the responsible mediators have been hampered by a lack of available human tumor cell systems suitable for study of the pathogenesis of the humoral hypercalcemia syndrome. We have established a transitional-cell carcinoma (TCC) line in vitro from a patient with humoral hypercalcemia. These cells are tumorigenic and cause hypercalcemia in athymic nude mice. Culture medium conditioned by TCC cells contains potent bone-resorbing activity in vitro, the physical and biological properties of which are similar to those of bone-resorbing activity present in the original patient's urine. The bone-resorbing activity of the TCC factor is accompanied by increased prostaglandin release from bone and is blocked by indomethacin and calcitonin. The TCC-derived bone-resorbing activity coelutes with prostaglandin-stimulating activity during gel filtration with an approximate molecular weight of 15,000. This activity is nondialyzable, stable to concentrated urea and reducing agents, and destroyed by boiling. The TCC factor does not increase cyclic AMP production in bone or kidney bioassays and does not exhibit transforming growth factor activity. We conclude that a unique macromolecular factor released by TCC cells causes bone resorption by a mechanism dependent upon stimulation of bone cell cyclooxygenase, and that this factor is the probable cause of the hypercalcemia in vivo. The TCC cell line provides a new model for study of the human humoral hypercalcemia syndrome.

Introduction

Hypercalcemia occurs frequently in advanced malignancies and often severely compromises management of the underlying neoplasm (1-3). Accelerated net bone resorption is believed to be the source of the hypercalcemia in such patients (1, 4, 5), although understanding of the mechanisms whereby malignant cells alter the functions of normal bone cells remains incomplete. Direct osteolysis by tumor cells metastatic to bone may occur (6). Often, however, circulating tumor-derived bone-resorbing

factors appear to cause osteolysis, most clearly so in those patients whose hypercalcemia occurs in the absence of skeletal metastases (7). The pathogenesis of this humoral hypercalcemia syndrome appears to be heterogeneous. Secretion by tumor cells of a variety of bone-resorbing factors has been postulated, including parathyroid hormone (PTH)¹ (8-10), prostaglandins (11-13), osteoclast-activating factor and other leukocyte products (14-17), transforming growth factors (1, 18, 19), and agents with PTH-like actions on bone and kidney (20-22).

Efforts to purify and characterize unknown bone-resorbing factors derived from human tumors have been impeded by the relative insensitivity of available in vitro bone resorption bioassays and, more importantly, the limited availability of human tumor tissue as a source of active factors. We have recently established a continuous tumor cell line from a patient with transitional-cell carcinoma (TCC) and humoral hypercalcemia. These tumor cells cause hypercalcemia when implanted in athymic nude mice and release a potent bone-resorbing factor in vitro that exhibits properties distinguishing it from agents previously implicated in the genesis of the humoral hypercalcemia of malignancy syndrome. The studies reported here provide the basis for a new model of human hypercalcemia of malignancy.

Methods

Clinical summary

The patient was a 54-year-old Caucasian woman who was found to be hypercalcemic (12.6 mg/100 ml) when admitted for a hypertensive lacunar stroke in February 1983. She had a history of alcoholism and was taking hydrochlorothiazide for mild hypertension. In 1981, serum calcium and phosphorus had been noted to be 10.6 and 1.7 mg/100 ml, respectively. Evaluation of the hypercalcemia revealed an undetectable plasma amino-terminal immunoreactive PTH (iPTH) (<8 pg/ml), low serum 1,25(OH)₂D (15 pg/ml), elevated carcinoembryonic antigen (45 ng/ml), and urine cytology positive for TCC. A ^{99m}Tc diphosphonate bone scan showed a generalized increase in skeletal uptake but no focal lesions. 24-h urinary calcium excretion was 160 mg. The following studies were normal: blood urea nitrogen, creatinine, alkaline phosphatase, urinary Bence-Jones protein, serum immunoelectrophoresis, chest x-ray, and liver-spleen scan. Serum calcium and phosphorus ranged between 12.6 and 13.7 mg/100 ml and between 3.3 and 5.7 mg/100 ml, respectively, and creatinine gradually rose to 2.5 mg/100 ml. Hypercalcemia and azotemia failed to respond to discontinuation of the diuretic, intravenous hydration, or oral orthophosphate but were successfully managed with mithramycin. Diffuse retroperitoneal node involvement was found at laparotomy, and a portion of an excised lymph node was used to establish the cell line reported here.

1. *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; PGE₂, prostaglandin E₂; PTH, parathyroid hormone; bPTH, bovine PTH; iPTH, immunoreactive PTH; ROS, rat osteosarcoma (cell); TCC, transitional-cell carcinoma; TGF, transforming growth factor.

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Tumor burden and hypercalcemia responded to repetitive courses of intravenous methotrexate, but urinary frequency ultimately prompted readmission in September 1983, when cystoscopy showed the bladder to be filled with tumor. Renal function had remained normal since her previous discharge, however, and urine cultures were negative. During this admission, serum calcium and phosphorus ranged between 9.7 and 12.4 mg/100 ml and between 2.1 and 2.8 mg/100 ml, respectively, a third bone scan was unchanged, and a total of eight 24-h urine collections were performed for measurement of urinary cyclic AMP excretion, the mean \pm standard error of the mean of which was 4.8 ± 0.8 nmol/100 ml glomerular filtrate. These and others were also concentrated for study of urinary bone-resorbing activity, as described below. Over the next 3 mo, further progression of locally metastatic disease occurred despite chemotherapy, renal failure resulting from ureteral obstruction ultimately supervened, and the patient expired. Permission for an autopsy was not granted.

Culture of tumor cells

Tumor tissue obtained at surgery was minced to 1–2-mm fragments in 100-mm plastic petri dishes containing 15 ml of Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal bovine serum ("5% DME"). Explants (10–20 fragments per dish) were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air with medium changes once or twice each week. Homogeneous populations of polygonal tumor cells grew out from these explants and were maintained in 5% DME with serial subculturing by trypsinization at 3–4-wk intervals. 3 d after feeding confluent monolayers of these tumor cells in 150-cm² flasks with 5% DME, the medium was discarded and replaced with 25 ml of DME containing 0.5% horse serum. The conditioned medium was then harvested after 2 d and the procedure was repeated once before returning the cells to 5% DME. The conditioned medium was centrifuged at 2,000 g for 15 min and stored at –20°C until further processing.

Tumor growth in athymic nude mice

Freshly trypsinized tumor cells, washed once with 5% DME, were re-suspended in serum-free DME at $2-4 \times 10^6$ cells/ml and administered subcutaneously (0.5 ml per animal) to athymic nude mice (nu AF, Harlan Sprague Dawley, Inc., Indianapolis, IN). Animals were maintained on standard mouse laboratory diet in germ-free cages and were observed weekly for evidence of tumor growth, which occurred in ~4 wk. Thereafter, blood was obtained at 1–2 wk intervals for measurement of calcium and creatinine. At the time of sacrifice, tumor-bearing mice were carefully examined for evidence of visceral or bony metastases. In some cases, ^{99m}Tc diphosphonate bone scans of normal and hypercalcemic tumor-bearing nude mice were kindly performed by Dr. Frank Castronova (Radiation Safety Unit, Massachusetts General Hospital).

Preparation of medium and urine concentrates

Cell-conditioned medium was thawed and centrifuged at 2,000 g prior to concentration. Urine samples were filtered serially through glass depth prefilters and 1.2- and 0.45- μ m membrane filters (Gelman Sciences, Inc., Ann Arbor, MI) upon arrival in the laboratory and immediately concentrated at 4°C. All samples were concentrated 100-fold by ultrafiltration under nitrogen using thin-channel and stirred-cell devices fitted with YM5 membranes (nominal mol wt cutoff, 5,000) (Amicon Corp., Danvers, MA). Preliminary studies showed no increase in recovery of bone-resorbing activity when UM2 membranes (1,000 mol wt) were used. Supernatants obtained after centrifugation of the concentrates at 50,000 g for 2 h and stored at –20°C exhibit no loss of bone-resorbing activity over periods of several months.

Bone resorption bioassay

Bone resorption was quantitated by the release of previously incorporated ⁴⁵Ca from newborn mouse calvarial bones in vitro. Half-calvaria from 5-d-old mice (CD-1 strain, Charles River Breeding Laboratories, Inc., Wilmington, MA) were obtained as previously described (23) after maternal administration of 50 μ Ci of ⁴⁵CaCl₂ (New England Nuclear, Boston, MA) subcutaneously on the 19th day of gestation. The bones were pre-

cultured in 2 ml of DME containing 1 mM calcium, 2 mM phosphate, 5% heat-inactivated horse serum, and 1% antibiotic/antimycotic solution (Gibco, Grand Island, NY) on a rocking platform at 50 oscillations/min in a 37°C incubator under 5% CO₂ in air. After 24 h, the medium was replaced with 3 ml of medium that contained test substances (or vehicle alone) sterilized by membrane filtration (0.45 μ m, Gelman Sciences, Inc.). Synthetic bovine PTH(1-34) (bPTH) (6,000 U/mg) was dissolved in 0.005 M acetic acid, and cortisol (Steraloids Co., Wilton, NH) and indomethacin (Sigma Chemical Co., St. Louis, MO) were stored as concentrated stocks in methanol at –20°C. The acetic acid and methanol vehicles, at concentrations of 0.5% and 0.1%, respectively, had been found not to affect the release of ⁴⁵Ca from control bones. After an additional 72 h, the bones were removed, rinsed in saline, and placed in plastic scintillation vials containing 0.4 ml of 2 N HCl. Aliquots of culture medium (1 ml) were transferred to separate vials containing 4 ml of scintillation fluid (Scint-A, Packard Instrument Co., Inc., Downers Grove, IL) for determination of released radioactivity. Bone extracts were counted similarly, and the percentage of total bone ⁴⁵Ca released into the medium during the 72-h treatment period was calculated. Results were expressed as the mean \pm standard error of the mean of the percentage of ⁴⁵Ca released for groups of five or six bones, and the statistical significance of differences between groups was evaluated by Student's *t* test. Epidermal growth factor (EGF), at doses between 0.1 and 100 ng/ml, did not significantly increase resorption in this system after 72 h in the absence of added calf serum.

Adenylate cyclase activity

Adenylate cyclase activity in isolated canine renal membranes was measured as the rate of conversion of [³²P]ATP to [³²P]cyclic AMP in the presence of guanyl nucleotides, as described by Nissenson et al. (24) with minor modifications. The final concentrations of ATP and guanylimidodiphosphate in the assay were 0.52 mM and 70 μ M, respectively. Test samples of PTH standard were added in a volume of 0.01 ml to provide a final incubation volume of 0.1 ml. Recovery of [³H]cyclic AMP after Dowex and alumina chromatography was typically 75–85%. Results were expressed as picomoles per milligram of membrane protein/30 min for triplicate determinations.

Cyclic AMP production

Rat osteosarcoma (ROS) cells. Monolayers of ROS 17/2.8 clonal ROS cells, kindly provided by Dr. Gideon Rodan (Department of Oral Biology, University of Connecticut, Farmington) were grown to confluence in Ham's F-12 medium with 5% fetal bovine serum and 1% kanamycin (Gibco) in 16-mm wells. Cells were incubated first at 37°C in air for 30 min in 0.5 ml of PAG buffer (phosphate-buffered saline with 2 mg/ml of glucose and 4 mg/ml of heat-inactivated bovine albumin), this buffer was discarded, and test agents were then added in 0.2 ml of PAGI buffer (PAG with 1 mM isobutylmethylxanthine). Incubations were terminated after 10 min by addition of cold methanol (1 ml/well), the cell layers were extracted twice more with methanol, the combined extracts were evaporated by vacuum centrifugation, and the dried extracts were re-dissolved in acetate buffer for measurement of cyclic AMP by a specific radioimmunoassay (New England Nuclear). Significant stimulation of cyclic AMP in this bioassay was routinely observed at concentrations of bPTH(1-34) $> 0.5 \times 10^{-9}$ M. Concentrates were also tested for stimulation of conversion of [³H]adenine to [³H]cyclic AMP in intact ROS cells (after 7 d of pretreatment with dexamethasone), as kindly performed by Dr. Gideon Rodan. The sensitivity of this bioassay for bPTH is $\sim 0.01-0.03 \times 10^{-9}$ M (25).

Calvarial bones. Neonatal mouse calvaria were obtained as above and incubated at 37°C in PAG buffer for up to 1 h before transfer of individual bones to siliconized (Sigmacote, Sigma Chemical Co.) glass tubes containing test agents in 0.25 ml of PAGI buffer. Reactions were conducted for 10 min at 37°C and terminated by addition of 1 ml of cold methanol. Bones were sonicated for 30 min at room temperature, the methanol extraction was repeated twice, and the combined extracts were then evaporated and processed for cyclic AMP radioimmunoassay as described above. Significant stimulation of calvarial cyclic AMP was

usually evident at concentrations of bPTH(1-34) between 0.5 and 1.0×10^{-9} M. In some experiments, cyclic AMP was measured directly in aliquots of medium obtained at 72 h from the bone resorption bioassay.

Prostaglandin synthesis

Prostaglandin E₂ (PGE₂) in medium from cultures of mouse calvaria was determined by specific radioimmunoassay (New England Nuclear) after appropriate dilution of the media in assay buffer. Control culture media at these dilutions were found not to interfere with measurement of added standard PGE₂ in this assay.

Epidermal (EGF) and transforming (TGF) growth factor activities

Binding of ¹²⁵I-labeled murine EGF to human A431 cells was studied as described by DeLarco et al. (26) except that cells, grown in 5% DME, were plated at 1.25×10^4 cells/cm² in 16-mm plastic wells 24 h before study. Radioiodinated murine EGF (170 μCi/μg, Collaborative Research Co.) was added (30,000 dpm/0.35 ml) to wells containing 0.5 ml of test samples or standard EGF (Biomedical Technologies Inc., Cambridge, MA), and specific binding was determined after 60 min at 20°C.

Induction of anchorage-independent growth of non-neoplastic rat cells was assessed essentially as described by Roberts et al. (27). Briefly, normal rat kidney cells (clone 49F) were plated at a density of 500 cells per microtiter well in 0.2 ml of DME containing 0.3% agarose and 10% calf serum (Flow Laboratories, Inc., McLean, VA) with or without EGF (5 ng/ml, Collaborative Research Co., Lexington, MA). Cells were incubated for 7 d in a humidified atmosphere of 10% CO₂ in air and then stained with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (0.5 mg/ml) for enumeration of colonies containing >20 cells. Purified β-TGF was prepared by acid/ethanol extraction of normal human platelets and subsequent chromatography on Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, CA) as previously described (28). This purified factor elicited a half-maximal increase in colony formation at a protein concentration of 25 ng/ml.

Column chromatography

Concentrates (5 ml) were applied to 2.5 × 90-cm columns of Ultrogel AcA 44 (LKB Co., Gaithersburg, MD) and eluted by ascending flow with 0.1 N acetic acid in 50 mM ammonium acetate, pH 4.1, at a flow rate of 22 ml/h. Fractions (4 ml) were collected, optical density at 280 nm was determined, and selected fractions were lyophilized and redissolved in medium for direct bioassay of bone-resorbing activity.

Other procedures

Immunoreactive PTH was measured in plasma, concentrated cell medium, or urine using chicken antiserum CK-67, which exhibits specificity for the amino-terminal region of the PTH sequence (29). The sensitivity of the PTH immunoassay for human PTH(1-34) was ~1–2 pg/tube. Serum 1,25(OH)₂D was measured by specific radioimmunoassay in the laboratory of Dr. Thomas Clemens at the Regional Bone Center, Helen Hayes Hospital, West Haverstraw, NY, where normal levels were 20–65 pg/ml. Cyclic AMP was measured in appropriately diluted (1:100 to 1:1000) urine by a commercial radioimmunoassay (New England Nuclear) that was highly specific for cyclic AMP. Serum and urinary creatinine were measured in the hospital's Clinical Chemistry Laboratory. Values for total urinary cyclic AMP (nmol/100 ml of glomerular filtrate) measured by these techniques in normal subjects (3.1 ± 0.4 , $n = 6$) and in patients with primary hyperparathyroidism (9.9 ± 0.8 , $n = 7$), hypercalcemia owing to solid tumors not metastatic to bone (7.0 ± 1.2 , $n = 12$), and hypercalcemia owing to hematologic malignancy (3.1 ± 1.4 , $n = 3$) were in agreement with data provided by the supplier of the cyclic AMP kits and with results previously published by others (30, 31).

For organic extraction, aliquots of medium and urine concentrates were diluted to 1 ml with saline, acidified to pH 3.5 with glacial acetic acid, and extracted three times with 3 vol of ethyl acetate. In control experiments, this procedure resulted in over 98% extraction of [³H]PGE₂ added as an indicator of the efficiency of the separation. The combined

ethyl acetate extracts were evaporated to dryness under a stream of nitrogen and redissolved in medium for bioassay. The aqueous phases were dialyzed against 140 mM NaCl, 1 mM sodium phosphate, pH 7.4, and diluted with medium prior to bioassay.

Results

Cultured tumor cells from this patient ("TCC cells") were tumorigenic when administered subcutaneously to athymic nude mice. Moreover, the majority of tumor-bearing mice developed hypercalcemia (Fig. 1) by 8 wk in the absence of bony metastases either at autopsy or by radionuclide bone scanning (four mice). Renal function remained normal in the tumor-bearing animals.

Concentrates of both the patient's urine and of conditioned medium from her cultured tumor cells contained potent bone-resorbing activity when tested in the mouse calvarial resorption assay (Fig. 2). Moreover, the biologic and chemical properties of the activity in conditioned medium and the patient's urine were similar by a variety of independent criteria. Thus, in both cases, the activity was nondialyzable and was not destroyed by exposure to denaturing (8 M urea) or reducing (65 mM dithiothreitol) agents (Table I). Activity was stable upon heating to 56°C for 45 min but was lost upon boiling for 10 min. No PTH immunoreactivity was detectable in either urine or medium concentrates at dilutions active in the resorption bioassay, and levels of PGE₂ were <100 pM, which is 100-fold lower than the detection limit of the resorption assay for PGE₂ (Fig. 2). The possibility that other lipid-soluble substances (other prostaglandins, vitamin D metabolites, vitamin A) might be responsible for the bone-resorbing activity in TCC medium or urine concentrates was addressed by extracting these concentrates with ethyl acetate and then testing the organic extracts and residual aqueous phases for bone-resorbing activity. As shown in Table I, all of the bone-resorbing activity in both preparations remained

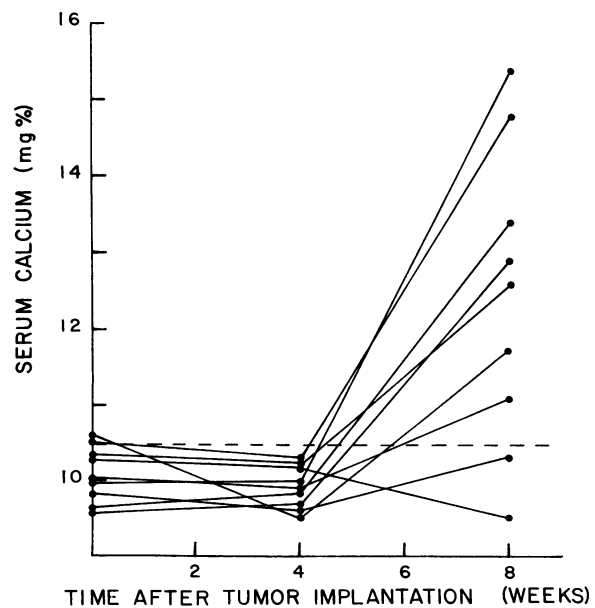


Figure 1. Hypercalcemia in athymic nude mice bearing TCC tumors. Tumor cells ($1-2 \times 10^6$) were injected subcutaneously into the flanks of nude mice, and blood was obtained via tail vein at 4-wk intervals. The upper limit of normal (mean + 2 SD) is indicated by the dashed line. Serum creatinine (mean ± SEM) was 0.61 ± 0.04 mg/dl initially and 0.55 ± 0.03 mg/dl at 8 wk.

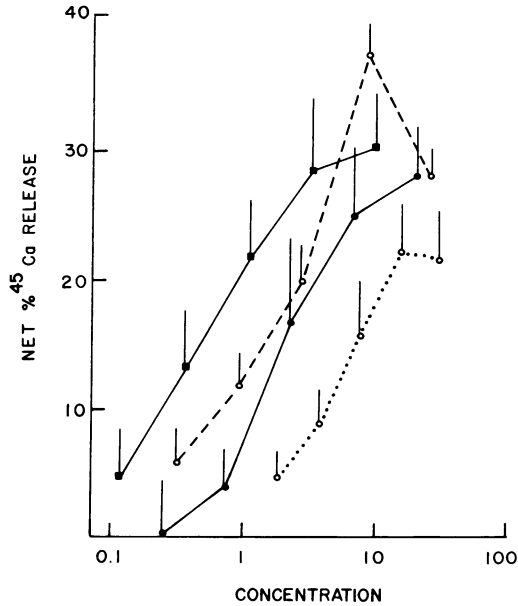


Figure 2. Bone resorption induced by concentrates of TCC medium and patient urine. Concentrates (100-fold) prepared by ultrafiltration of TCC cell medium (■) or the patient's urine (●) were assayed for bone-resorbing activity in 72-h cultures. Results are expressed as the increment in percent total bone radiocalcium released over that of untreated control bones for groups of five bones (mean±SEM) in each experiment. Doses of medium or urine concentrate are given as microliters/milliliter of concentrate in the final assay medium. The effects of bPTH(1-34) (nanomolar) (○---○) and PGE₂ (nanomolar × 10⁻¹) (○···○) in this assay are also shown. Control resorption in the experiments with TCC medium, patient urine, bPTH(1-34), and PGE₂ was 23±3, 18±2, 17±1 and 25±3%, respectively.

in the aqueous phases, and none was found in the corresponding organic extracts.

In addition, resorption induced by both concentrates was almost completely blocked by calcitonin, and neither preparation caused increased release of radiocalcium from bones previously devitalized by repetitive freeze-thawing (Table I). Gel filtration chromatography of both patient urine and TCC medium concentrates demonstrated a single major peak of bone-resorbing activity that eluted with an apparent molecular weight of ~15,000 (Fig. 3).

Both urine- and cell-derived resorbing activities were associated with increased release of immunoreactive PGE₂ by the calvarial bones, and, moreover, in both cases the bone-resorbing activity was inhibited by addition of indomethacin to the bone resorption bioassay (Table II). By contrast, PTH also increased PGE₂ release from these bones (Table II), but the increased resorption induced by this hormone was unaffected by indomethacin, despite virtually complete blockade of PGE₂ release by the drug. The possibility that bone-resorbing and prostaglandin-stimulating activities in the TCC medium were properties of the same molecule was further supported by the demonstration of coelution of these two activities during gel-filtration chromatography (Fig. 3 A and B).

Concentrated urine and cell medium were tested for evidence of PTH-like adenylate cyclase-stimulating activity using several in vitro bioassays. When cyclic AMP was measured in the medium of cultured calvarial bones undergoing resorption 3 d after

addition of maximal doses of TCC medium, no evidence of delayed stimulation of cyclic AMP production was observed, whereas increases in medium cyclic AMP were seen when bones were treated with doses of PGE₂ causing comparable bone resorption (see legend to Table III). At doses causing maximal activity in the bone resorption assay, neither TCC medium nor patient urine concentrate increased adenylate cyclase activity acutely in isolated canine renal membranes (Fig. 4) or in intact ROS 17/2.8 cells (22, 25) (data not shown). Similarly, when PTH and TCC medium concentrates were tested at doses that caused maximal bone resorption, no acute increase in release of cyclic AMP by calvarial bones or rat osteosarcoma cells was observed in response to TCC medium concentrates, whereas

Table I. Chemical and Biologic Properties of TCC-derived Bone-resorbing Activity

Experiment	Bone resorption*		
	Control medium	TCC medium	Patient urine
No. 1			
Untreated	17±1	46±4	30±4
Dialyzed (3,500 mol wt)	18±1	45±3	39±1
Urea (8 M × 4 h)	18±2	49±2	44±4
No. 2			
Untreated	18±3	37±3	50±5
Heated (56°C × 45 min)	13±3	38±3	43±4
Boiled (100°C × 10 min)	11±1	13±1‡¶	13±1‡¶
No. 3			
Untreated	10±2	23±3	17±2
Ethyl acetate extraction			
Aqueous phase	12±1	29±4	30±4
Organic phase	13±1	14±1‡¶	11±1‡¶
No. 4			
Untreated	25±3	40±4	41±2
Dithiothreitol (65 mM)	27±3	36±2	40±4
No. 5			
Untreated	13±2	37±3	35±4
Devitalized bones	8±1	8±1‡¶	8±1‡¶
Calcitonin (2 U/ml)	8±1	14±1¶	12±1¶

Aliquots of concentrates were diluted to 1 ml with 140 mM NaCl/1 mM sodium phosphate, pH 7.4 ("saline phosphate") and treated as indicated prior to further dilution with assay medium and addition to bone cultures. Samples incubated in 8 M urea or 65 mM dithiothreitol (37°C × 2 h) were dialyzed against saline phosphate before dilution and bioassay. Bones were devitalized by three cycles of repetitive freeze-thawing. Final dilutions in the bioassay of control medium, TCC cell medium, and patient urine concentrates were 1:100, 1:100, and 1:500, respectively. In experiment 5, resorption induced by 50 ng/ml of bPTH(1-34) (42±2%) was reduced to 12±1% in the presence of calcitonin (*P* < 0.05). Experiments shown were repeated at least three times each using different preparations of medium and urine concentrates, with similar results.

* Values shown are mean±standard error of the mean of the percent of total bone ⁴⁵Ca released in 72 h for groups of five bones. Unless indicated (‡), all results with TCC medium or patient urine are significantly different from those for the corresponding control media (*P* < 0.05).

¶ Significantly different from untreated controls (*P* < 0.05).

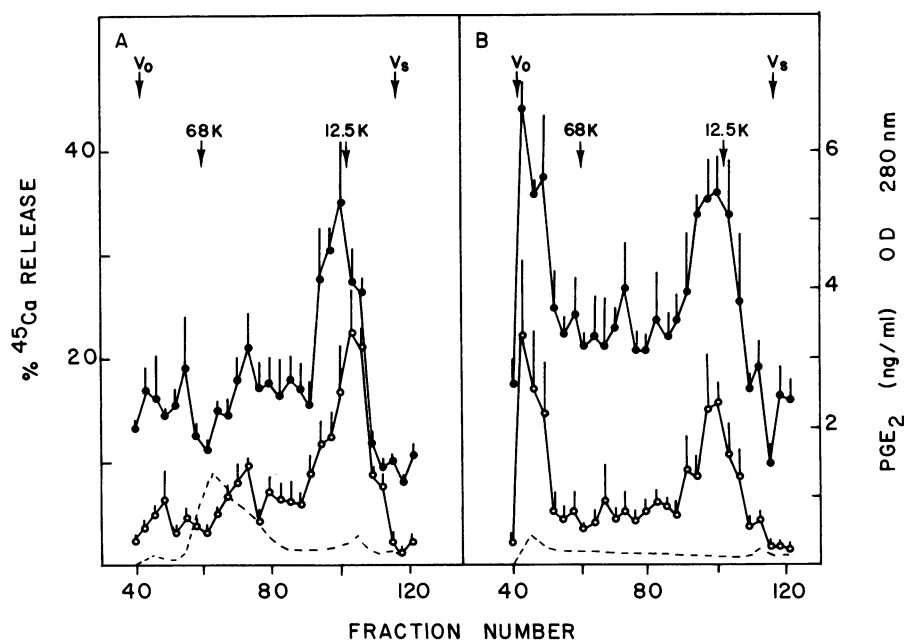


Figure 3. Ultragel AcA 44 chromatography of concentrated TCC medium and patient urine. (A) Concentrated TCC medium (5 ml) was applied to a 2.4 × 90-cm column and eluted with 0.1 M acetic acid in 50 mM ammonium acetate, pH 4.1. Selected fractions (4 ml) were lyophilized, redissolved in 0.2 ml of 10 mM acetic acid, diluted to 16 ml with medium, and added to the calvarial bioassay for measurement of bone resorption (●), expressed as of percent ⁴⁵Ca (mean±SEM) released in 72 h for groups of five bones. Absorbance at 280 nm is shown (---), as is medium PGE₂ at 72 h (mean±SEM for groups of five bones) (○). Elution positions (K, thousands in mol wt) of blue dextran (V₀), bovine albumin, cytochrome c, and ⁴⁵CaCl₂ (V_s) are indicated. (B) As in A, except that the sample was 5 ml of concentrated patient urine and only 1 ml of selected fractions was lyophilized for bioassay. When the remaining eluate in fractions 40–55 was pooled, lyophilized, and incubated in 8 M urea for 2 h

before reapplication to the column, ~60% of the resorbing activity eluted in a peak at fraction 100 and the remainder again eluted near the void volume of the column.

PTH elicited the expected large increments in cyclic AMP (Table III). Because slight inhibition of basal renal membrane adenylate cyclase occurred at higher doses of TCC medium (Fig. 4), we studied the response of these membranes to bPTH(1-34) in the presence and absence of maximal levels of TCC medium concentrate (10 μl/ml). As shown in Fig. 4, the adenylate cyclase response to bPTH(1-34) was inhibited slightly by added crude TCC medium concentrate. Comparable results were observed with the osteosarcoma cells (Table III). This apparently non-specific inhibition seems inadequate to account for the complete absence of adenylate cyclase responsiveness to TCC medium alone, however.

Active TCC medium concentrates were also tested for the presence of constituents that might bind to receptors for EGF (Fig. 5). Although binding was reduced slightly below that of control wells in the presence of TCC concentrate, this was also true of control medium concentrate, and no dose-response relation was evident in either case. These data indicate that TCC medium concentrates, at dilutions (1:50 or more) maximally

active in the bone resorption assay, must contain <0.1 ng/ml of EGF-equivalent receptor binding activity. By contrast, EGF elicits maximal resorption of mouse calvaria in vitro only at doses higher than 1 ng/ml (32), and then only in the presence of fetal bovine or calf serum (not used in our studies). The presence of TGF activity in medium concentrates was also sought by testing for induction of anchorage-independent growth of normal rat kidney fibroblasts in soft agar in the presence and absence of EGF. At doses as high as 100 μl/ml, however, TCC-cell medium concentrates failed to stimulate significantly any growth of fibroblast colonies after 7 d, whereas purified platelet-derived β-TGF elicited up to 200 colonies per well (Table IV). The response to platelet β-TGF was not inhibited in the presence of 20 μl/ml of TCC medium (not shown).

Discussion

Urothelial cancers are frequently associated with humoral hypercalcemia (3), and the patient reported here exhibited the

Table II. Prostaglandin Dependence of TCC-derived Bone-resorbing Activity

Preparation	Untreated		Indomethacin	
	Resorption	Medium PGE ₂	Resorption	Medium PGE ₂
	%	pg/ml	%	pg/ml
Control medium	17±1	130±30	11±1*	18±2*
bPTH(1-34) (50 ng/ml)	45±2	690±180	39±2	8±8*
Patient urine (1:500)	42±1	1800±100	12±1*	1±1*
TCC medium (1:100)	38±3	1100±130	14±2*	(Undetect.)*

Bones were cultured as indicated in the presence or absence of indomethacin (10 μM). Values for bone resorption are percent ⁴⁵Ca release (mean±SEM), as in legend to Table I. Concentrations of PGE₂ (pg/ml) in the medium of the bone cultures after 72 h are expressed as mean±SEM for the groups of five bones. * Significantly different from untreated controls (*P* < 0.05).

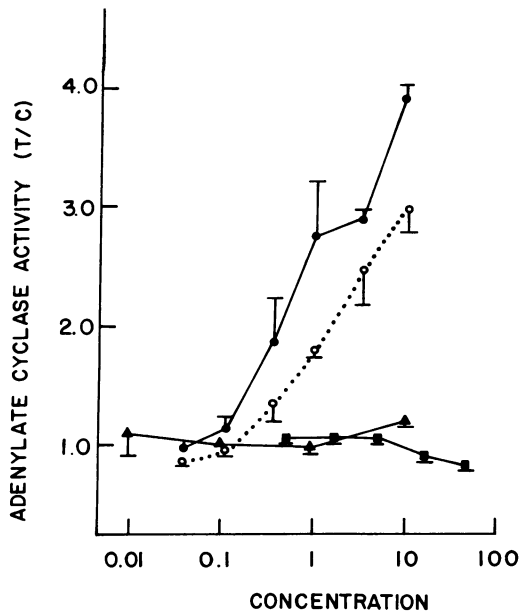


Figure 4. Lack of stimulation of canine renal membrane adenylate cyclase by TCC factor. Adenylate cyclase activity, expressed as treated-to-control ratios (T/C), is shown in response to graded doses of concentrated TCC medium alone (■), concentrated patient urine (▲), standard bPTH(1-34) (●), and bPTH(1-34) in the presence of 10 µl/ml of TCC medium concentrate (○). Concentrations of TCC medium or urine are given as microliters per milliliter and of PTH as nanomolar. Results shown are mean±standard error of the mean for triplicate determinations and are representative of five separate experiments.

characteristic clinical features of the humoral hypercalcemia syndrome (1, 7). Thus, hypercalcemia occurred in the absence of radiographically demonstrable bone metastases, serum phosphorus and 1,25(OH)₂D were low, and plasma iPTH was undetectable, in spite of which urinary cyclic AMP excretion was not suppressed. The severity of the hypercalcemia fluctuated with treatment of the patient's underlying malignancy, and therapy with glucocorticoids and orthophosphate were not clearly successful in relieving the hypercalcemia. The virtual replacement of the patient's bladder by invasive tumor provided a unique opportunity to collect directly the products of tumor-cell secretion in vivo. Moreover, the establishment of the patient's tumor cells as a continuous cell line in vitro permitted direct study of the nature of the cellular product(s) potentially responsible for the humoral hypercalcemia.

The similarity, both physicochemical and biologic, of the bone-resorbing factors identified in concentrates of this patient's urine and of culture medium conditioned by exposure to her tumor cells in vitro suggests that these TCC cells have continued to release the same active factor in vitro as had been produced previously in vivo. Moreover, the occurrence of humoral hypercalcemia in athymic nude mice bearing TCC tumors strongly supports the hypothesis that the bone-resorbing factor released by these cells in vitro causes hypercalcemia in vivo and was likely responsible for the patient's hypercalcemia. Nevertheless, direct proof of this hypothesis awaits the availability of highly purified bone-resorbing factor.

The bone-resorbing factor released by TCC cells exhibits properties that distinguish it from several other factors previously

Table III. Effect of TCC Medium on Bone Cell Cyclic AMP Production

Preparation	Mouse calvarial bones pmol/bone per 10 min	Rat osteosarcoma cells pmol/well per 10 min
Control	15±5	21±10
bPTH(1-34)		
1 × 10 ⁻⁸ M	93±15	442±86
1 × 10 ⁻⁷ M	346±48	741±163
TCC medium I		
No additions	10±32	5±3
+bPTH(1-34) (1 × 10 ⁻⁸ M)	79±14	253±74
TCC medium II		
No additions	9±3	14±12
+bPTH(1-34) (1 × 10 ⁻⁸ M)	91±17	216±54*

Incubations were conducted at 37°C as described in Methods. Two different preparations of TCC medium concentrate were tested (I and II), both with and without added bPTH(1-34). Results are expressed as the mean±standard error of the mean for groups of four bones or six wells of ROS 17/2.8 cells. When calvaria were incubated for 72 h with TCC medium (10 µl/ml), PGE₂ (100 ng/ml), or control medium alone, percent ⁴⁵Ca release was 33±4, 40±4, and 14±2, respectively, whereas medium cyclic AMP (pmol/ml) was 10±1, 21±2 and 10±1.

* Significantly different from bPTH(1-34) (10⁻⁸ M) alone (*P* < 0.05).

implicated in the pathogenesis of humoral hypercalcemia of malignancy. Direct measurements of PTH and PGE₂ in active medium concentrates indicate that these agents could not be responsible for the resorbing activity observed in such preparations. That the resorbing activity is retained by 3,500-mol wt dialysis membranes and partitions into the aqueous phase during organic extractions also provides evidence against a role for other prostaglandins, retinoids, or vitamin D metabolites. The TCC factor is distinguished from osteoclast-activating factor (OAF) purified from tonsillar tissue by its larger apparent molecular weight (15,000 vs. 9,000) and the lack of stimulation of bone-cell cyclic AMP production (16). OAF derived from leukocytes is similar in size to the TCC factor but, unlike the TCC factor, leukocyte OAF does not cause bone resorption via a prostaglandin-dependent mechanism (33). Resistance to denaturing concentrations of urea also indicates that a noncovalent subunit structure is probably not required for biologic activity of the TCC factor.

Bone resorption induced by the TCC factor in vitro appears to be dependent upon stimulation of prostaglandin synthesis in the bone tissue, in that large increments in medium PGE₂ occur in these cultures and pretreatment of the bones with indomethacin effectively blocks resorption induced by this material. We have previously reported similar observations in the case of another human tumor-cell line (melanoma) (34), and others have reported prostaglandin-dependent bone-resorbing activity in extracts of a number of tumors associated with hypercalcemia (35). Although only measurements of PGE₂ are reported here, preliminary experiments in our laboratory with radiolabeled arachidonic acid show that PGE₂ and 6-keto-PGF₁α, the major metabolite of prostacyclin, are the principal prostaglandins syn-

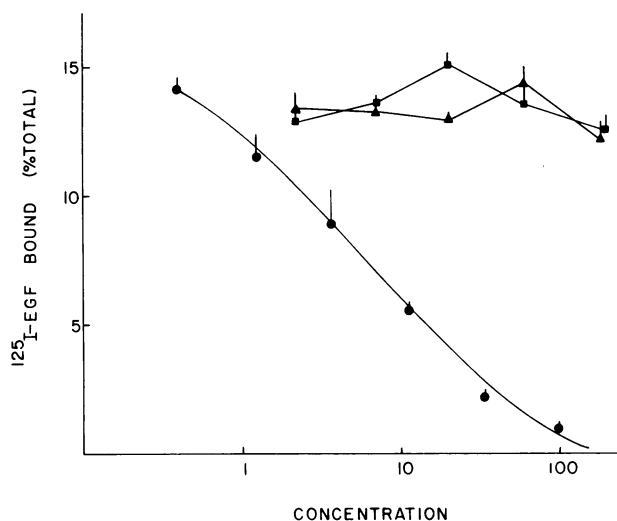


Figure 5. Absence of EGF receptor-binding activity in TCC medium concentrate. Incubations of TCC medium concentrate (■), control medium concentrate (▲), and purified murine EGF (●) with ^{125}I -EGF and A431 cells were conducted as described in Methods. Each point represents the percent total ^{125}I -EGF bound (mean \pm SEM) for groups of four wells each. Control binding in the absence of added EGF was $15.2 \pm 0.7\%$. The concentrations of test agents are given as nanograms per milliliter for EGF and as microliters per milliliter for the medium concentrates (100-fold). When tested for bone resorbing activity (at 20 $\mu\text{l/ml}$), these preparations of control and TCC medium concentrates elicited 25 ± 3 and $51 \pm 3\%$ ^{45}Ca release, respectively (assay control, $25 \pm 2\%$).

thesized by calvarial bones in response to the TCC factor. These are the same prostaglandins observed by others in cultures of mouse bones resorbing in response to phorbol esters or EGF (32, 36). Because of the heterogeneity of cell populations within the calvarial tissue, the precise cellular origin of the prostaglandins measured in the medium of bones treated with TCC factor remains unknown. Moreover, the mechanism(s) whereby increased prostaglandin release initiates accelerated bone resorption induced by this material is also unclear. It is of interest that PTH also causes increased medium PGE_2 in these bone cultures (Table II) but that indomethacin, although it blocks the increment in PGE_2 , fails to inhibit the resorption induced by this hormone.

Table IV. Absence of TGF Activity in TCC Medium Concentrate

Preparation	Colony-stimulating activity*	
	-EGF	+EGF
Control	0	12
β -TGF		
25 ng/ml	0	110
2,500 ng/ml	0	200
TCC medium		
10 $\mu\text{l/ml}$	0	11
100 $\mu\text{l/ml}$	0	8

This preparation of TCC medium concentrate elicits maximal bone resorption in vitro at 30 $\mu\text{l/ml}$.

* Colonies (>20 cells) per microtiter well after 7 d (mean of duplicates). Concentration of EGF is 5 ng/ml.

These observations indicate that the nature of the biologic consequences of increased prostaglandin production in bone may depend upon the particular agent stimulating this production, the identity of the cells responsible for the augmented prostaglandin synthesis, or, possibly, the pattern of arachidonate metabolites produced. The potent inhibitory effect of calcitonin seen in our studies suggests that increased osteoclastic activity underlies the resorptive effects of the factor, although the synthesis of prostaglandins and, possibly, of a prostaglandin-dependent regulator of osteoclast activity might occur in adjacent osteoblasts in the calvarial tissue (37). The increased release of prostaglandins by bone in response to this or similar factors secreted by tumors in patients with humoral hypercalcemia could account for the elevated levels of plasma prostaglandins often seen in such patients (13), the occasional response of humoral hypercalcemia to indomethacin or aspirin (1, 11, 13), and the observation that tumor prostaglandin content often correlates poorly with hypercalcemia (38).

The involvement of prostaglandins in the resorptive response of mouse calvaria to TCC factor is analogous to the effects of some bone-resorbing growth factors, including EGF and platelet-derived growth factor, in this tissue (32, 39). This is of particular interest in light of the hypothesis of Mundy et al. (1, 18, 19) that tumor-derived growth factors may mediate the humoral hypercalcemia syndrome. We were unable to detect either EGF receptor-binding activity or β -TGF activity in concentrated cell medium, however, even at doses much higher than those required to elicit maximal bone resorption in vitro. Moreover, calvarial bone resorption induced by TCC medium occurs in the absence of added calf serum, whereas EGF stimulates resorption in this system only in the presence of calf serum (unpublished observations). Finally, other common features of tumor-derived growth factors, including inactivation by reducing agents and stability to boiling (40), are not shared by the TCC factor. It therefore seems unlikely that the TCC-derived resorbing factor is also a transforming growth factor, although further studies with more purified factor will be necessary to exclude this possibility definitively.

Recently, PTH-like macromolecular factors have been described in extracts of tumors and in culture medium from tumor cell lines obtained from hosts with humoral hypercalcemia (20–22). These factors, like PTH, stimulate adenylate cyclase activity in renal membranes or osteoblast-like bone cells, an effect that can be blocked by synthetic inhibitory analogs of PTH (20–22). Analogous observations have been made using a highly sensitive renal cytochemical bioassay (20). It has thus been proposed that such factors, although immunologically and chromatographically distinct from PTH, might nevertheless interact with PTH receptors in bone and kidney to initiate increased bone resorption and decreased renal tubular phosphate reabsorption by cyclic AMP-mediated mechanisms (20). Such effects might account for hypercalcemia, hypophosphatemia, and high nephrogenous cyclic AMP excretion seen in humoral hypercalcemia, even though other common features of the syndrome, including low levels of serum $1,25(\text{OH})_2\text{D}$, elevated fractional excretion of calcium, and histologically apparent uncoupling of bone resorption and formation rates (5), would still not be readily explained. In light of these reports, we carefully examined the possibility that such a PTH-like factor might be present in our TCC medium or urine concentrates. Extensive studies of the possible effects of these concentrates on cyclic AMP production in a variety of

PTH-responsive bioassays, including the calvarial bone cultures used for the resorption measurements, failed to reveal any evidence of such a factor, however. This was true even at doses well above those that elicit maximal activity in the bone resorption bioassay. The absence of adenylate cyclase-stimulating activity appears not to be due to the presence in these concentrates of nonspecific inhibitors of the bioassay. These observations clearly indicate that the TCC factor induces bone resorption *in vitro* by a mechanism not involving activation of PTH-receptor-linked adenylate cyclase. The source of the abundant cyclic AMP present in our patient's urine is not known with certainty. Although her total urinary cyclic AMP excretion (4.8 nmol/100 ml glomerular filtrate) is such that we cannot unambiguously classify this patient into either the high- or low-cyclic AMP groups proposed by Stewart et al. (7), this amount of urinary cyclic AMP seems inappropriate in the setting of persistently undetectable serum iPTH. It is possible that the tumor cells in her urinary tract may have released large amounts of cyclic AMP directly into the urine, although we have not observed high concentrations of cyclic AMP in fresh medium from the TCC cell cultures. Because plasma cyclic AMP was not measured, it is possible that much of the cyclic AMP present in the patient's urine derived from glomerular filtration. Finally, others have reported elevations of nephrogenous cyclic AMP in normocalcemic cancer patients (31, 41), which suggests that this abnormality may be a general feature of some malignancies that is unrelated to coexisting abnormalities in calcium metabolism.

In summary, we have identified a potent macromolecular bone-resorbing factor released *in vitro* by a tumor cell line established from a patient with humoral hypercalcemia of malignancy. This cell line, which is tumorigenic and causes humoral hypercalcemia in athymic nude mice, provides a new experimental model for study of the human humoral hypercalcemia syndrome. The factor released by these cells *in vitro* is physically and biologically similar to activity recovered from the patient's urine, which had been in direct contact with a large mass of tumor tissue. Our studies suggest that this factor is different from others previously implicated as causes of the humoral hypercalcemia syndrome and that its effects on bone resorption are mediated by an increase in prostaglandin synthesis in bone cells. Purification and further study of the mechanism of action of this factor, both *in vivo* and *in vitro*, should lead to better understanding of the pathogenesis of humoral hypercalcemia of malignancy.

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