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Research Article

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Factors Associated with Humoral Hypercalcemia of Malignancy Stimulate Adenylate Cyclase in Osteoblastic Cells

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ABSTRACT The culture media of three cell lines, a human prostate carcinoma (PC3), a rat Leydig cell tumor (Rice-500), and a rat carcinosarcoma (WRC-256), that were derived from tumors associated with humoral hypercalcemia of malignancy (HHM), were examined for stimulation of adenylate cyclase in ROS 17/2.8 osteoblastic cells and for bone resorptive activity in culture. Cells from a nonhypercalcemic variant of the WRC 256 tumor served as control. Extracts from three solid human tumors, a lung adenocarcinoma from a patient with HHM and two adenocarcinomata from normocalcemic patients (lung and colon), were also examined for adenylate cyclase stimulation. We found excellent correlation between stimulation of cyclic AMP accumulation in ROS 17/2.8 cells and bone resorbing activity in culture, or production of HHM in vivo. Stimulation of adenylate cyclase by HHM factors was inhibited by the parathyroid hormone competitive inhibitor, [^{8}norleucyl, ^{18}norleucyl, ^{34}tyrosinyl] bovine parathyroid hormone (3-34) amide.

INTRODUCTION

Clinical and histomorphometric studies indicate that humoral hypercalcemia of malignancy (HHM)[1] is primarily due to augmented osteoclastic bone resorption (1-3). The cellular mechanisms for humoral control of bone resorption remain unclear (4). Parathyroid hormone (PTH), a potent stimulator of osteoclastic bone resorption, is known to stimulate cyclic (c) AMP accumulation in osteoblastic cells (5). Receptors to PTH have been demonstrated on osteoblasts by radioligand binding studies (6). These and other observations suggest that cells belonging to the osteoblastic lineage may participate in the control of bone resorption (7). Several HHM factors were recently reported to increase renal adenylate cyclase activity in vitro (8, 9). This effect was inhibited by the PTH analogue, [^{8}norleucyl, ^{18}norleucyl, ^{34}tyrosinyl] bovine PTH (3-34) amide ([^{8}Nle, ^{18}Nle, ^{34}Tyr]bPTH(3-34) amide); therefore, interaction with the PTH receptor is suggested. It was of interest to examine if HHM factors stimulate

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[1] *Abbreviations used in this paper:* FCS, fetal calf serum; HHM, humoral hypercalcemia of malignancy; [^{8}Nle, ^{18}Nle, ^{34}Tyr]bPTH(3-34) amide, [^{8}norleucyl, ^{18}norleucyl, ^{34}tyrosinyl] bovine PTH (3-34) amide; PTH, parathyroid hormone.

adenylate cyclase in osteoblastic cells, if this stimulation was also inhibited by [^{8}Nle, ^{18}Nle, ^{34}Tyr]bPTH(3-34) amide, and if it correlated with bone resorbing activity.

METHODS

Tumor cell culture and collection of tumor cell conditioned media. Cells from the hypercalcemic variant of Walker tumor were derived from explant cultures of the tumor that was kindly provided by Dr. R. Martodam of the Procter and Gamble Co., Cincinnatti, OH. All rats carrying these tumors subcutaneously developed hypercalcemia in the range of 13–18 mg/dl (10–12). Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum (FCS), penicillin, 100 U/ml, and streptomycin, 100 μg/ml, and were incubated at 37°C in a humidified 5% CO_2, 95% air atmosphere. Subconfluent cells were transferred into Dulbecco's modified Eagle medium containing 2% vol/vol FCS and at 48 h later, the tumor cell-conditioned medium was collected, centrifuged at 1,000 *g* for 15 min, and stored at −20°C.

The cell line LLC-WRC 256 from the normocalcemic variant of the Walker tumor was purchased from the American Type Culture Collection, Rockville, MD (CCL38). Cells were cultured in conditioned medium obtained as described above. The PC3 prostatic carcinoma cell line was also obtained from American Type Culture Collection and was cultured as described by Kaighn et al. (13). The tumor cells were obtained originally from the bone metastasis of a patient with disseminated prostatic cancer. 48-h conditioned media were assayed for bone resorption and adenylate cyclase stimulating activity.

The Rice 500 Leydig cell tumor that produces hypercalcemia in Fisher rats (3) was obtained from the National Cancer Institute Breast Cancer Task Force Bank and maintained by serial subcutaneous transplantation. The tumor was mechanically dispersed into single cell suspension and was cultured in RPMI 1640 media (Gibco Laboratories, Grand Island, NY) with 10% bobby calf serum. At confluence, cells were incubated in serum free medium which was collected for the assays described in the study.

Human tumor extracts. Tumor extracts were prepared as described by Stewart et al. (8). One adenocarcinoma of the lung was obtained from a patient with clinical and biochemical features that are characteristic of HHM. Control tumor tissue was obtained at surgery or autopsy from two normocalcemic patients with adenocarcinoma of the lung and adenocarcinoma of the colon, respectively.

Bone resorption assay. Bone resorptive activity was measured by following the release of ^{45}Ca that was previously incorporated into the forearm bones of 19-d-old rat embryos as previously described (14). Bones were incubated for 48 h in BGJb medium with 5% FCS (heat-inactivated) in the presence of conditioned media, diluted 1:2, or control media. Bone resorbing activity was expressed as treated-to-control ratios of ^{45}Ca release. Four bones were used in each treatment group.

Measurement of adenylate cyclase. ROS 17/2.8 cells were plated at 10,000 cells/cm^2 in 35-mm culture dishes in F-12 medium containing 5% FCS and 1% kanamycin (15). Medium was changed every 3–4 d, and at 10–12 d, cells were incubated with 1 μCi [^{3}H]adenine for 2 h. The incubation medium was discarded, cells were washed with 2 × 2 ml calcium-magnesium free Hanks' balanced salt solution and were incubated for 10 min with 0.5 ml F-12 medium with 2% FCS and 0.2 mM isobutylmethylxanthine. At that time, conditioned medium, tumor extract, PTH, isoproterenol, or forskolin were added at the appropriate dilution for 10 min, or the indicated time, at 37°C. The incubation volume was 1 ml and control samples contained appropriate amounts of control media. The reaction was stopped and cAMP extracted with 1.2 M trichloracetic acid. After centrifugation at 2,000 rpm, the supernatant was neutralized with 4 N KOH and a carrier solution containing 5 mM cAMP, adenine, adenosine, ATP, and ADP was added. About 3,000 cpm of ^{32}P-cAMP were added for calculating recovery. cAMP was separated on serial Dowex X50 and alumina column chromatography (16) and counted in a scintillation counter. [^{8}Nle, ^{18}Nle, ^{34}Tyr]bPTH(3-34) amide and human PTH (1-34) were kindly provided by Dr. M. Rosenblatt (Massachusetts General Hospital, Boston, MA).

RESULTS

Table I shows that conditioned media, which enhance bone resorption in culture and were derived from tumors that produce hypercalcemia in vivo, also stimulate cAMP accumulation in the ROS 17/2.8 osteoblastic cell line. These include two animal tumors, the Walker carcinosarcoma and the Rice 500 Leydig cell tumor, and one human tumor, the PC3 prostate carcinoma. On the other hand, the Walker carcinosarcoma variant that produces nonhypercalcemic tumors and has no bone resorptive activity in culture did not stimulate cAMP accumulation in osteoblastic cells. Furthermore, a tumor extract from a patient with HHM strongly stimulated cAMP accumulation, whereas extracts from similar or other tumors, which are not associated with HHM, did not. The most active materials tested were the Walker (hypercalcemic) conditioned medium, which stimulated resorption slightly over twofold and cAMP accumulation about 6- to 10-fold, and the carcinoma extract, which stimulated adenylated cyclase over 10-fold. The cAMP-stimulating activity of the HHM media corresponded to that of 0.3–3 nM PTH.

As seen in Table II, the PTH analogue [^{8}Nle, ^{18}Nle, ^{34}Tyr]bPTH(3-34) amide, at 1 μM, inhibited the stimulation of adenylate cyclase by PTH and by HHM tumor extracts and culture media, but did not inhibit stimulation by isoproterenol or forskolin. On the other hand, propranolol, the β-catecholamine antagonist, does not inhibit the effects of the HHM factors or PTH. Fig. 1 shows the effect of sequential dilution of the conditioned media obtained from the hypercalcemic Walker tumor on cAMP stimulatory activity. For comparison, the lower end of the PTH dose-response curve (which saturates at about 100 nM) is presented. At even a 16-fold dilution, this medium exhibited detectable activity in this assay, which was equivalent to that of 0.3 nM PTH. A similar dilution curve was obtained for the adenocarcinoma extract (data not shown). Fig.

TABLE I

Effect of Tumor Cell Culture Media and Tumor Extracts on ROS 17/2.8 Adenylate Cyclase and on Bone Resorption

Sample	cAMP accumulation		Bone resorption
	cpm/culture	*treated/control*	*treated/control ratios of ^{45}Ca release‡*
Cell culture conditioned media			
Walker hypercalcemic (H)	864±64*	6.0	2.17±0.30
Rice 500 Leydig cell tumor (H)	520±23	3.8	1.50±0.07
Prostate carcinoma (PC-3) (H)	341±41	2.7	1.36±0.09
Walker normocalcemic (N)	123±12	0.98	1.01±0.09
Human tumor extracts			
Adenocarcinoma of the lung (H)	2,310±302	10.2	ND
Adenocarcinoma of the lung (N)	289±55	1.04	ND
Adenocarcinoma of the colon (N)	332±151	1.20	ND

Culture media and tumor extracts were prepared as described in Methods and were assayed, as described, at a 1:2 dilution. Each sample was compared to its own control containing culture medium or extraction buffer. *H*, hypercalcemic, *N*, nonhypercalcemic; *ND*, not determined.

* Values are means±SD from three cultures.

‡ Values are means±SEM from four pairs of bones.

2 shows the time course of cAMP accumulation, which increased linearly for 10 min in the presence of a low concentration of hypercalcemic Walker medium and isobutylmethylxanthine.

DISCUSSION

The significance of these findings is threefold: (*a*) they support the notion that stimulation of cAMP accumulation in osteoblastic cells is related to activation of osteoclastic bone resorption; (*b*) they are consistent with the suggestion that certain HHM factors produce their effect by interaction with the PTH receptors; and (*c*) they provide a sensitive tool for assaying this activity, which could assist in purification and in studies of the HHM mechanism of action.

Previous studies (1–3, 11) indicate that the HHM syndrome is due principally to stimulation of osteoclastic bone resorption. The identification of receptors for bone resorbing agents (PTH, prostaglandin E, and $1,25(OH)_2$ Vitamin D_3) in osteoblastic cells has led to the hypothesis that cells of the osteoblastic lineage may mediate the resorbing effects of these agents (7). Moreover, it was shown that the bone resorbing activity of prostanoids in culture correlated closely with their cAMP stimulating activity in osteoblastic cells (17). The same pattern is seen for the HHM activities examined in this study; this suggests a similar mechanism

TABLE II

Effect of [^{8}Nle, ^{18}Nle, ^{34}Tyr]bPTH(3-34) amide on HHM Stimulation of Adenylate Cyclase

Adenylate cyclase agonist (factor)	Control	Agonist	Agonist + (3-34)PTH-analogue* (1 μM)	Agonist + propranolol (10 μM)
			cAMP/culture	
Carcinoma of lung	260±22	2,772±168	532±96	2,384±64
Walker, hypercalcemic	176±21	1,396±103	888±63	1,395±134
Rice 500 Leydig cell tumor	146±25	609±102	191±18	ND
Parathyroid hormone (10 nM)	128±5	5,370±323	1,182±47	5,234±187
Isoproterenol (100 nM)	144±16	4,803±463	5,876±303	128±8
Forskolin (5 μM)	373±85	2,123±228	2,393±121	ND

Culture media and tumor extracts were prepared and assays were run as described in Methods. The effects of [^{8}Nle, ^{18}Nle, ^{34}Tyr]bPTH(3-34) amide alone, varied between no stimulation and 20% stimulation. *ND*, not determined.

* [^{8}Nle, ^{18}Nle, ^{34}Tyr]bPTH(3-34) amide.

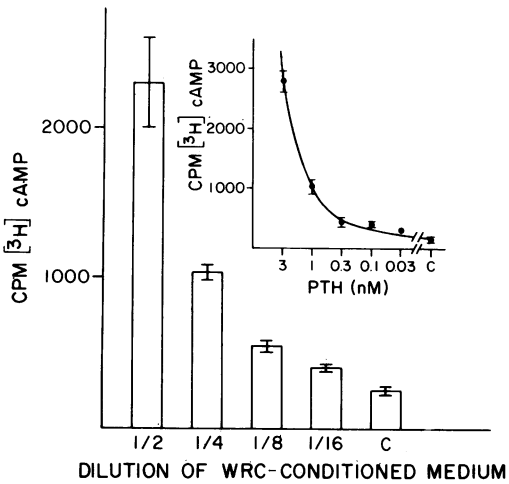

FIGURE 1 Effects of sequential dilution of WRC-conditioned media on cAMP accumulation in ROS 17/2.8 cells. WRC hypercalcemic conditioned medium was obtained as described in Methods and was assayed for 10 min for stimulation of cAMP accumulation, as described. (*Inset*) Dose-response curve for hPTH(1-34) stimulation of cAMP accumulation assayed for 5 min, as described.

of action at the level of bone. The means of communication between osteoblasts and osteoclasts are not known. Various possibilities, which were discussed elsewhere, include the release of humoral mediators or the blocking of humoral or anatomical inhibition (7).

The findings of this study also support the suggestion that the HHM factors may stimulate adenylate cyclase via PTH receptors. HHM factors have been reported to stimulate PTH-sensitive renal adenylate cyclase, and for at least two tumors (8, 9), this activity was inhibited by the PTH competitive inhibitor, [^{8}Nle, ^{18}Nle, ^{34}Tyr]bPTH(3-34) amide. In ROS cells, we found only two groups of hormones, PTH and β-catecholamines, which stimulate adenylate cyclase (18). On the basis of structural analogy and the data presented above (Table II), the most likely site of action of the 3-34 PTH analogue is the PTH receptor. The data are consistent with interaction of the HHM factors with the same site. However, the partial agonist nature of the 3-34 PTH analog and the lack of radioligand displacement data dictate caution in drawing this conclusion. We observed that the 3-34 PTH analogue was not equally effective in inhibiting the stimulation of the three HHM factors examined (Table II). Also, the time course of cAMP accumulation in the presence of the WRC factor was different from that for PTH (Fig. 2). Interpretation of such differences is premature in view of impurity of the material. However, it is possible that PTH receptors are not the primary receptors of the HHM factors, in analogy to somatomedin interaction with insulin receptors and interaction of tumor growth factors with epidermal growth factor receptors (19, 20). The affinity for the PTH receptor may vary among the HHM factors. The structural basis for interaction of these factors with PTH receptors is unknown. Several HHM factors were reported to be proteins with M_r > 35,000, which lacked immunological cross-reactivity with PTH (9, 21); however, there is no indication that the HHM activity from the different tumors is produced by the same material. Investigation of these questions requires purification of the HHM factors.

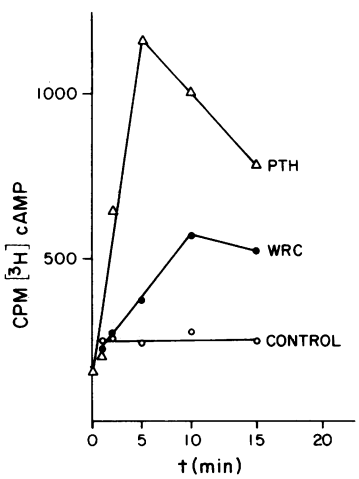

FIGURE 2 Time course of cAMP accumulation in ROS 17/2.8 cells in the presence of 1 nM PTH (△), and WRC hypercalcemic media diluted to 1:8 (●). Control cells (○) contained 0.001% acetic acid and 0.001% bovine serum albumin (PTH vehicle) in culture media. Assays were run as described in Methods.

The observations reported here have several practical implications. The cellular cAMP assay, like the renal adenylate cyclase assay, has a high signal-to-noise ratio for this HHM activity. This assay is more rapid than the bone resorption assay (4–5 h vs. 3–6 d), uses a permanent stable cell line rather than animals, and is less expensive. In addition, if osteoblastic cells are indeed target cells of the HHM factors in vivo, certain aspects of the mechanism of action of these factors could be investigated in this system.

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